Understanding Mechanisms of
Cellular Injury in the
Antiphospholipid Syndrome

Katie Suzanne Poulton
Department of Medicine
University College London

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**Declaration**

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

Patients with the Antiphospholipid Syndrome (APS) have circulating antiphospholipid antibodies (aPL) which cause vascular thrombosis (VT) and/or pregnancy morbidity (PM). Previously we have shown that IgG isolated from patients with APS and VT alone (APS-VT) caused activation of p38 MAPK and NFκB signalling pathways and up-regulation of tissue factor (TF) activity in monocytes. These effects were not seen with IgG from patients with APS and PM alone (APS-PM) or healthy controls. TF up-regulation caused by the APS-VT samples was reduced by p38 MAPK, NFκB, and TLR4 inhibitors, thus implicating a TLR4-MyD88 dependent signalling mechanism. Therefore, my PhD aimed to examine whether IgG isolated from patients with different manifestations of the APS have differential effects upon activation of a pregnancy related cell type; trophoblast cells and a thrombotic related cell type; endothelial cells (EC).

IgG was purified from the serum of APS-VT patients, APS-PM patients and two control groups. Using a human first trimester trophoblast cell line, HTR-8 cells, I identified that APS-PM but not APS-VT increased TLR4 and TRIF mRNA expression. HTR-8 cell migration was significantly inhibited in cells treated with APS-PM but not APS-VT and this inhibited migration was restored after pre-treatment with a TLR4 inhibitor. I also identified that both APS-VT and APS-PM increased protease-activated receptor (PAR)-1 and PAR-2 mRNA expression in HTR-8 cells, which was not seen in control IgG treated cells. Work carried out in HUVEC identified that only APS-VT phosphorylated p38 MAPK and not APS-PM or healthy controls. When investigating p38 MAPK phosphorylation in HTR-8 cells however, neither APS-PM nor APS-VT phosphorylated p38 MAPK in this pregnancy related cell type.

The results obtained in this thesis identify that IgG purified from patients with different clinical manifestations of the APS have differential effects on a pregnancy related cell type than they do on a thrombotic related cell type.
Acknowledgments

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Publications

Work described in this thesis has been included in the following published material:


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aCL – Anti-cardiolipin antibodies
ANOVA – Analysis of variance
Anti-β2GPI – Anti-β2-glycoprotein-1 antibodies
APASS – Antiphospholipid antibody and stroke study
APC – Antigen presenting cell
aPL – Antiphospholipid antibodies
aPL+/APS- - Patients with antiphospholipid antibodies who do not have the antiphospholipid syndrome
APLASA – Antiphospholipid antibody acetylsalicylic acid study
aPPT – Activated partial thromboplastin time
APS – Antiphospholipid syndrome
APS ACTION – Antiphospholipid syndrome alliance for clinical trials and international networking
BCA – Bicinchoninic acid
BCR – B cell receptor
BSA – Bovine serum albumin
CAMS – Cell adhesion molecules
CAPS – Catastrophic antiphospholipid syndrome
CCL5 - Chemokine (C-C motif) ligand 5
CD – Cluster of differentiation
cDNA – Complementary DNA
CpG DNA - Cytosine pairing guanine containing DNA motifs
CRTF – Clinical research task force
Ct – Cycle threshold
CVA – Cerebrovascular accident
DAF – Decay acceleration factor pathway inhibitor
DEA – Diethanolamine
DEPC - Diethlypyrocarbonate
DI – Domain I
dRVVT – Dilute Russell’s viper venom test
dsRNA – double stranded RNA
DTT - Dithiothreitol
DVT – Deep vein thrombosis
EC – Endothelial cell
ELISA – Enzyme linked immunosorbent assay
EMP – Endothelial microparticle
EU – endotoxin units
EVT – Extravillous cytotrophoblast
F - Factor
FCS – Foetal calf serum
GAPDH – Glyceraldehyde 3-phosphate dehydrogenase
GnRH – Gonadotrophin-releasing hormone
GPLU – Immunoglobulin G phospholipid units
GRO-α - Growth related oncogene-alpha
hCG - Human chorionic gonadotrophin
HUVEC – Human umbilical vein endothelial cells
ICAM-1 – Intercellular cell adhesion molecule-1
IFN - Interferon
Ig – Immunoglobulin
IL – Interleukin
INR – International normalised ratio
IRAK - Interleukin-1 receptor associated kinase
IRF – Interferon regulatory factor
IUGR – Intrauterine growth restriction
LA – Lupus anticoagulant
LDL – Low-density lipoprotein
LMWH – Low molecular weight heparin
lpr – Lymphoproliferative gene
LPS - Lipopolysaccharide
MAL - Myeloid differentiation primary-response gene 88 – adaptor like
MAPK – Mitogen activated protein kinase
MCP-1 – Monocyte chemotactic protein-1
MHC – Major histocompatibility complex
MI – Myocardial infarction
MPLU – Immunoglobulin M phospholipid units
MyD88 - Myeloid differentiation primary-response gene 88
NFκB – Nuclear factor kappa B
P - Passage
PAMP – Pathogen associated molecular pattern
PAPS – Primary antiphospholipid syndrome
PAR – Protease-activated receptor
PBS – Phosphate buffered saline
PE – Pulmonary embolism
Pen/Strep – 100 units/mL penicillin / 100 mg/mL streptomycin
PFA - Paraformaldehyde
PI3K – Phosphadylinositol-3-kinase
PM – Pregnancy morbidity
p-NPP – p-nitrophenylphosphate
PRL - Prolactin
PRR – Pattern recognition receptor
qRT-PCR – Quantitative real time polymerase chain reaction
RAPS – Rivaroxaban in antiphospholipid syndrome trial
RCT – randomised control trial
RITAPS – Rituximab in antiphospholipid syndrome trial
SARM - Sterile α- and armadillo-motif-containing protein
SDS – PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM – Standard error of the mean
SLE – Systemic lupus erythematosus
SN-APS – Seronegative antiphospholipid syndrome
ssRNA – Single stranded RNA
Stat – Signal transducer and activator of transcription
ST-PL - Second trimester pregnancy loss
SU – Standard units
TBS-T – Tris buffered saline – 0.1% tween
TC – Tissue culture
TCR – T cell receptor
TEMED – Tetramethylethylenediamine
TF – Tissue factor
TFPI – Tissue factor pathway inhibitor
TGFβ = Transforming growth factor beta
TIA – Transient ischaemic attach
TIR – Toll/interleukin-1 receptor
TLR – Toll like receptor
TNF – Tumour necrosis factor
TRAM – Toll/interleukin-1 receptor-domain-containing adaptor protein inducing interferon-β receptor adaptor molecule
TRIF – Toll/interleukin-1 receptor-domain-containing adaptor protein inducing interferon-β
TT-PL - Third trimester pregnancy loss
UFH – Unfractionated heparin
VCAM-1 – Vascular cell adhesion molecule-1
VEGF - Vascular endothelial growth factor
VT – Vascular thrombosis
VT+/PM- - aPL positive patients with a history of vascular thrombosis
VT-/PM+ - aPL positive patients with a history of pregnancy morbidity
WARSS – Warfarin aspirin recurrent stroke study
Chapter I

Introduction
Introduction

1.1 General Immunology

1.1.1 Innate and adaptive immunity
The English word Immunity comes from the Latin term *immunis* meaning “exempt”, which developed from the observation that individuals who had recovered from certain infectious diseases were thereafter protected from that disease (1). Immunity has both less specific and more specific components in order to protect against invading pathogens. The less specific component, innate (or ‘natural’) immunity provides the first line of defence against infection. The innate immune response is a broad response primarily carried out by phagocytic cells, such as neutrophils, monocytes and macrophages, cells that release inflammatory mediators, such as basophils, mast cells and eosinophils, and natural killer cells. The molecular components of the innate immune response include complement, acute-phase proteins and cytokines such as the interferons (IFN) (2, 3). The specific component of the immune response, adaptive (or ‘acquired’) immunity, does not come into play until some time after the initial exposure to an antigen. This adaptive immune response however reacts to an antigenic challenge with a high degree of specificity as well as the remarkable property of memory. Adaptive immune responses involve the proliferation of antigen-specific B and T cells, which occurs when the surface receptors of these cells bind to antigen. Specialised cells, known as antigen presenting cells (APC), display the antigen to lymphocytes and collaborate with them in the response to antigen. Innate and adaptive responses usually work together to eliminate pathogens (4).

1.1.2 Key components of the adaptive immune system

1.1.2.1 B lymphocytes
B lymphocytes, or B cells, are produced and mature within the bone marrow. When they leave the bone marrow each expresses a unique antigen-binding receptor, or B cell receptor (BCR) on its membrane. This BCR is a membrane bound antibody molecule. When a naïve B cell first encounters an antigen that matches its BCR the binding of the antigen to the antibody causes the cell to divide rapidly; its progeny differentiate into memory B cells and effector B cells called plasma cells. Memory B cells have a longer life span than naïve cells and they express the same membrane bound antibody as their parent B cell. Plasma cells produce the antibody in a form that can be secreted and have little or no membrane-bound antibody. When an antigen is coated with an antibody it
can be eliminated in several ways. For example, antibody can cross link several antigens, forming clusters that are more readily ingested by phagocytic cells. Binding of antibody to antigen on a microorganism can activate the complement system, resulting in lysis of the foreign organism. Antibody can also neutralise toxins or viral particles by coating them, which prevents them from binding to host cells (1, 4, 5).

Antibodies are glycoproteins that consist of two identical heavy polypeptide chains and two identical light polypeptide chains. Each heavy chain is joined to a light chain by disulfide bonds and additional disulfide bonds hold the two pairs together (6) (Figure 1.1). The amino-terminal variable ends of the pairs of heavy and light chains form a cleft within which antigen binds. The C terminal domains of the heavy and light chains form the constant regions, which define the class and subclass of the antibody. The amino acid sequence of the constant region of the heavy chains specifies five classes of immunoglobulins (Ig) (IgG, IgA, IgM, IgD and IgE), four subclasses of IgG and two subclasses of IgA. These classes and subclasses have different functions, outlined in Table 1.1 (1, 4, 5).
Figure 1.1: Antibody structure

Diagram illustrates the structure of an antibody, highlighting the constant region, which determines the class of the antibody and the variable domains, which bind to antigen.

<table>
<thead>
<tr>
<th>Class</th>
<th>Subclass</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>IgA</td>
<td>α1, α2</td>
<td>Accounts for 10-15% of total serum Ig. The predominant Ig class in external secretions such as breast milk, saliva, tears and mucus of the bronchial, genitourinary and digestive tracts. IgA thereby serves an important effector function at mucous membrane surfaces, which are the main entry sites for most pathogenic organisms.</td>
</tr>
<tr>
<td>IgD</td>
<td>None</td>
<td>Constitutes 0.2% of total serum Ig. No biological effector function has been identified for IgD however, together with IgM it is the major membrane bound Ig expressed by mature B cells.</td>
</tr>
<tr>
<td>IgE</td>
<td>None</td>
<td>Found in very low levels in serum they mediate the immediate hypersensitivity reactions that are responsible for the symptoms of hay fever, asthma, hives and anaphylactic shock.</td>
</tr>
<tr>
<td>IgG</td>
<td>γ1, γ2, γ3, γ4</td>
<td>Constitutes 80% of total serum Ig. IgG antibodies are produced by B cells when the body is attacked by the same microorganism in a subsequent invasion i.e. part of the adaptive immune response. IgG serves to activate the complement system and is frequently involved in opsonisation. It is the only Ig that can cross the placental barrier.</td>
</tr>
<tr>
<td>IgM</td>
<td>None</td>
<td>Accounts for 5-10% of total serum Ig. IgM is the first Ig class produced in a primary response to an antigen and is the first Ig synthesised by a neonate. Can also activate complement.</td>
</tr>
</tbody>
</table>

Table 1.1: Immunoglobulin class, subclass and function

Table displaying the class, subclasses and function of the 5 different immunoglobulins (Ig) produced by humans.
Antibodies produced in a response to a particular antigen are heterogeneous. Most antigens are complex and contain a number of different antigenic determinants, so the immune system usually responds by producing antibodies to several sites, also known as epitopes, of an antigen. This response requires the recruitment of several clones of B cells. The output of one B cell clone is monoclonal antibodies, each of which have the same antigenic specificity. Monoclonal antibodies from each B cell clone together make up the polyclonal and heterogeneous serum antibody response to a particular antigen (1).

1.1.2.2 T lymphocytes
T lymphocytes, better known as T cells, similar to B cells are produced in the bone marrow, but unlike B cells, T cells migrate to the thymus to mature (7). During maturation the T cell develops to express a unique antigen binding molecule on its membrane: the T cell receptor (TCR). Unlike B cells that can recognise antigen alone, TCRs can only recognise antigen that is presented to the T cell bound to cell membrane proteins called major histocompatibility complex (MHC) molecules. There are two types of MHC molecules; class I MHC molecules which are expressed by nearly all nucleated cells and class II MHC molecules which are only expressed by APCs. When a naïve T cell encounters antigen combined with a MHC molecule on a cell, the T cell proliferates and differentiates into memory or various effector T cells (1, 4, 8).

There are two well defined subpopulations of T cells: T helper (Th) cells and cytotoxic T lymphocytes (CTLs) which are distinguished from one another by the presence of either cluster of differentiation (CD)4 or CD8 membrane glycoproteins on their surfaces. T cells that express the membrane glycoprotein molecule CD4 are restricted to recognising antigen bound to class II MHC molecules, whereas T cells expressing CD8 are restricted to recognition of antigen bound to class I MHC molecules. However, the classification of CD4 MHC class II restricted cells as Th cells and CD8 MHC class I restricted cells as CTLs is not absolute, there can be ambiguous functional activities (1, 4).

Several linages of Th cells can differentiate from naïve CD4 T cells, defined by their pattern of cytokine production and function, including Th1, Th2, Th17 and induced regulatory T cells (iTreg). Other types of CD4 T cells have been recognised, including natural killer T cells (NKT) and natural Tregs (nTregs). These cells are not derived
from naïve T cells that give rise to Th1 and Th2 cells, but develop in the thymus, distinct from the cells undergoing parallel thymic differentiation to become the naïve “conventional” CD4 T cell that are progenitors of Th1 and Th2 cells (9).

1.1.2.3 Antigen presenting cells

APC are specialised cells, which include macrophages, B lymphocytes and dendritic cells. These cells are distinguished by two properties; firstly they express class II MHC molecules and secondly they are able to deliver a co-stimulatory signal that is necessary for CD4 T cell activation. APC first internalise antigen, either by phagocytosis or by endocytosis, and then display a part of that antigen in their membrane bound to a class II MHC molecule. The CD4 T cell recognises and interacts with the antigen-class II MHC molecule complex on the membrane of the APC. An additional co-stimulatory signal is then produced by the APC leading to activation of the CD4 T cell (1).

1.1.3 Toll like receptors

The receptors of innate immunity recognise broad structural motifs that are highly conserved within microbial species but are generally absent from the host, known as pathogen associated molecular patterns (PAMP). These PAMP are recognised by host receptors known as pattern recognition receptors (PRR), one of these PRR being the toll-like receptors (TLR) (1), which are a large focus of my PhD. TLRs are type I transmembrane proteins that are evolutionarily conserved between insects and humans. Of the 10 TLRs known to exist in humans, TLR2 heterodimerises with either TLR1 or TLR6 and recognises bacterial lipopeptides. Other TLRs homodimerise, with TLR3 recognising synthetic or viral double-stranded (ds)RNA, TLR4 recognising gram-negative bacterial lipopolysaccharides (LPS), TLR5 recognising bacterial flagellin and TLR9 recognising unmethylated cytosine pairing guanine containing DNA motifs (CpG DNA), which occur in bacterial and viral DNA. TLR7 and TLR8, recognising single-stranded (ss)RNA, have been shown to dimerise with each other or TLR9 (10, 11). No ligand has been identified for human TLR10, although it appears to be a functional receptor (12). TLRs are expressed by various immune cells, including macrophages, dendritic cells and even on non-immune cells such as trophoblasts (13) and endothelial cells (EC) (14). Expression of TLRs is not static but is modulated rapidly in response to pathogens, a variety of cytokines or environmental stresses. Furthermore, TLRs may be expressed extra- or intra-cellularly: certain TLRs (TLRs 1, 2, 4, 5 and 6) are expressed
on the cell surface while others (TLR 3, 7, 8, and 9) are found almost exclusively in intracellular compartments such as endosomes (Figure 1.2) (10, 11).

TLR signalling involves a family of five TLR adaptor proteins, which couple to downstream protein kinases that ultimately lead to the activation of transcription factors such as nuclear factor kappa B (NFκB) and members of the IFN-regulatory factor (IRF) family. The key signalling domain is the toll/interleukin-1 (IL-1) receptor (TIR) domain, which is located on each TLR and their adaptors (15). There are five known TLR adaptor proteins: myeloid differentiation primary-response gene 88 (MyD88), MyD88-adaptor-like (MAL), TIR-domain-containing adaptor protein inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM) and sterile α- and armadillo-motif-containing protein (SARM). All TLR adaptor proteins couple to downstream protein kinases that ultimately lead in the positive regulation of transcription factor activation except SARM, which inhibits TRIF mediated activation (11). The TLR and adaptor proteins are illustrated in Figure 1.2. MyD88 is the most extensively studied of the adaptor proteins. It is the integral signalling adaptor for all TLR other than TLR3 and some TLR4 signals (11). TRIF is the sole adaptor protein for TLR3 signalling in response to dsRNA, it is also involved in TLR4 signalling by coupling through the bridging adaptor TRAM (16). The activation of two different adaptor protein pathways (MyD88 and TRIF/TRAM) in TLR4 signalling is considered to be related to the induction of early (MyD88) and late (TRIF) signalling pathways, culminating in the activation of the transcription factor NFκB (17).

The adapter protein MyD88 was first named in 1990 (18) and by 1997 its function was uncovered, with the crucial evidence coming from MyD88 deficient mice (19, 20). These mice were shown to be profoundly unresponsive to ligands for TLR2, TLR4, TLR5, TLR7 and TLR9. The second adaptor protein to be discovered was MAL (21, 22). MAL is required for signalling by TLR2 and TLR4 serving as a bridge to recruit MyD88. Again, MAL-deficient mice studies have shown the importance of MAL in TLR2 and TLR4 signalling (23, 24). MAL was initially thought to control the TLR4-mediated MyD88-independent pathway leading to IRF-3 and delayed NFκB activation. The discovery that MAL was in fact a bridging adaptor in the MyD88 dependent pathway left a gap in the understanding of how TLR4 could mediate IFN production. TRIF was then discovered and identified to control the TLR4-induced MyD88 independent pathway and also be the exclusive adaptor used by TLR3. Similar to
MAL, TRIF was identified by database screening for TIR-domain-containing-proteins (25) and also independently by a yeast two-hybrid screen with TLR3 (26). TRIF-deficient mice were impaired in TLR3 and TLR4 induced IFN-β production and activation of IRF-3 (16). TRAM was the fourth adaptor protein to be identified using bioinformatics, followed by a combination of overexpression studies, dominant-negative mutant analysis, interaction studies with TRIF and studies in TRAM deficient cells. The results of these studies identified that TRAM functions exclusively in the TLR4 pathway, acting as a bridging adaptor for TRIF recruitment (11). Finally, SARM was the last of the adaptor proteins to be assigned a role in TLR signalling. It was recognised by Liberati et al. (27) that human SARM, in contrast to the other four adaptor proteins, did not induce NFκB activation when overexpressed. It has now been identified that SARM is a negative regulator of NFκB and IRF activation. Its expression has been shown to block TRIF-dependent transcription factor activation and gene induction without affecting the MyD88 dependent pathway (28). The mechanisms by which SARM inhibits TRIF function remain unclear (11).
Figure 1.2: Overview of the TLRs and their adaptor proteins
A schematic diagram showing the TLR and TLR adaptor protein signalling cascade. Abbreviations: CpG DNA, unmethylated cytosine pairing guanine containing DNA motifs; dsRNA, double stranded RNA; LPS, lipopolysaccharide; MyD88, myeloid differentiation primary-response gene 88; MAL, MyD88-adaptor-like; TLR, toll-like receptor; TRIF, toll/interleukin-1 receptor-domain-containing adaptor protein inducing interferon-β, TRAM, TRIF-related adaptor molecule; SARM, sterile α- and armadillo-motif-containing protein; ssRNA, single stranded RNA.
1.1.4 Autoimmunity
Autoimmunity is the failure of an organism to recognise its own constituent parts as self, which allows an immune response against its own cells and tissues. Any disease that results from such an aberrant immune response is termed an autoimmune disease. Human autoimmune diseases can be divided into organ specific and systemic diseases. The organ specific diseases involve an autoimmune response directed primarily against a single organ or gland. Examples include Hashimoto’s thyroiditis and insulin-dependent diabetes mellitus. The systemic diseases are directed against a broad spectrum of tissues and have manifestations in a variety of organs resulting from cell mediated responses and cellular damage caused by autoantibodies or immune complexes. Examples include systemic lupus erythematosus (SLE), rheumatoid arthritis and the antiphospholipid syndrome (APS) (1).

Autoimmune diseases, such as insulin-dependent diabetes mellitus, are major causes of morbidity and mortality throughout the world. Many of the diseases are complex and tend to be difficult or impossible to cure, due to the fact that the focus of the immune response – self-antigens – cannot be eliminated. Understanding the activation of various different cell types in the autoimmune disease APS has been the focus of my PhD thesis.

1.2 The Antiphospholipid Syndrome
The APS is a systemic autoimmune disorder clinically characterised by vascular thrombosis (VT) and/or pregnancy morbidity (PM) (29). The syndrome is now recognised as the most common cause of acquired hypercoagulability in the general population (30) and the most important treatable cause of recurrent miscarriage (31). APS is caused by the persistent presence of pathogenic antiphospholipid antibodies (aPL), detectable by the anticardiolipin (aCL) and/or anti-β2-Glycoprotein I (anti-β2GPI) and/or lupus anticoagulant (LA) assays (32). β2GPI is a protein of approximately 53 kDa, composed of 5 protein modules, which are termed domains I through to V, with domain V being responsible for binding anionic phospholipid (33-35). APS was first described in patients with SLE; however, it has now been established that it may develop independently from any underlying disease and is then called primary (P)APS (36). When the syndrome occurs in the presence of an
underlying autoimmune disease (most commonly SLE) the associated disorder is reported, such as SLE/APS (32). The most severe and fortunately infrequent form of APS is catastrophic (C)APS. CAPS represents less than 1% of all patients with APS and is characterised by widespread small vessel thrombosis with multi organ failure. It is a life-threatening situation as patients have a 50% mortality rate (37, 38).

1.2.1 Classification criteria of the APS

The preliminary classification criteria for the APS were proposed in 1998 at an international workshop in Sapporo, Japan (39). The purpose of these criteria are to define the essential features of the APS, to facilitate studies of treatment and causation. It was hoped that the preliminary classification criteria would be tested in prospective multicentre studies so that modifications or additions to the criteria could be made at subsequent workshops. The criteria outlined that definite APS is considered to be present in a patient when at least one clinical manifestation (as outlined in Table 1.2) together with a positive test for circulating aPL, including LA, aCL or both, which is detected at least twice, 6 weeks apart.

In 2006 the classification criteria were revised and updated (32) and the revised criteria are outlined in Table 1.2. Essentially, the clinical criteria remained unchanged; however, two important modifications to the laboratory criteria were made. The time between two positive laboratory tests was extended to 12 weeks to ensure the detection of persistent antibodies only; and anti-β2GPI (both IgG and/or IgM) were added to the laboratory criteria.
### Clinical Criteria

| Vascular Thrombosis | • One or more clinical episodes of arterial, venous or small vessel thrombosis, in any tissue or organ.  
|                     | • Thrombosis must be confirmed by objective validated criteria. |
| Pregnancy morbidity | • One or more unexplained deaths of a morphologically normal foetus at or beyond the 10th week of gestation, with healthy foetal morphology documented by ultrasound or examination of foetus.  
|                     | • One or more premature births of a morphologically normal neonate before the 34th week of gestation because of: eclampsia or severe pre-eclampsia defined according to standard definitions or recognised features of placental failure.  
|                     | • Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation, with maternal anatomical or hormonal abnormalities and paternal and maternal chromosomal causes excluded. |

| Laboratory Criteria | • The presence of LA in plasma on two or more occasions at least 12 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Haemostasis.  
|                     | • The presence of aCL of IgG and/or IgM isotype in the serum or plasma of the patient, present in medium or high titres (ie, above 40 G/MPLU or greater than the 99th percentile) measured by a standardised ELISA on two or more occasions at least 12 weeks apart.  
|                     | • The presence of the anti-β2GPI antibody of IgG and/or IgM isotype in the serum or plasma of a patient on two or more occasions at least 12 weeks apart, measured by a standardised ELISA. |

### Table 1.2: Clinical and laboratory criteria for the APS

This table outlines the clinical and laboratory classification criteria of the APS as defined in the ‘International consensus statement on an update of the classification criteria for definite APS’ (32).
Although these criteria help to define a homogeneous population for research studies some patients who appear clinically to have the APS may not meet the modified criteria. A retrospective analysis identified that out of 81 aPL-positive patients who met the 1999 Sapporo laboratory and clinical APS classification criteria, only 58% (n=47) met the 2006 revised criteria. Thirty-four (42%) patients did not fulfil the revised 2006 classification criteria because of the following new requirements: aCL medium-to-high titre cut-off $\geq 40$ GPLU (n=15); two positive aPL, but not within the recommended time frame of $>12$ weeks apart (n=2); and $>5$ year time gap between the aPL test and the clinical event (n=17) (40). A large retrospective study (described in the clinical manifestations section below) identified heart valve disease, livedo reticularis and thrombocytopenia as some of the most frequent clinical manifestations seen in a cohort of 1,000 APS patients (36). In the updated APS classification criteria however, these clinical manifestations are stated to be non-criteria clinical findings associated with aPL (32).

1.2.2 Clinical manifestations of the APS

There are two distinct hallmarks of the APS: VT, encompassing both venous or arterial thrombosis, and PM. The more common clinical manifestations of the APS are summarised in Table 1.3 (41).

<table>
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<th>Clinical Manifestations of the APS</th>
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<td><strong>Frequent</strong> (&gt;20% of cases)</td>
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*Table 1.3: Clinical manifestations of the APS*

This table highlights the more frequent clinical manifestations of the APS (36).

The largest study to date describing the key clinical manifestations of the APS was reported by Cervera *et al.* (36). This retrospective analysis of 1,000 APS patients (820 female and 180 male) was a multicentre study involving 20 tertiary referral university
hospitals from 13 European countries, all with substantial experience in the management of the APS. Of the 1,000 patients investigated 53.1% had PAPS, 47.1% APS associated with another autoimmune rheumatic disease (most commonly SLE in 36.2% of the patient total), and 0.8% CAPS. Within the cohort studied a number of different clinical manifestations were seen, the most common of which are highlighted in Table 1.3. Venous thrombosis was the most frequent manifestation with deep vein thrombosis (DVT) affecting 31.7% of those studied. Patients also presented with arterial thrombotic complications, the commonest being stroke (13.1%). Of the 820 women in the study, 590 experienced 1 or more pregnancies (1,580 pregnancies in total), with 437 of these women succeeding in having one or more live births. Using a cut-off point of 10 weeks gestation, out of the 1,580 total pregnancies, 560 resulted in early foetal loss, making it the most common foetal complication, and 267 in late foetal loss. Out of the 753 total live births 80 of these were premature births (at or before 34th week of gestation). Pre-eclampsia (9.5% of pregnant women), eclampsia (4.4%) and abruption placentae (2%) were the most common obstetric complications that occurred in the mother.

A number of comparisons were made by Cervera et al. (36) in their retrospective analysis. One of these comparisons was between primary APS and APS associated with SLE. They found that both groups had similar profiles, except that patients with APS associated with SLE had more episodes of arthritis and livedo reticularis and more frequently exhibited thrombocytopenia and leukopenia. They also compared differences related to gender. From their 5:1 ratio of females to males both groups had similar clinical profiles with the following exceptions. Female patients had more frequent episodes of arthritis, livedo reticularis and migraine; while male patients more frequently had myocardial infarction, epilepsy and arterial thrombosis in the lower legs and feet. A final comparison Cervera et al. made was between the age at onset of APS. They found that those patients with childhood-onset APS (before the age of 15) presented with more episodes of chorea and jugular vein thrombosis, whereas those patients with older-onset APS (after the age of 50) were more frequently male and had more strokes and angina pectoris (36).

These patients were prospectively followed up for five years to investigate the main causes of morbidity and mortality in the APS. During follow-up 20% of the patients developed APS-related clinical manifestations, of these, 16.6% experienced recurrent
thrombotic events despite treatment with either warfarin and/or aspirin in the majority (82%) of these individuals. The most common recurrent thrombotic event was stroke (2.4%). Other common recurrent thrombotic events included transient ischemic attacks (2.3%), DVT (2.1%) and pulmonary embolism (PE) (2.1%). Of 121 women with only obstetric APS manifestations at the beginning of the study, 3 (2.5%) developed a thrombotic event during the follow-up period. A total of 77 women on the study experienced one or more pregnancies (105 pregnancies in total), with 63 succeeding in having one or more live births (80 live births in total). Similar to the previous study, the most common foetal complications included early pregnancy loss, late pregnancy loss, premature birth and intrauterine growth restriction (IUGR). A total of 5.3% patients died during the study period with no statistical difference detected in the cause of death depending on the underlying disease (PAPS or SLE) or the treatment that the patient was receiving. The authors concluded from this study that patients with APS still develop significant morbidity and mortality despite current treatment. Although I believe this statement to be true the actual percentage of individual APS related clinical events in the follow-up study were small. For example DVT affected 31.7% in the initial study but there were only 2.1% experiences of DVT in the follow-up period. Despite that the initial study listed clinical manifestations from disease onset whereas the follow-up study was over a smaller 5-year time frame, these data would suggest that current treatment regimes are working well in the majority of patients; however, APS related clinical events still occur despite treatment.

Despite the two hallmarks of the APS; VT and PM, women who are aPL positive may experience VT only, some only PM whilst others may experience both VT and PM. The studies of Cervera et al. (36, 42) highlight the distribution of VT and PM within female patients with the APS. Of the 1,000 patients enlisted in this study 820 were females. Of the female cohort 121 patients (15%) presented with only foetal loss. It is unclear from the data presented how many women only experienced VT (36). Of the 121 women with only obstetric APS manifestations at the beginning of the study, only 3 (2.5%) developed a thrombotic event during the follow-up period (42), identifying that a number of APS patients have only obstetric manifestations. It has yet to be identified why some women with APS experience only VT and why some only experience PM.
1.3 Management of aPL positive patients

Prevention of both thrombosis and pregnancy loss is the major goal of therapy in patients with aPL. There can be two different clinical settings: patients with aPL who have had a clinical event and patients with aPL who have not had a clinical event (asymptomatic). From this the management of aPL positive patients can be split into three distinct groups: secondary prophylaxis of VT, secondary prophylaxis of PM and primary prophylaxis. Furthermore, I will also discuss the treatment of seronegative patients, that is patients who fulfil the clinical criteria of APS but do not have laboratory evidence of aPL.

1.3.1 Secondary prophylaxis of VT

The current recommendation for the prevention of recurrent thrombosis in persistently aPL positive individuals is long-term treatment with the anticoagulant warfarin (43). Long-term anticoagulation is widely used for secondary thromboprophylaxis in the APS due to the high risk of recurrent events after withdrawing treatment (44-46). Warfarin, administered orally, inhibits synthesis of vitamin K-dependent coagulation factors (factor II, VII, IX, X), as well as protein C and protein S (47). The International Normalised Ratio (INR) is used to express the intensity of anticoagulation therapy needed. The duration and intensity of warfarin treatment is a matter of debate. At the 13th International Congress on aPL 7 task forces were set up to discuss topics of interest in the APS among experts in the APS field. One of these task forces, set up to make evidence-based recommendations for the prevention and long term management of thrombosis in aPL positive patients, made recommendations for indefinite anticoagulant therapy in patients with definite APS and thrombosis to a target INR 2.0-3.0 (43). Indefinite anticoagulation therapy is recommended due to the evidence that the frequency and risk of recurrent thrombosis rises after withdrawal of anticoagulation therapy (48, 49). The APS task force recommend that patients with definite APS and arterial thrombosis should be treated with warfarin at an INR >3.0 or combined antiaggregant-anticoagulant (INR 2.0-3.0) therapy after an estimation of the patients risk of bleeding. Due to the low quality of evidence that exists supporting this intensity of warfarin in APS patients with arterial thrombosis, not all members of the task force agreed with this recommendation. In cases of first venous event with a low-risk aPL profile (isolated, intermittently positive aCL or anti-β2GPI at low-medium titres) and a known transient precipitating factor, the task force recommendations are that
anticoagulation therapy could be limited to 3-6 months, in line with the treatment of venous thrombosis in non-APS patients. The task force also recommend that patients with either arterial or venous thrombosis and aPL who do not fulfil criteria for APS be managed in the same manner as aPL-negative patients with similar thrombotic events (43).

There is much debate over the intensity and duration of anticoagulation therapy especially in the treatment of venous compared with arterial thrombosis. Two systematic reviews have tried to overcome these debates, by analysing the data from relevant trials, but with contradictory outcomes. Firstly a systematic review by Lim et al. (45) analysing results of randomised clinical trials (RCT) only, recommended that patients with aPL and venous thrombosis should be treated indefinitely with warfarin at a target INR 2.0-3.0. They also recommended treatment of patients with aPL and a first episode of stroke (arterial thrombosis) with moderate intensity warfarin (INR 1.4-2.8) or aspirin, with aspirin being preferred because of its ease of use and lack of need for laboratory monitoring.

Lim et al. (45) based their recommendation that patients with aPL and venous thrombosis should be treated indefinitely with warfarin at a target INR 2.0-3.0 on two RCTs, a Canadian trial by Crowther et al. (50) and a European trial by Finazzi et al. (51). Both studies compared high versus moderate intensity warfarin and came to the conclusion that high intensity warfarin is no better than moderate intensity warfarin in preventing recurrent thrombosis in APS patients. Close examination of these RCTs however, reveals certain limitations. For instance, Crowther et al. (50) enrolled 114 individuals in their study, 58 individuals were treated with moderate intensity warfarin (INR 2.0-3.0) and 56 individuals were treated with high intensity warfarin (INR 3.0-4.0). Eight of these 114 patients experienced recurrent thrombosis, 6 in the high intensity warfarin group and 2 from the moderate intensity warfarin group. It would be easy to assume from this finding that high intensity warfarin is no better, in fact worse than moderate intensity warfarin. Close examination however, of the INR of the patients with recurrent thrombosis in the high intensity warfarin group at the time of the clinical event reveals that 3 out of 5 patients had an INR <2.0 and one other patient had discontinued warfarin 137 days before the recurrent thrombosis. Furthermore, the INR of warfarin is difficult to maintain and patients in the moderate intensity warfarin group were at target INR range 71% of the time, but the patients in the high intensity warfarin
group were only in target INR range 40% of the time. In fact overall 43% of the time patients in the high intensity warfarin groups actual INR was below the target range of INR 3.0-4.0.

Equally the study of Finazzi et al. (51) has limitations. This study was similar to the previous study with patients randomised to be treated with either high intensity warfarin therapy (INR 3.0-4.0) (54 patients) or treatment with either moderate intensity warfarin therapy (INR 2.0-3.0) (52 patients) or aspirin alone (3 patients). This trial only reports mean INR levels during follow-up of these patients, which was 3.2 in the high intensity warfarin group and 2.5 in the moderate intensity warfarin or aspirin alone group. Recurrent thrombosis was observed in 6 patients given high intensity warfarin and 3 patients in the moderate intensity warfarin or aspirin alone group: this finding was not significant. More importantly this trial did not report the INR of patients who experienced recurrent thrombosis at the time of their event. Given that the mean INR reported was only 3.2 it is possible that at the time of the event the INR in the high intensity warfarin group was below the target range of 3.0-4.0.

The intensity of warfarin therapy is one debate, another controversy discussed by Lim et al. (45) in their systematic review is the treatment of arterial thrombosis. Their recommendations for the treatment of arterial thrombosis are based on one study, the Antiphospholipid Antibodies and Stroke Study (APASS) (52). This study is a prospective cohort study within a RCT: the Warfarin Aspirin Recurrent Stroke Study (WARSS) (53). Patients were classified into two groups based on the presence or absence of aPL and treatment by warfarin (INR 1.4-2.8) or aspirin for the prevention of recurrent stroke or death and these groups were compared. They concluded that there was no altered risk of thrombotic events in patients treated with warfarin compared with aspirin. In addition, they concluded that the presence of aPL did not predict an increased risk of subsequent vascular occlusion events. A substantial limitation however, of this study is that none of the patients included in APASS met the classification criteria for the APS (32) since only one test for aPL (either LA or aCL) was carried out on samples stored at the time of enrolment and persistence of aPL was not confirmed. Furthermore, baseline aPL immunoreactivity indicated 96.6% of patients selected had low or moderate titres of aCL and only 6.7% of aPL positive patients tested positive for both aCL and LA.
A different systematic review by Ruiz-Irastorza et al. (46) released after the review by Lim et al. (45) took a different approach and included observational studies as well as the RCTs reported by Lim et al. to increase the level of evidence, reviewing a total of 16 reports. Ruiz-Irastorza et al. (46) evaluated the data from cohort studies, sub-group analyses and RCTs, citing both the overall findings and limitations, including studies where patients do not fulfil the APS classification criteria. Similar to Lim et al., Ruiz-Irastorza and co-workers conclude that the two RCTs (50, 51) demonstrate no advantage of high-intensity anticoagulation (INR 3.0-4.0) versus standard anticoagulant treatment (INR 2.0-3.0) but highlighted the fact that both trials frequently failed to achieve target INR in the high-intensity treatment group and that in the study by Crowther et al. (50) 6 out of 8 events occurred while the INR was <3.0. Ruiz-Irastorza and colleagues (46) also highlight the limitations of the APASS study (52) pointing out that this study used patients who did not fulfil the APS classification criteria with only a single positive aPL test being confirmed, LA not being performed according to international recommendations and low titre aCL reported in a high number of patients. Ruiz-Irastorza et al. (46) combined the data of ten studies that measured the INR at the time of the recurrent thrombotic event and the data of three studies where thrombosis only occurred among patients who were not receiving anticoagulant treatment. They reported that 57% of recurrent thrombosis events occurred when patients were not taking any anticoagulant or anti-aggregant drug and an additional 15% of recurrent events occurred among patients that were taking aspirin only. The remaining 27% of recurrent events were seen in patients treated with warfarin, with 86% of these cases occurring when the actual INR at the time of the event was <3.0. This finding highlights their recommendation that patients with APS with arterial thrombosis and/or recurrent venous events should be treated with warfarin at an INR >3.0.

One major concern with increasing the INR of warfarin therapy is the risk of bleeding in these patients. Long-term high intensity anticoagulation carries a major risk of bleeding at a frequency of 2-3%, which is similar to the bleeding rates of patients without the APS but receiving anticoagulation therapy (45). Ruiz-Irastorza and colleagues (46) investigated bleeding complications in seven studies selected in their review where the INR was reported at the time of bleeding. Of 93 bleeding episodes (including both minor and major bleeding) 26% occurred when INRs were below 3.0 whereas the remaining 74% occurred when INRs were above 3.0. The proportion of major bleeding within both groups however, were similar: 20% when INRs were below 3.0 versus 29%
when INRs were above 3.0. They conclude that the risk of recurrent thrombosis is higher than the risk of major bleeding and the mortality rate associated with thrombosis is much higher than that secondary to haemorrhages (46). The increased risk of bleeding associated with warfarin treatment at an INR >3.0 was discussed by a task force at the 13th International Congress on aPL. They recommended that an estimation of a patient's bleeding risk should be performed before high-intensity anticoagulant or combined antiaggregant-anticoagulant therapy is prescribed (43).

Although I have no clinical experience, based on my reading, especially of the systematic reviews of Lim et al. (45) and Ruiz-Irastorza et al. (46), I believe that patients with APS with arterial thrombosis and/or recurrent venous events should be treated with warfarin at an INR >3.0. The RCTs carried out so far, examining warfarin intensities in APS patients with recurrent thrombosis, have several limitations, as highlighted above, making it difficult to accept their conclusions for recurrent thrombosis treatment. This finding is highlighted by the fact that when the data from cohort studies, sub-group analyses and RCTs were evaluated by Ruiz-Irastorza et al. (46) different recommendations for the treatment of recurrent thrombosis were made. More stringent RCTs comparing intensities of warfarin in recurrent thrombosis patients (both venous and arterial) where patients entirely fulfil the clinical criteria for definite APS and where patients INR are maintained within target range a greater percentage of the time would help to solidify the argument for treatment of APS patients with arterial thrombosis and/or recurrent venous events at an INR >3.0.

Another task force, the APS Clinical Research Task Force (CRTF), was developed at the 13th International Congress on aPL. This group discussed the limitations of APS clinical research and developed guidelines for researchers to help improve the quality of APS research. They listed some important clinical questions without evidence-based answers in the management of aPL-positive patients. Some of these questions are directed at answering the optimal therapy for the prevention of recurrent thrombosis and included: what should be the intensity of warfarin (INR 2.0-3.0 versus INR 3.0-4.0) in separate cohorts of APS patients with venous or arterial thrombosis. The APS CRTF also discussed the development of collaborative, large-scale, clinical research projects that will help generate evidence-based recommendations for prevention and management of aPL-related clinical events. Following this meeting the AntiPhospholipid Syndrome Alliance for Clinical Trials and InternationaNetworking
(APS ACTION) has been set up (54). The founding steering committee of APS ACTION is composed of 28 members from 20 international centres, with the number of APS ACTION members/centres increasing yearly. In early 2012 APS ACTION launched two collaborative international projects: 1.) A RCT of hydroxychloroquine in the primary thrombosis prevention of persistently aPL-positive but thrombosis-free patients without other systemic autoimmune disease and 2.) A web-based registry of aPL-positive patients with or without systemic autoimmune diseases, which will also include annual blood collection for aPL-testing and future basic science studies (54). I consider the setting up of APS ACTION to be an exciting opportunity, bringing together expertise in the field of APS across the globe, to further our understanding of APS and its management.

1.3.2 Secondary prophylaxis of PM
Current management of obstetric APS involves low-dose aspirin and/or heparin. Low-dose aspirin is often given preconception or at the time of a positive pregnancy test due to its beneficial effect on early stages of implantation (55). Heparin therapy is initiated early in the first trimester after the presence of a live embryo is shown by ultrasonography (41).

Pregnant patients with APS without having had a previous thrombotic event are classified into one of two groups for the benefit of treatment: (1) patients with three or more recurrent early (pre-embryonic or embryonic) miscarriage and no other feature of APS or (2) those with one or more previous foetal deaths (at more than 10 weeks gestation) or previous early delivery (at less than 34 weeks gestation) because of severe pre-eclampsia or placental insufficiency. These patients are treated with either: group (1) Low-dose aspirin alone or in combination with either low-dose unfractionated heparin (UFH) (5000-7500 IU) or low molecular weight heparin (LMWH) (at prophylactic doses), or group (2) low-dose aspirin plus either intermediate-dose UFH (7500-10000) or LMWH (at prophylactic doses). Patients with a history of thrombosis, irrespective of pregnancy history are treated with low-dose aspirin plus either UFH (adjusted to maintain the mid-interval activated partial thromboplastin time (aPTT) or heparin concentration (anti-Xa activity) in the therapeutic range) or LMWH (at therapeutic dose) (56).
Aspirin and/or heparin therapy post-partum is recommended in all women with APS, with or without previous thrombosis. Women with previous thrombosis are switched back to warfarin therapy after the patient is clinically stable after delivery (57). In patients with no previous thrombosis however, there is variation amongst experts with regard to post-partum treatment. The port-partum care of APS patients was discussed amongst an obstetric task force at the 13th International Congress on aPL. A pre-meeting survey found that among experts attending the 13th International Congress some experts recommended treating a patient who had been on low-dose aspirin only during pregnancy for 6-8 weeks post-partum; whereas others recommend long-term low-dose aspirin treatment. Similarly, the duration of continued UFH or LMWH treatment in patients treated with heparin during pregnancy varied among experts from several days to 6-8 weeks post-partum. Other experts recommend these patients be swapped from heparin to warfarin treatment post-partum. The task force agreed that due to the debate surrounding the postpartum care of APS patients without a history of thrombosis, with little evidence to guide care, they recommended the setting up of a registry of postpartum patients stratified according to the presentation with obstetric APS and laboratory criteria (58).

There is continuing controversy regarding the best treatment strategy for obstetric APS. The use of heparin (either UFH or LMWH) in combination with low-dose aspirin has been investigated by many different groups, predominantly in women with recurrent early miscarriage, with conflicting results. Some groups report that combination therapy with both heparin and low-dose aspirin significantly reduces pregnancy loss and results in higher birth rates (59, 60) compared to treatment with low-dose aspirin alone. Kutteh and co-workers, in a prospective single centre trial of 50 patients assigned to either low-dose aspirin (25 patients) or low-dose aspirin plus UFH (25 patients) treatment groups, found that more viable infants were delivered to patients treated with low-dose aspirin and UFH (80%) compared to those treated with aspirin alone (44%). A RCT by Rai et al. (60) reported that treatment with low-dose aspirin and UFH led to a significantly higher rate of live births (71%) than that achieved with low-dose aspirin alone (42%).

In contrast, reports by two other RCTs, both using LMWH, (61, 62) suggest that combination therapy of both heparin and low-dose aspirin is no better than low-dose aspirin alone. Farquharson and co-workers (61) randomised, before 12 weeks gestation,
98 aPL positive patients with a history of recurrent pregnancy loss to receive low-dose aspirin daily (47 patients) or low-dose aspirin plus LMWH (51 patients). No benefit in LMWH with low-dose aspirin treatment was seen with 13 pregnancy losses and 34 live births in patients treated with low-dose aspirin only (live-birth rate 72%) and 11 pregnancy losses and 40 live births in patients receiving low-dose aspirin and LMWH (live birth rate 78%). Similarly, Laskin et al. (62) identified no difference in live births between patients with a history of recurrent pregnancy loss of which 43 patients received low dose aspirin only (live-birth rate 79.1%) and 45 received low-dose aspirin and LMWH (live birth rate 77.8%). Only half of the patients in this study had aPL however, and so by definition the APS: divided between 20 patients receiving low-dose aspirin only and 22 patients receiving low-dose aspirin and LMWH. Although no difference in live birth rate was seen between aPL positive patients in the two treatment groups, the sample size in each group was too small for any conclusions regarding the significance of this observation to be made.

Overall, comparing the early trials that state heparin plus low-dose aspirin is better than low-dose aspirin alone (59, 60) to the later trials that state heparin plus low-dose aspirin is no better than low-dose aspirin alone (61, 62) treatment with heparin (either UFH or LMWH) plus low-dose aspirin identified similar live-birth rates 70-80%. It was treatment with low-dose aspirin alone that differed in the earlier compared to the later trials with live-birth rates of 40-50% in the earlier trials compared to live-birth rates of 70-80% in the later trials.

The obstetric task force at the 13th International Congress on aPL mentioned earlier, discussed why such differences in findings were reported between the use of heparin and low-dose aspirin compared to low-dose aspirin alone (58). The trials suggesting that heparin and low-dose aspirin are better than low-dose aspirin alone were published pre 2000 whereas the trials suggesting heparin plus low-dose aspirin are no better than low-dose aspirin alone were published post 2000. A common problem with both pre 2000 and post 2000 trials is that different assays for aPL detection and different definitions for positivity were used in each of the studies, with each one not fulfilling the current laboratory APS classification guidelines, that is at least two positive tests for a circulating aPL, including LA, aCL or anti-β2GPI (IgG or IgM), at medium or high titres detected at least 12 weeks apart (32). Another significant difference between the two sets of trials is that UFH were used in the early trials but LMWH in the two later
trials. Two studies have investigated whether UFH or LMWH is better in combination with low-dose aspirin. Noble et al. (63) reported no differences in pregnancy outcome when comparing UFH to LMWH, both in combination with low-dose aspirin whereas Stephenson et al. (64) reported that LMWH is more effective than UFH for the treatment of APS in pregnancy. However, both studies had relatively small patient sample sizes, 50 and 26 respectively.

A recent meta-analysis of RCTs assessed whether combination of heparin and low-dose aspirin are more effective than low-dose aspirin alone for aPL positive patients with recurrent pregnancy loss. Data from five trials (59, 61, 62, 65, 66), of which 4 have been described previously, involving 334 patients were analysed. The overall live birth rates were 74.27% in the combination treatment group and 55.83% in the low-dose aspirin alone group, concluding that combination of heparin and low-dose aspirin is superior to low-dose aspirin alone in achieving more live births in aPL positive patients with recurrent pregnancy loss (67). A limitation of this analysis is that the data from trials where UFH has been used have been analysed alongside trials where LMWH have been used. Despite this limitation I think the evidence described in this section for treatment of PM in the APS with heparin and low-dose aspirin is the most convincing.

1.3.3 Primary prophylaxis

Treatment of patients with aPL who have yet to have a clinical event requires consideration. Current guidelines for the treatment of non-SLE aPL positive individuals without prior thrombosis recommend long-term thromboprophylaxis with low-dose aspirin in those with a high-risk aPL profile, especially in the presence of other thrombotic risk factors, such as smoking, arterial hypertension, hypercholesterolemia and estrogenic oral contraceptive use, and in high-risk situations, such as surgery. High-risk aPL profile encompasses LA positive individuals, patients with triple positivity (LA + aCL + anti-β2GPI) and persistently positive aCL at medium-high titres. It is also recommended that patients with SLE and LA or isolated persistent aCL at medium-high titres receive primary thromboprophylaxis with hydroxychloroquine and low-dose aspirin (43, 68).

Researchers disagree whether low-dose aspirin should be prescribed in aPL positive patients in order to prevent the clinical manifestation of the APS. The Antiphospholipid Antibody Acetylsalicylic Acid (APLASA) study, a randomised 3-year prospective
controlled trial of aspirin versus placebo, indicated there was no benefit of aspirin for persons with moderate to high titre aPL but who had no prior thrombosis (69). A limitation of this study however, is the actual number of thrombotic events that occurred during the trial period was low. Of the 48 patients randomised to receive aspirin only 3 patients developed thrombosis (2 venous events and 1 arterial event) as compared to none of the 50 patients receiving placebo. Patients randomised to receive aspirin or placebo had similar aPL profiles, that is a high-risk aPL profile in 58% of patients in both groups, defined as aCL of IgG, IgM or IgA isotype ≥40 units tested on two occasions at least 6 weeks apart and/or positive LA test results. In a parallel nonrandomised observational study, out of 61 patients receiving aspirin 4 patients developed thrombosis (3 venous events and 1 arterial event) compared to none of 13 patients not receiving aspirin. In the observational study however, there were nearly five times as many patients observed receiving aspirin compared to those not receiving aspirin and more patients had a high-risk aPL profile that were receiving aspirin (70%) compared to those not receiving aspirin (54%). All patients that had a thrombotic event, whether in the randomised or observational study, had a high-risk aPL profile. Another limitation of this study is that the aPL tests used were not based on the updated APS classification criteria (32), that is anti-β2GPI antibodies were not tested for and the aCL was not tested at least 12 weeks apart.

In contrast, other studies show that low-dose aspirin is beneficial in the treatment of primary prophylaxis of the APS (70, 71). In a retrospective study of 103 asymptomatic aPL positive carriers taking aspirin (n=75) or not (n=28) Hereng and co-workers (70) conclude that aPL-positive SLE and autoimmune thrombocytopenia patients should take aspirin to prevent APS manifestations. Among the aPL-positive patients in this study 37 had SLE, 20 had prolonged aPTT, 19 had other connective tissue diseases (including rheumatoid arthritis, systemic sclerosis and Sjögren’s syndrome), 16 had autoimmune thrombocytopenia and 11 had diverse diseases. Nineteen patients experienced 24 APS related clinical manifestations during the 64 ± 24.7 months follow-up including, 12 arterial thrombosis, 9 venous thrombosis and 3 obstetrical events. Of the 19 patients who experienced an APS related clinical event 9 were taking aspirin (12% of the aspirin taking patients) and 10 were not taking aspirin (35.7% of the non aspirin taking patients). Of the patients with SLE 4 out of the 10 patients not taking aspirin (40%) developed thrombosis compared with 3 out of the 27 patients taking aspirin (11%). Of the patients with autoimmune thrombocytopenia 4 out of the 6
patients not taking aspirin (67%) developed thrombosis compared to 1 out of the 10 patients taking aspirin (10%). Limitations of this study include the fact that this study was not randomised, more patients were in the aspirin treated group than in the non aspirin treated group and patients receiving aspirin had higher frequencies of IgG and IgM aCL and therefore a higher risk of thrombosis. Despite the limitations this study supports the use of aspirin in aPL-positive patients, particularly in aPL positive patients with SLE and autoimmune thrombocytopenia, to prevent thrombotic events. In a retrospective study by Ruffatti and co-workers (71), analysing risk factors for a first thrombotic event in aPL carriers, 30 out of 370 patients developed a first thrombotic event during a 59.3 month (range 5-239 months) follow-up period. Only 8 of these events (26.7%) occurred during prophylactic treatment with either low dose aspirin or heparin. Primary prophylaxis administered for long intervals and during high-risk periods had the highest association with the prevention of thrombosis. Patients with hypertension and medium/high levels of IgG aCL and receiving prophylaxis had a 9.2% probability of developing a thrombotic event compared with 61% probability when no prophylaxis was being administered. Despite the retrospective and observational nature of this study it still highlights the protective effects of primary prophylaxis in aPL positive patients.

Overall, the most convincing evidence is that aspirin should be used for the prevention of thrombosis in aPL positive patients, especially in patients with a high-risk aPL profile or patients with underlying autoimmune diseases such as SLE. The evidence for the use of hydroxychloroquine however is lacking but this will hopefully be addressed by a RCT proposed by the newly formed APS ACTION group. Their primary objective is to determine the efficacy of hydroxychloroquine in the primary thrombosis prevention of 1,000 persistently aPL-positive but thrombosis free patients (age 18-60 years) with no other systemic autoimmune disease over five year study period. Secondary objectives include determining the thrombosis incidence rate, the effect of hydroxychloroquine on mortality rate and the effect of hydroxychloroquine on aPL profile (54).

1.3.4 Treatment of seronegative patients

The term ‘seronegative APS’ (SN-APS), first introduced by Hughes and Khamashta, has been devised to describe a subset of patients with clinical manifestations suggestive of APS who are persistently negative for the criteria APS assays that detect LA, aCL
and anti-β2GPI antibodies (72). A recent retrospective study has described the clinical manifestations in a series of patients with SN-APS and compared their characteristics with a subset of patients fulfilling both the clinical and laboratory criteria for definite APS. The authors assessed clinical manifestations of the APS in 87 patients who fulfilled the clinical and laboratory criteria for definite APS and 67 patients with SN-APS and found no significant differences in the frequency of thrombotic or obstetric morbidity between the two groups of patients (73). The question is why is it important to define these SN-APS patients and why does it matter? The answer relates to the treatment of these patients as patients who are aPL positive with the clinical features of APS are treated differently to patients without aPL but with similar clinical features of the APS, in order to prevent recurrent events. Both in SLE and rheumatoid arthritis seronegative subsets of patients have been recognised and so help to solidify the clinical observation of SN-APS.

1.3.5 New therapies

Despite the fact that warfarin, heparin and aspirin are the treatments of choice for the prevention of recurrent thrombosis and PM they have a number of disadvantages and side effects. For instance, long-term use of LMWH can cause osteoporosis and heparin-induced thrombocytopenia and also some patients dislike its administration via a subcutaneous injection (74). Long-term high intensity anticoagulation carries a major risk of bleeding at a frequency of 2-3% per year (45) and despite anticoagulation therapy, some patients may re-thrombose demonstrating a lack of efficacy (75, 76). Therefore, there is a need to find better treatments.

1.3.5.1 New use of old drugs

Several potential new therapeutic approaches for APS are emerging. Firstly, the potential to use drugs that are already available and have been used to treat other diseases, including other autoimmune diseases, such as statins, hydroxychloroquine and B-cell depletion therapies, are being explored. Statins are known to lower cholesterol and are used to treat patients with cardiovascular disease. The antithrombotic properties of statins in APS patients have also been explored. In cultured human EC (77), on monocytes isolated from APS patients (78) and in murine models (79) fluvastatin has been shown to inhibit tissue factor (TF) production induced by aPL. The increased adhesiveness of EC induced by β2GPI, shown by an increase in the number of monocytes adhering to EC and an increase in the expression of cell adhesion molecules
(CAMs) by EC, is also prevented by statins (80). In contrast the statin, pravastatin does not prevent the effects of aPL on human first trimester trophoblast cell function in vitro (81). In a pilot trial for fluvastatin 9 patients with APS were given 40 mg fluvastatin per day for 30 days. These patients given fluvastatin showed decreased serum concentrations of the inflammatory and thrombogenic mediators vascular endothelial growth factor (VEGF), soluble TF and tumour necrosis factor (TNF)-α (82). After further clinical trials statins may be prescribed to APS patients due to their antithrombotic effects. Despite their antithrombotic effects statins have a number of side effects including muscle pain, fatigue and abdominal pain. Statin drugs are also contraindicated in pregnancy because of teratogenicity, placental disruption (83, 84) and theoretical long-term foetal neurological damage (85). Statins may disrupt gonadal stem cell development in foetuses, potentially leading to later infertility or other problems (86).

Hydroxychloroquine, an antimalarial drug, has both antithrombotic and anti-inflammatory properties and is widely used in the treatment of patients with SLE, due to its beneficial effects on lupus activity and damage (87). A cross-sectional study of 133 patients of which 77 had definite APS and 56 were aPL positive without clinical manifestations of APS, reported that the probability of a thrombotic event decreased when patients with the APS or aPL positive (but not APS) SLE patients received hydroxychloroquine (88). The question of whether hydroxychloroquine should be used in primary thrombosis prevention will hopefully be answered by the international RCT of hydroxychloroquine launched by APS ACTION (54).

B-cell depletion therapy with the chimeric monoclonal antibody rituximab has been shown to be effective in a number of autoimmune diseases, such as rheumatoid arthritis and SLE. Rituximab works by depleting the immune system of B cells, thus preventing further production of pathogenic autoantibodies. Ioannou and co-workers (89) have shown that aCL can be significantly lowered by rituximab treatment in patients with SLE (n=7) and known persistent aCL positivity (at least 2 positive tests, more than 12 weeks apart). Six out of the 7 patients tested were depleted to undetectable levels of aCL post-rituximab, none of these patients however had APS and only had low to moderate levels of aCL at the beginning of the study (mean aCL 20.6 GPLU) (89). An open-label Phase II pilot study, known as the Rituximab Antiphospholipid Syndrome (RITAPS) trial has recently assessed the effectiveness of rituximab in aPL-positive
patients who do not have criteria manifestations of the APS as stated by the updated Sapporo classification criteria for definite APS (32). The number of patients with a complete response, a partial response, no response and recurrence for the clinical outcome measures at 24 weeks were as follows: for thrombocytopenia, 1, 1, 2 and 0 respectively, for cardiac valve disease, 0, 0, 3 and not analysed respectively, for skin ulcer, 3, 1, 0 and 1 respectively, for aPL nephropathy 0, 1, 0 and 0 respectively and for cognitive dysfunction 3, 1, 1 and not analysed respectively. The authors conclude that despite there being no substantial change in aPL profiles seen over the 12 month study period rituximab may be effective for controlling some but not all non-criteria manifestations of APS (90).

1.3.5.2 New anticoagulants

New oral anticoagulants which are rapidly absorbed and have been shown to be effective in the management of venous thrombosis are now available. Whereas LMWH and warfarin inhibit many targets within the coagulation cascade, the novel anticoagulants dabigatran and rivaroxaban function by directly inhibiting thrombin and factor Xa respectively, resulting in early termination of the coagulation cascade and also do not require laboratory monitoring (56). Both of these drugs have been tested in randomised, double-blinded clinical trials in patients with venous thromboembolism (91, 92); however, the efficacy and safety of these new oral anticoagulants remains unknown in the APS. Haematologists and rheumatologists from different centres in London have developed a prospective phase II/III RCT of rivaroxaban versus warfarin in patients with thrombotic APS, with or without SLE, known as the RAPS (rivaroxaban in APS) trial, which is due to start in 2013. The hypothesis of this trial is that in patients with thrombotic APS, who have had venous thromboembolism and are being maintained on warfarin at a target INR of 2.5, rivaroxaban can induce more predictable anticoagulation and therefore a greater sustained reduction in thrombin generation than would warfarin, with additional benefits to patients because there is no requirement for regular laboratory monitoring of anticoagulation (93).

1.3.5.3 Novel therapies

The decoy domain I (DI), is being developed as a novel therapeutic concept for blocking pathogenic aPL. $\beta_2$GPI is the most important serum cofactor used by aPL to bind anionic phospholipids. It is comprised of five domains, of which the N-terminal domain – DI – has emerged as the immunodominant region (94-96) (see Figure 1.3).
Figure 1.3: Structure of $\beta_2$GPI

This figure illustrates the structure of $\beta_2$GPI and its five domains including Domain I, the antibody binding domain and Domain V which binds to anionic phospholipids.
Two studies by de Laat and colleagues (95, 97) have demonstrated the clinical significance of anti-DI antibodies in APS patients who have anti-β2GPI antibodies. The first study showed anti-DI antibodies to be associated with thrombosis (predominantly venous) more than antibodies targeted to other domains of β2GPI (95). Furthermore, in a double-blinded multicentre study, 243 patients of 442 patients tested (55%) were identified to have anti-domain I antibodies, of which 83% had a history of thrombosis. Anti-DI antibodies were also found to be associated with pregnancy morbidity (97).

Ioannou and co-workers (98) have developed a bacterial based expression system to produce large amounts of DI. This DI binds purified IgG from patients with the APS better than disease and healthy control IgG in the solid phase and inhibits antibody-antigen interactions in the fluid phase (98). These authors have also identified a variant of DI with two amino acid substitutes at positions 8 and 9, called DI(D8S/D9G), that binds IgG from APS patients better than native DI in both solid and fluid phase in vitro assays (96). Both native DI and DI(D8S/D9G) abrogated in vivo aPL-mediated thrombosis in a dose-dependent manner, while at low concentrations of inhibitor, DI(D8S/D9G) was superior. DI also inhibited TF expression in ex vivo monocytes and expression of CAMS in ex vivo aortic EC. Ioannou et al. conclude, since DI does not affect the basal levels of thrombosis seen in this mouse model in the absence of aPL, that the anti-thrombotic effect observed is specifically due to the formation of aPL-DI complexes, which are subsequently cleared from the circulation (99). The work of Ioannou and co-workers supports the development of recombinant DI and potentially the high-binding mutant DI (D8S/D9G) as a very specific targeted inhibitor of pathogenic anti-DI antibodies in patients with APS who harbour such antibodies. This is now progressing through pre-clinical development as a novel targeted therapeutic for APS (100).

1.4 Antiphospholipid antibodies

The aPL are a family of autoantibodies that exhibit a broad range of target specificities and affinities, all recognising various combinations of phospholipids, phospholipid-binding proteins or both (101). The term aPL can be somewhat misleading. Initially, it was thought that the aPL bound directly to phospholipids. It is now known however, that most pathogenic aPL do not bind directly to phospholipids but proteins complexed
to anionic phospholipids (102, 103). These proteins include prothrombin, annexin V, protein S and C and β2GPI (104), a 53-kDa plasma glycoprotein which constitutes the major antigenic target for aPL circulating in the plasma of APS patients. Even though it is a misnomer the term aPL as a generic term persists when referring to antibodies that specifically target β2GPI or one of these other protein cofactors.

Not all aPL are pathogenic, aPL can be found in healthy adults and patients with infectious, malignant or drug-related disorders who do not go on to develop APS (105). It is now understood that there are two distinct populations of aPL; non-pathogenic aPL, which bind neutral or anionic phospholipid and do not require serum cofactors and pathogenic aPL, which occur in the APS and bind negative phospholipid, requiring the presence of serum cofactors such as β2GPI to do so (105).

1.4.1 How pathogenic aPL are detected
1.4.1.1 Current aPL assays
According to the revised classification criteria for the APS (32) there are three criteria assays that can be utilised to test for the presence of the following aPL; LA, aCL and anti-β2GPI assays. The presence of LA precipitates the formation of thrombi in vivo yet paradoxically prolongs clotting times in coagulation assays. LA is detected when a patient’s plasma causes prolongation of a phospholipid dependent clotting assay, such as the aPTT or diluted Russell’s viper venom test (dRVVT), which fails to correct upon mixing with healthy plasma. The LA test involves three sequential steps, defined in the International Society of Thrombosis and Hemostasis criteria (106, 107) as follows. Firstly, a screening test: to demonstrate presence of prolongation of a phospholipid dependent clotting time. Secondly, a mixing test: achieved by mixing 1:1 pooled normal and patient plasma. If prolongation of clotting time is the result of a coagulation factor deficiency the clotting time will correct to normal with the addition of normal plasma, whereas with LA, correction requires larger volumes of normal plasma. Finally, a confirmatory test: to confirm that the prolongation is phospholipid dependent (108).

Positivity to aCL and anti-β2GPI is tested in an enzyme-linked immunosorbent assay (ELISA). The aCL ELISA involves the assessment of diluted patient serum for binding to a CL-coated plate in the presence of bovine serum. As well as detecting antibodies that bind CL alone it will also detect antibodies that bind CL-bound bovine β2GPI. An
inherent weakness of this assay is that there remains potential to miss patients with antibodies that bind human but not bovine \(\beta_2\)GPI (108). A study by Arvieux et al. (109) found that out of 76 sera that tested positive to IgG and 64 sera that tested positive to IgM human or bovine \(\beta_2\)GPI antibodies, 43.7% of the IgM positive sera and 7.9% of the IgG positive sera reacted only to human but not bovine \(\beta_2\)GPI. Hence, some commercial assays now use human serum containing \(\beta_2\)GPI in the CL ELISA (108). IgG and IgM aCL are expressed in international standardised Immunoglobulin G Phospholipid Units (GPLU) and Immunoglobulin M Phospholipid Units (MPLU) respectively, derived using standardised polyclonal or monoclonal IgG and IgM aCL calibrators. These polyclonal calibrators are standardised and have been validated in different laboratories around the world. They are obtained by mixing various proportions of sera from IgG aCL high positive or IgM aCL high positive and pooled normal human serum (110).

The \(\beta_2\)GPI ELISA involves coating purified native \(\beta_2\)GPI directly onto an irradiated plate. There is however, a lack of a formal universally accepted method for performing this ELISA which coupled with a lack of standardised calibrators, has resulted in intra-assay variability. Effort has been made, nonetheless, to resolve this issue with discussions and recommendations of a ‘critical’ aPL test task force at the 13th International Congress on aPL (111, 112). At a pre-conference wet workshop the performance of different aCL and anti-\(\beta_2\)GPI ELISAs were tested. Aliquots of 26 unidentified APS serum samples and 21 controls (14 from healthy individuals and 7 from patients with infectious diseases) were given to participating groups to be tested in various aCL and anti-\(\beta_2\)GPI ELISAs. Despite the known problems with the anti-\(\beta_2\)GPI ELISA, the correlation of positive titres was shown to be excellent, with all healthy control samples correctly identified by all groups as negative. All aCL and anti-\(\beta_2\)GPI tests showed excellent clinical sensitivities, specificities and positive predictive values and good agreement with respect to the levels of the IgG and IgM antibodies, regardless of assay type or whether tests were done using automated or manual systems (111). This finding demonstrated that despite the lack of standardisation of the anti-\(\beta_2\)GPI assay, encompassing both the performance of the assays and a lack of universal units of measurement for anti-\(\beta_2\)GPI antibodies, the inter-assay variations were acceptable. With the collaborative work of this aPL task force and the development of an
international consensus guideline (112), standardisation will be improved in the near future.

A number of retrospective analyses have shown a strong correlation between triple positivity (LA, aCL and anti-β2GPI) and thrombosis and miscarriage, compared to patients who test positive to either two or only one assay (113-116). Pengo and co-workers showed that triple positivity was a strong independent risk factor for thrombosis related events, retaining its significance when only venous or arterial thromboembolism was considered, in a 6-year study period of 618 subjects referred to a Thrombosis Centre for aPL screening (115). A retrospective analysis of 600 women with recurrent pregnancy complications by Ruffatti et al. (116) identified 53 patients with APS obstetric manifestations and more than one APS laboratory criterion present (among LA, aCL and anti-β2GPI antibodies). During a mean follow-up of 6.3 years (range 0.5-15 years), patients positive for anti-β2GPI and aCL antibodies with no history of thrombosis who had experienced only obstetric manifestations of APS, suffered from no VT event and succeeded in bringing new pregnancies to term when treated with antithrombotic drugs. On the other hand, patients with obstetric and thrombotic manifestations and/or with triple positivity (LA, aCL and anti-β2GPI) showed frequent VT events in the follow-up period and a high percentage of unsuccessful new pregnancies (116). This study shows a clear association between triple positivity and poor clinical outcome.

1.4.1.2 Non-criteria aPL assays

Several autoantibodies have shown to be directed to negatively charged phospholipids other than CL, such as phosphatidic acid, phosphatidylinositol and phosphatidylycerine, or to other proteins of the coagulation cascade, such as prothrombin and/or phosphatidylycerine-prothrombin complexes. Autoantibodies have also been shown to be directed to DI of β2GPI or to interfere with the anticoagulant activity of annexin A5. All of these autoantibodies have been proposed to be relevant to APS (117). The clinical usefulness of these autoantibodies, the newly developed assays to detect them and their diagnostic value have not been fully characterised. A non-criteria aPL task force at the 13th International Congress on aPL discussed and analysed critical questions related to non-criteria aPL tests in an evidence based manner. After reviewing studies of these non-criteria aPL the task force acknowledged that several studies suggest non-criteria aPL may have clinical significance but the current level of evidence did not
warrant a change to the 2006 criteria for the diagnosis of APS. The task force highlighted the importance of these non-criteria aPL tests and their possible future use in APS diagnosis (118).

Although currently the aCL and anti-β₂GPI criteria assays detect either IgG or IgM antibodies there is emerging evidence that IgA antibodies are important, however they remain absent from the APS classification criteria. A recent systematic review investigated the prevalence of IgA aCL and anti-β₂GPI in patient populations and their association with APS clinical manifestations, as the sole antibody or in combination with other aPL. Out of 31 relevant articles selected 17 studies performed in an autoimmune population suggested usefulness of IgA aPL testing. They noted that only 6 out of 17 studies found a statistically significant association between different APS-related clinical manifestations and IgA aPL in the absence of other antibodies/isotypes. The authors conclude that based on the data published there is not enough evidence to recommend testing for IgA aCL and/or IgA anti-β₂GPI to increase the diagnostic accuracy of the APS and that prospective studies to establish the clinical significance of these antibodies are required (119).

1.4.2 Aetiology of aPL

There are a number of proposed mechanisms suggested to contribute to autoantibody production in the APS, however the exact mechanism for the generation of aPL remains unknown. Proposed mechanisms that are thought to contribute to aPL production, which will be discussed in this section, include impaired cell clearance, molecular mimicry and genetic factors.

**Impaired cell clearance**

One possible mechanism is the impaired cell clearance hypothesis. In SLE it has been proposed that there may be impaired clearance of apoptotic cells, which may predispose a genetically susceptible individual to mount an immune response against the molecules bound to the apoptotic blebs, leading to autoantibody production against these antigens (120). Similarly in the APS it has been suggested that β₂GPI and prothrombin may bind to exposed phosphatidylserine on apoptotic blebs. If impairment in apoptotic cell clearance occurs or there is an increase in the generation of apoptotic cells, this may predispose an individual to develop antibodies directed against the β₂GPI and prothrombin bound to the apoptotic blebs (120-122).
**Molecular mimicry**

Molecular mimicry between epitopes displayed by infectious agents, such as bacteria and viruses, that immunologically resemble host determinants is thought to contribute to the development of autoimmune disease. Once an immune response is induced to the pathogens epitope this immune response can cross-react with the host’s epitope, breaking tolerance to the host’s epitope. This enables cross-reactive T or B cells to induce a pathogenic autoimmune response, leading to disease (123).

The strongest evidence suggesting molecular mimicry drives aPL development comes from studies with antigens sharing sequence and/or structural similarity to $\beta_2$GPI. Gharavi et al. (124) demonstrated that TIFI, a 20-amino acid peptide derived from the sequence of cytomegalovirus and sharing sequence similarity to Domain V of $\beta_2$GPI, could induce aPL production in mice. Furthermore, some of these aPL were pathogenic in an experimental murine model of thrombotic APS. Blank and co-workers (125) identified 3 hexapeptides that specifically reacted with and inhibited in vivo properties of anti-$\beta_2$GPI antibodies and found a high homology of these hexapeptides with bacterial products in particular from *Haemophilus influenza*, *Neisseria gonorrhoea* and *Tetanus toxoid*. Mice immunised with microbial preparations related to one of these hexapeptides had the highest anti-$\beta_2$GPI and anti-peptide antibody levels compared to mice immunised with microbial preparations unrelated to this hexapeptide. Resulting anti-peptide antibodies were then able to induce experimental APS in murine model of pregnancy loss (126).

**Genetic factors**

Genetic studies have shown evidence in favour of a genetic link with the presence of circulating aPL or the APS. These studies however have small sample sizes so do not allow for firm conclusions to be drawn. A genetic inheritance of aPL was first suggested by Exner et al. (127) who described the presence of LA in two pairs of siblings. Mackworth-Young and co-workers (128) then discussed the higher incidence of aCL in 101 first-degree relatives of 22 patients with SLE compared to controls. A further study by Radway-Bright et al. (129) described the higher incidence of various IgG aPL in 25 first-degree relatives of 14 patients with APS and SLE compared to healthy controls.
A few family studies analysing genetic predisposition of aPL inheritance have looked at common patterns in human leukocytes antigen (HLA) alleles, which encode for a large proportion of genes involved in the immune system. A study of 14 members of an English-Canadian family, where one member had SLE and aPL and another had a history of thrombosis and aPL, deduced that aPL were associated with HLA haplotypes including class II antigens DR4, DRw53 and DQw3 (130). A family of identical twins and their mother, all of whom had SLE and different manifestations of the APS, all shared the same HLA-DR4; DRw53; DQw3 class II antigens (131). Asherson and colleagues (132) have reported an association with HLA-DR4 and DRw53 and the APS in 13 UK PAPS patients. Camps et al. (133) also identified similar findings in a study of 19 Spanish patients with PAPS. Genome-wide studies would be useful to better understand the genetic predisposition to developing APS.

1.4.3 Evidence of aPL interactions
There exists both clinical data, describing an association, and experimental evidence, showing a direct pathogenic effect between the presence of aPL and the clinical manifestations of thrombosis, venous or arterial, and recurrent foetal loss. The exact mechanisms however, through which aPL mediate thrombosis or foetal loss are yet to be clearly defined. This next section will highlight some of the key clinical and experimental evidence demonstrating the pathogenicity of aPL.

1.4.3.1 Association of aPL and thrombosis

Clinical evidence
Hughes and colleagues were the first, between 1983-86, to describe a clinical syndrome with widespread arterial and venous thrombosis, associated with antibodies directed against phospholipids (29, 134, 135). The association between aPL and both venous and arterial thrombosis is now widely accepted. A number of clinical studies identify a statistically significant association between raised aPL levels and venous thrombosis (49, 136, 137). Ginsberg et al. (136) reported a significant association between positive LA but not aCL (>30 GPLU) in 65 patients with a confirmed history of venous thromboembolism compared to 179 patients with a non-thrombotic event. The mean age of the individuals studied however was 55 years. This is problematic as aCL titres rise in asymptomatic controls after 50 years of age (138). The authors state that a lack of association with aCL in this study was due to patients without venous thromboembolism testing positive in this assay. Schulman et al. (49) compared the risk
of recurrent venous thrombosis in patients with and without aCL. Among 412 patients with a first episode of venous thromboembolism, who were treated for 6 months with anticoagulation therapy, the risk of recurrence after stopping anticoagulation therapy was 29% in patients with aCL and 14% in those without aCL. The risk of recurrence increased with aCL titre levels and there was an increased risk during the first 6 months after cessation of anticoagulation in patients with aCL. These data indicate that the presence of elevated titres of aCL 6 months after an episode of venous thromboembolism is a predictor for an increased risk of recurrence. Galli and co-workers (137) identified in a group of 57 patients with a previous history of either venous or arterial thrombosis and consistently positive LA test that aCL above 40 GPLU was strongly associated with venous thrombosis.

Evidence for the association of aPL and arterial thrombosis also exists (138-141). In a prospective study Vaarala et al. (139) compared 133 patients who developed a cardiac end point, defined as cardiac death or non-fatal myocardial infarction (MI), to 133 control subjects. No patients had coronary artery disease at the time of entry into this study. Patients who developed a cardiac end point had higher levels of aCL (measured in optical density units) as compared to control subjects. In a study of 524 patients with acute stroke and 1,020 controls Tuhrim and co-workers (140) identified that both aCL and anti-phosphatidylserine antibodies were independent risk factors for stroke. In a study of 374 men with a history of MI and 259 men with a history of stroke compared to 1,360 controls Brey and co-workers (138) showed a significant association between \( \beta_2 \)GPI-independent aCL and MI and \( \beta_2 \)GPI-dependent aCL and stroke. Finally, a study by Meroni et al. (141) reported a significant association of anti-\( \beta_2 \)GPI antibodies and MI when comparing 172 women with a history of MI with 172 women with no history of thrombosis. These authors also measured aCL titres but did not find an association with MI. However, the cut-off for positivity used in the aCL assay was low (>10 G/MPLU) allowing for the inclusion of false-positive tests in the analysis.

Other studies have investigated the association of aPL with both venous and arterial thrombosis (142-145). A prospective study in 360 unselected aPL positive patients in an Italian cohort found that high aCL titre (>40 GPLU) as well as previous thrombosis were independent predictors of arterial or venous thrombosis (142). They did not however identify the LA test as a predictor for recurrent thrombotic events. This may have been because LA testing was not uniform between patients and strict International
Society of Thrombosis and Hemostasis criteria were not followed. In a meta-analysis Galli and co-workers (143) assessed the association of LA and aCL with thrombosis. They found that out of 25 prospective studies, LA was a strong risk factor for thrombosis (both venous and arterial, first event or recurrent). aCL was not found to be as strong a risk factor as LA unless medium or high aCL titres of the IgG isotype were considered. In a further study Galli and co-workers (144) identified the presence of anti-β₂GPI antibodies were significantly associated with any thrombosis (when no distinction was possible between venous or arterial) in 17 of 22 studies examined, with venous thrombosis in 12 of 21 studies and arterial thrombosis in 5 of 17 studies. Forastiero et al. (145), in a study of 194 patients with persistent aPL (LA and/or aCL), 119 of which had APS, followed up for a median period of 45 months, identified IgG anti-β₂GPI antibodies (p=0.001) and anti-prothrombin antibodies (p=0.019) as risk factors for recurrent venous or arterial thrombosis. Patients with positive LA and anti-β₂GPI antibodies or anti-prothrombin antibodies had a significantly higher risk of thrombosis (p=0.08 and 0.004 respectively), however the presence of LA alone was not a predictor of thrombosis.

A ‘two hit hypothesis’ has been suggested to explain the clinical observation that thrombotic events occur only occasionally in spite of the persistent presence of aPL. According to this principle the antibody (representing the first hit) induces a thrombophilic state, but clotting only takes place in the presence of another thrombophilic condition (the second hit). It has been suggested that infectious processes might constitute the second hit, as in some cases they precede full-blown APS (146).

**Experimental evidence**

The pathogenic mechanisms that are potentially responsible for thrombus formation have been demonstrated using both *in vivo* and *in vitro* models. The *in vivo* models published in mice, hamsters and rats have confirmed that aPL are able to increase both venous and arterial thrombus formation (147-149). The most extensively utilized is the *in vivo* murine model of thrombosis developed by Pierangeli and co-workers (147). This murine model of thrombosis was first used to show that infusion of polyclonal IgG isolated from patients with the APS promotes the development of thrombosis. CD1 mice were treated by intraperitoneal injection of IgG from APS patients (number = 17), normal pooled sera (number = 9) or saline solution (number = 12), followed by a
subsequent injection of the same preparations 48 hours later. The mice were anesthetised 72 hours after the first injection and the right femoral vein was exposed and a standardised mechanical pinch injury performed to induce the formation of a thrombus. Clot formation and dissolution in the transilluminated vein was visualised with a microscope equipped with a closed-circuit video system. Thrombus size was measured after the pinch injury by freezing the digitised image and tracing the outer margin of the thrombus; the times (in minutes) of formation (from appearance to maximum size) and disappearance (from maximum size to disappearance) of the thrombus were also measured. Results showed that average clot size was significantly larger in mice immunized with IgG from APS patients compared to those treated with saline. In addition, the thrombus persisted longer in a significantly higher number of mice immunized with IgG from APS patients (10/17) compared to mice immunized with normal pooled sera (1/9) or saline (2/12). This murine model of thrombosis has been extensively used by these authors to not only show that polyclonal aPL (150-152) but also to show that patient derived monoclonal aPL (153) induce thrombus formation in vivo.

Jankowski et al. (148) investigated the ability of three murine monoclonal antibodies directed against human β2GPI, which display cross-reactivity with hamster β2GPI, to promote thrombus formation using photochemically induced arterial vessel damage in a hamster. In short, male hamsters were anesthetised and a venous catheter inserted into their right jugular vein. The left carotid artery was mounted on a transilluminator, following which monoclonal antibodies were injected into the venous catheter. Next the dye rose-bengal was injected into the hamster and the exposed artery was irradiated for 2 minutes with green light. Quantification of thrombi in the hamster carotid artery was performed using a camera mounted on a microscope and a transmitted light intensity versus time curve was made. The murine monoclonal antibody 5H2, which has LA activity, promoted thrombus formation dose dependently. The monoclonal antibody 11E8, which has only aCL properties, and the control monoclonal antibody 27A8 did not significantly promote thrombus formation. In a second set of experiments the authors compared intact 5H2 to its F(ab’)2 and Fab’ fragments. Intact 5H2 and its F(ab’)2 fragment promoted thrombus formation equally well; however, the 5H2-derived Fab’ fragments were inactive. The authors concluded that bivalent immune complex formation plays an important role in the generation of arterial thrombosis by certain aPL but cellular activation via the Fc portion of these immune complexes is not essential.
Fischetti et al. (149) have identified that passive infusion of human aPL IgG together with a small amount of LPS triggers clotting in a rat mesenteric microcirculation. These authors examined the number of intravascular platelet-leukocyte aggregates and thrombotic occlusions using intravascular microscopy in rats injected with aPL IgG purified from patients with APS (6 APS patients in total) with or without pre-treatment 3 hours before with LPS. Results showed that injection with APS IgG alone did not promote a procoagulant effect but pre-treatment with LPS caused rapid endothelial deposition of fibrinogen, followed by platelet-leukocyte aggregates and thrombotic occlusions when compared to effects caused by IgG purified from healthy controls, indicating that a proinflammatory stimulus is required for an aPL-dependent procoagulant effect. When the authors treated rats with anti-β2GPI depleted aPL IgG failed to induce platelet-leukocyte aggregates and thrombotic occlusions indicating that both antibodies to β2GPI and a proinflammatory stimulus is required for a procoagulant effect to be stimulated in rats.

*In vitro* models have been used to demonstrate that aPL interact with and activate EC, monocytes and platelets resulting in thrombus formation. Studies have shown that EC express significantly higher amounts of the CAMS intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin when incubated with aPL antibodies and β2GPI *in vitro* (154). Similarly, the incubation of EC with antibodies reacting with β2GPI has been shown to induce EC activation with upregulation of CAMS, IL-6 production and alteration in prostaglandin metabolism (155). Evidence of up-regulation of TF by aPL in both EC and monocytes has been reported by several investigators (156). Activated platelets increase expression of glycoprotein 2b-3a and synthesis of thromboxane A2 (156). *In vitro* effects of aPL on thrombotic cell types will be discussed in detail later in sections 1.5 and 1.6.

1.4.3.2 Association of aPL and PM

*Clinical evidence*

In pregnancy aPL have been implicated as the cause of IUGR, preeclampsia, preterm delivery and foetal death as well as pregnancy loss in any trimester and placental alteration in the third trimester (157). Approximately 15-20% of all women with recurrent miscarriage are aPL-positive and when no treatment is given these women have a live birth rate as low as 10% in subsequent pregnancies (158). Considerable
clinical evidence exists supporting the idea that aPL are significantly associated with adverse pregnancy outcome (159-162).

A prospective study by Polzin et al. (159) concluded a statistically significant association between the presence of circulating maternal aCL and IUGR. In this study 55 pregnant women with suspected IUGR were followed throughout their pregnancy, with 37 new-borns confirmed by birth weight to be at or below the tenth percentile and all 55 new-borns below the 45th percentile. Fifteen of the 55 women (27%) were positive for aCL, as were 9 of the 37 (24%) with correctly diagnosed IUGR. None of these patients however tested positive for LA. In another prospective study, Lynch and co-workers (160) investigated the association between the presence of aPL at the first prenatal visit and adverse pregnancy outcome in healthy pregnant women. They found that aPL positivity at first prenatal visit was significantly associated with foetal loss. Of the aPL positive patients (n=95) 15.8% had foetal loss compared with 6.5% of aPL negative women (n=294), indicating an association between aPL positivity and PM. The aPL measured included aCL (IgG, IgM and IgA) and LA however, the only single aPL test that was significantly associated with foetal loss was IgG aCL. Yasuda et al. (161) again in a prospective study, measured aCL activity and found a strong association with spontaneous abortion (25% of aCL-positive women (n=60) versus 9.8% of aCL-negative women (n=800)), preeclampsia (11.7% versus 1.9%) and IUGR (11.7% versus 1.9%). In a retrospective study Yetman and colleagues (162) identified that 17.3% of women with recurrent pregnancy loss (n=866) tested positive for aCL compared to only 4% of control women who have borne one or more viable offspring (n=288), while 10.1% of women with a history of foetal loss carried antibodies against other anionic PL, such as phosphatidylinositol. All aPL identified were more frequently of the IgG isotype.

**Experimental evidence**

Several different animal models have shown that infusion of monoclonal or polyclonal aPL induces foetal loss and/or growth retardation (163, 164, 65, 66). The first reported animal model of APS features was in the MRL/lpr autoimmune strain of mice, which carry the lymphoproliferative gene (lpr) (163). After the age of two months, MRL/lpr mice developed high levels of autoantibodies including IgG aPL, in association with thrombocytopenia and reduced litter size.
Animal models in which APS has been induced in normal mice by infusion with polyclonal IgG from APS patients include the work by Branch and colleagues (164), Blank and colleagues (165) and Bakimer and colleagues (166). Branch and colleagues (164) investigated whether purified IgG from patients with aPL and a history of foetal loss caused foetal loss in pregnant mice. On day 8 of pregnancy BALB/c mice were given an intraperitoneal injection of IgG. Mice treated with APS IgG aborted within 48 hours but mice injected with control IgG did not. The clinical relevance of these data is indeterminable as the mice were injected with APS IgG after pregnancy was established. Patients with APS most likely will already have circulating aPL before pregnancy is established. Alternatively, Blank et al. (165) passively transferred either a murine monoclonal IgG aCL antibody or IgG and IgM polyclonal antibodies isolated from a patient with PAPS or control monoclonal and polyclonal antibodies into the tail vein of ICR mice. They found that mice infused with the monoclonal IgG aCL antibody had a higher rate of reabsorbed foetuses compared to mice infused with a control monoclonal antibody. Mice infused with IgG antibodies from a PAPS patient resulted in fewer vaginal plugs and a lower incidence of pregnancy compared to mice infused with control IgG. In addition, the mean weights of embryos and placentae were significantly smaller than those of controls in mice treated with IgG from the PAPS patient. These effects were also seen, but less impressive, in mice infused with IgM antibodies from a PAPS patient compared to IgM controls (165). Similarly, Bakimer et al. (166) immunised BALB/c mice with a human monoclonal aPL. Four-6 months following this, mice developed higher aPL levels, low fecundity rates, higher reabsorption index of foetuses and low weight embryos and placentae.

Animal studies have identified a role for complement in aPL-mediated pregnancy loss. A fully active complement system is needed to protect both mother and developing foetus from infectious and other toxic agents. When pregnant BALB/c mice were injected with polyclonal IgG from a patient with APS (characterised by multiple cerebrovascular accidents, livedo reticularis, LA and high titres of IgG aCL), an increased foetal reabsorption and deposition of C3 in the decidua compared to mice injected with healthy control IgG was identified (167). Inhibitors of C3 and C5 of the classical complement cascade were able to protect against aPL-mediated foetal loss and growth restriction in these mice, an observation also seen when pregnant mice deficient in C3, C4 or C5 were employed (167-169). In the same model, pregnant mice injected with F(ab’)2 fragments prepared from a pathogenic APS IgG source did not cause foetal
loss, also mice deficient in factor B (a serine protease of the alternative complement pathway) were protected from aPL mediated foetal loss (169). This identified that aPL were able to activate both the classical and alternative complement pathways leading to pregnancy loss in mice.

There is evidence for a direct in vitro aPL effect on trophoblast and endometrial cells. As shown in trophoblasts by a reduction of invasiveness, human chorionic gonadotrophin (hCG) release, proliferation and differentiation and increased apoptosis. These will be discussed extensively in sections 1.7 and 1.8.

1.5 The role of endothelial cells in APS mediated thrombosis

EC are a type of epithelial cell that form the endothelium, lining the inside of all blood vessels, providing an interface between blood circulating in the lumen and the inner layer of the blood vessel. As well as forming the structural basis of blood vessels and providing an anti-thrombogenic surface, they also contribute to numerous metabolic functions including coagulation and thrombolysis, control of blood pressure through vasoconstriction and vasodilation and antigen presentation as well as basement membrane and growth factor synthesis. They affect inflammation by producing cytokines and expressing adhesion molecules to control the trafficking, or moving, of circulating leukocytes within the blood vessel. Quiescent EC have various phenotypes depending on which vascular bed they are part of and what their designated function is, and therefore comprise a very heterogeneous population of cells. They can comprise a tight, continuous monolayer such as in the brain or lungs, where they perform important barrier functions, or a discontinuous monolayer with gaps in between, for example allowing certain molecules to enter or leave the bloodstream, such as in the kidney (170).

1.5.1 Effects of aPL on EC

Pathogenic aPL have shown to have direct effects on EC both in vivo and in vitro (152, 153, 171) leading to up-regulation of CAM and TF at the cell surface as well as endothelial microparticle (EMP) production. EC activation can then promote adhesion and activation of other cells, notably monocytes and platelets, which further contribute to hypercoagulability in the APS. This next section will describe the evidence that
exists that polyclonal and monoclonal aPL activate EC through upregulation of TF, CAMS and EMP.

1.5.1.1 TF expression
Interactions of aPL with EC lead to the up-regulation of TF, which is the major physiological trigger of blood coagulation. After vessel injury activated platelets aggregate to form a plug in the vessel in order to limit blood loss. Vessel injury causes TF expressing EC to be exposed to the circulation, which triggers a sequence of reactions culminating in thrombin generation and fibrin formation that helps to stabilise the platelet plug. Blood coagulation has three main phases: (1) Initiation; where TF is exposed to the circulation to generate trace amounts of thrombin, through the activation of factor (F) VII. (2) Amplification, where thrombin is central to overcoming the inhibitory effect of the natural regulator of the TF pathway, tissue factor pathway inhibitor (TFPI). This process is achieved by activation of platelets and other coagulation factors, such as FXI, V, and VIII, that are associated with phosphatidylserine on damaged EC or activated platelets to generate further thrombin. (3) Propagation, where thrombin generation leads to the formation of fibrin monomers that help to stabilise the platelet plug and form a clot (172, 173).

There is increasing evidence to suggest that changes to the TF pathway of blood coagulation contribute to the development of thrombosis in the APS. A number of researchers have shown that aPL induce TF expression and/or activity on EC in vitro (174-176) thereby activating the process of blood coagulation. Branch et al. (174) demonstrated that sera and IgG preparations obtained from patients with APS were able to increase TF activity significantly on cultured human umbilical vein endothelial cells (HUVEC) compared to sera and IgG preparations from healthy controls. Pierangeli and co-workers (175) examined the up-regulation of TF expression on the surface of cultured EC and showed that in the presence of IgG isolated from 3 APS patients, all with a history of thrombosis, TF was significantly up-regulated compared to controls. TF expression and activity in HUVEC after treatment with IgG purified from 8 APS patients was also investigated by Vega-Ostertag et al. (176). These authors show that both TF expression and activity were significantly increased in HUVEC treated with IgG from 8 aPL positive patients (4 with a history of thrombosis, 3 with a history of thrombosis and PM and 1 with no APS clinical event) compared to HUVEC treated with IgG from 4 healthy controls. In addition, they identified IgG from 2 of these APS
patients, both with a history of thrombosis and PM, increased IL-6 and IL-8 production from HUVEC compared to cells treated with healthy control IgG.

1.5.1.2 Expression of CAMS

When incubated with aPL and β2GPI in vitro, EC have been shown to up-regulate their expression of the CAMS; ICAM-1, VCAM-1 and E-selectin (154, 177). CAMS cause circulating leukocytes to adhere to EC and then transmigrate through the vessel lining to sites of inflammation (178). Simantov et al. (154) incubated HUVEC for 4 hours with IgG purified from patients with aCL activity and examined the expression of CAMS by immunofluorescence microscopy. HUVEC incubated with IgG with aCL activity expressed surface E-selectin, VCAM-1 and ICAM-1 while HUVEC incubated with healthy control IgG expressed only low levels of ICAM-1. When blocking antibodies to all of these CAMS were added before incubation with aCL, IgG adhesion of a monocyte cell line (Mono Mac 6) to HUVEC was inhibited. E-selectin blocking antibody alone blocked monocyte adhesion to HUVEC by 80% whereas the blocking antibody to VCAM-1 alone had no effect in this system. Del Papa and co-workers (177) identified that aPL activation of CAMS on EC was β2GPI dependent. Using affinity purified anti-β2GPI antibodies they identified that the cell surface expression of E-selectin on EC occurred only in the presence and not in the absence of bovine serum as a source of β2GPI.

Pierangeli and co-workers have extensively researched expression of CAMS by EC treated with polyclonal and monoclonal aPL both in vitro and in vivo (152, 153, 179, 180). In their in vitro experiments they initially identified that HUVEC monolayers exposed to IgG purified from 6 aPL positive patients (3 with a history of thrombosis only, 1 with PM only, 1 with both thrombosis and PM and 1 with no APS clinical events) displayed a significant 2.3- to 4.4-fold increase in VCAM-1 expression for 5 IgG patient samples tested. Only 1 IgG sample, from a patient with a history of both thrombosis and PM, significantly increased the expression of E-selectin by 3.8-fold compared to IgG purified from healthy controls. In unstimulated conditions ICAM-1 was constitutively expressed in the HUVEC and the addition of APS IgG did not increase ICAM-1 expression above this constitutive expression (152). Next, Pierangeli and colleagues investigated the effects of 7 patient derived IgG monoclonal aCL antibodies on HUVEC expression of CAMS. These IgG monoclonal aCL antibodies were derived from 2 APS patients; 1 female PAPS patient with a history of venous
thrombosis and no PM (IS) and 1 male APS patient with SLE with a history of venous thrombosis (CLE). Five of the 7 monoclonal aPL activated HUVEC to express more E-selectin and VCAM-1 and only 2 monoclonal aPL, both derived from the same APS patient (CLE), also up-regulated ICAM-1 above its constitutive expression (153).

Pierangeli and co-workers have also shown the importance of E-selectin, VCAM-1 and ICAM-1 in aPL induced EC activation and thrombus formation in vivo (179, 180) using their murine model of thrombosis described in the association of aPL and thrombosis, experimental evidence section (1.4.3.1). In this same murine model, the effects of aPL upon leukocyte adhesion, an indicator of EC activation, were also tested. This process was carried out by exposing the cremaster muscle, made up of a single layer of striated muscle cells, and assessing the number of leukocytes adhering to the endothelium. The same IgG purified from aPL positive patients were used in these experiments as previously used to assess the expression of CAMS on HUVEC monolayers described in the previous paragraph. Five out of 6 aPL positive IgG increased leukocyte adhesion compared to control IgG and this result correlated with enhanced thrombus formation (152). It is of interest to note that the only aPL positive IgG that did not increase leukocyte adhesion was that purified from a patient with PM only and no history of thrombosis. Using this murine model of thrombosis Pierangeli and colleagues investigated thrombus formation and leukocyte adherence in ICAM-1, ICAM-1/P-selectin (179) and E-selectin (180) knock out mice. A reduced ability of aPL to induce thrombosis was seen using these knock out mice (179, 180). Administration of anti-VCAM-1 with IgG from APS patients also inhibited leukocyte adherence (179).

1.5.1.3 EMP production

Many cell types produce microparticles, including platelets, leukocytes and EC. MP are produced and released as a result of activation or apoptosis upon remodelling of the cell membrane and externalisation of membrane anionic phospholipids (181). They are found at low concentrations in the peripheral blood of healthy individuals but elevated levels are detected in disease states, including patients with atherosclerosis, inflammatory and autoimmune disorders (182, 183). EMP production is considered a hallmark of vascular cell damage and elevated levels of EMP have been found in patients with APS. Combes et al. (184) showed that EMP production was increased in 30 patients with LA activity (13 of which also had a history of thrombosis and so were classified as having APS) compared to 30 healthy controls. They found that EMP were
significantly more abundant in LA positive patients that also had a history of thrombosis compared to patients with only LA positivity. These authors also demonstrated that EMP express CAMS, showing that in addition to their procoagulant activity, they may also have an adhesive potential. A further study by the same group (185) measured circulating EMP levels in 35 aPL-positive patients with thrombotic APS, 28 aPL-positive patients with SLE but no APS, 23 aPL-negative patients with SLE, 25 patients with thrombosis but no aPL and 25 healthy controls. They showed that aPL positive patients (with either thrombotic APS or SLE but no APS) had significantly elevated circulating EMP levels compared to aPL negative patients and healthy controls. Plasma from patients with APS stimulated cultured HUVEC to produce EMP with procoagulant activity, which prolonged an in vitro coagulation assay, the dRVVT. In contrast, EMP generated from HUVEC treated with plasma from patients with SLE and aPL who lacked clinical features of the APS did not have this procoagulant effect.

Recent work in my group (186) has shown that EMP are produced as a direct effect of aPL on EC. EMP production in the supernatant of HUVEC was measured by flow cytometry after incubation with either IgG purified from APS patients (n=28) or healthy controls (n=8). Significantly more EMP were generated in the presence of IgG from APS patients compared to healthy control IgG. Of the 28 APS IgG, 11 were from patients with only thrombotic APS and 8 were from patients with only obstetric APS, the other 9 were from patients with both thrombotic and obstetric APS. Significantly more EMP were released from HUVEC exposed to IgG from patients with a history of thrombosis compared to healthy control IgG as opposed to IgG from patients with a history of obstetric APS only.

Overall, there is extensive evidence that aPL activate EC through the increased expression of TF, CAMS and EMP, promoting a prothrombotic state. The mechanisms by which aPL exert their effects on EC, through the activation of signalling pathways will be discussed next.

1.5.2 How do aPL exert their effects on EC – activation of signalling pathways

Human monoclonal and polyclonal aPL have been demonstrated to induce a signalling cascade similar to that seen when EC are activated by LPS or IL-1 (187). In this next
section the TLR and associated signalling pathways that have been implicated in aPL activation of EC will be discussed.

1.5.2.1 Activation of TLRs
There is evidence to suggest both TLR2 and TLR4 may be implicated in aPL activation of EC (188-190). Using two different types of mice; one type (C3H/HeJ) with a mutation in the TLR4 gene such that it was unable to respond to LPS and the other type (C3H/HeN) with no TLR4 mutation, hence was LPS-responsive, Pierangeli et al. (188) demonstrated that TLR4 is involved in the pathogenesis of aPL-induced thrombosis and aPL activation of EC. After injection of IgG purified from 2 patients with PAPS (1 with a history of thrombosis and 1 with a history of thrombosis and PM), 1 healthy control IgG and 1 polyclonal IgG from an aPL-negative patient with SLE, both LPS responsive and unresponsive mice were subjected to mechanical pinch induced thrombosis to their right femoral vein. The number of leukocytes adhering to endothelium in the cremaster muscle was also determined. The IgG purified from APS patients produced significantly larger thrombi and leukocyte adherence to venules than the non-APS samples in LPS responsive mice and these effects were reduced by depleting the samples of anti-β2GPI antibodies. LPS-unresponsive mice treated with APS IgG showed a significant reduction in thrombus size and adherent leukocytes compared to LPS-responsive mice, thus implicating the involvement of TLR4 in the pathogenic effects of APS IgG in this animal model.

Colasanti et al. (190) recently identified a peptide, 8 amino acids in length, in domain I of β2GPI that shares 88% identity with an epitope of the extracellular region of TLR4. Eleven out of 27 patients (41%) with APS (PAPS and APS secondary to SLE) and 22 of 44 patients (50%) with SLE presented serum IgG specific to this peptide but 30 healthy control subjects did not. Anti-β2GPI peptide antibodies induced activation of TLR4 and triggered Interleukin-1 receptor associated kinase (IRAK) phosphorylation and NFκB translocation and promoted VCAM-1 expression on HUVEC.

In contrast, Satta et al. (189) studied the ability of purified IgG from 31 aPL positive patients, 27 of which had PAPS, 3 had APS and associated SLE and 1 had SLE and no APS (and the anti-β2GPI affinity-purified sub-fraction from two patients) compared with 19 healthy control IgG to activate human monocytes and HUVEC. They found that 19 of the APS samples caused a modest increase in E-selectin mRNA expression in
HUVEC. This response was enhanced by pre-incubation of cells with TNF, which is known to cause up-regulation of TLR2. The effects of these 19 APS IgG samples on HUVEC were blocked by anti-TLR2 but not anti-TLR4 antibodies. The authors also used a human embryonic kidney cell line (HEK-293) that had been generated to express TLR2 or TLR4 and to respond to activation of these receptors by means of an alkaline phosphatase-mediated colorimetric change. They showed that the 19 IgG samples that were capable of activating monocytes and HUVEC would activate HEK-293 cells expressing TLR2 but not those expressing TLR4. Quantification of APS IgG binding to the cell surface revealed much stronger interactions with TLR2 than TLR4 in all three cell types used.

Indirect evidence that TLR2 may be important in the aPL-β2GPI-cell interaction has come from the work of Alard et al. (191). They demonstrated that HUVEC take up β2GPI - rather than synthesising and secreting it themselves - from fetal calf serum (FCS) through co-localisation with TLR2 and TLR4 in lipid rafts. The identity of the TLR was established by immunoblot and confocal microscopy of β2GPI-linked purified proteins. This study provides evidence that binding of β2GPI to the EC surface occurs through interaction with TLR2 rather than TLR4. It is important to note however, that this study did not examine the effects of aPL upon the β2GPI-TLR interaction and aPL-β2GPI complexes may engage other cell surface receptors such as annexin A2 that subsequently interact with TLR4 thus explaining some of the conflicting results of these studies implicating TLR2 or TLR4.

As well as TLR the TLR adaptor protein MyD88 has also been implicated in aPL activation of EC (187). MyD88 is the most extensively studied of the adaptor proteins in the APS. It is the integral signalling adaptor for all TLR other than TLR3 and some TLR4 signals (11). Raschi and co-workers (187) found that a human monoclonal IgM aPL anti-β2GPI and affinity purified polyclonal anti-β2GPI IgG isolated from 3 patients with APS (1 with a history of thrombosis, 1 with PM and 1 with both thrombosis and PM) activated intracellular signalling pathways in human microvascular EC in a manner comparable to that induced by LPS or IL-1, whereas control antibodies did not. These effects however, were abrogated in cells that had the MyD88 or TNF receptor-associated factor pathways knocked out. Both pathways are important in TLR4-mediated responses with MyD88 being a key TLR adaptor protein.
1.5.2.2 Activation of protein kinases

Vega-Ostertag et al. (171, 176) have extensively researched the involvement of the protein kinase, p38 mitogen activated protein kinase (MAPK), in aPL activation of EC. Firstly they demonstrated that treating HUVEC with IgG from 6 aPL positive patients, 2 with a history of thrombosis and PM, 3 with a history of thrombosis only and 1 with no APS clinical events, induced a 5.3- to 9-fold increase in p38 MAPK phosphorylation compared to IgG from 2 healthy controls. Pre-incubation with the p38 MAPK inhibitor, SB203580, inhibited aPL positive IgG-mediated increases in TF mRNA and protein expression and TF activity in HUVEC (176). In a subsequent paper (171), these authors showed that APS IgG purified from 1 male patient with PAPS with a history of thrombosis increased the adhesion of human monocyctic (THP-1) cells to cultured HUVEC compared with IgG purified from 1 healthy control and that the p38 MAPK inhibitor SB203580 significantly reduced this effect. SB203580 was then shown in a mouse model to inhibit the thrombotic effects of this APS IgG in vivo and reduce ex vivo; carotid artery and peritoneal macrophage TF activity; aortic vascular cell VCAM-1 expression and platelet aggregation.

As well as the work of Vega-Ostertag et al., other evidence suggesting a role for p38 MAPK in aPL activation of EC include the work of Tzang and co-workers (192). Using an immortalized HUVEC cell line they found that a commercial human anti-β2GPI IgG and rabbit anti-human parvovirus B19 IgG (with cross-reactivity against CL and β2GPI) mediated up-regulation of cell adhesion molecules and TNF-α expression correlated with p38 MAPK phosphorylation. Simoncini and co-workers (193) showed that IgG from 12 APS patients (5 with a history of thrombosis only, 2 with PM only and 5 with thrombosis and PM) but not IgG from 12 healthy controls added to HUVEC led to increased VCAM-1 expression, increased adhesion of THP-1 cells, and increased generation of reactive oxygen species. There was a rapid increase in p38 MAPK activation in APS IgG-treated cells, which was prevented by pre-incubation with antioxidants, which blocked the generation of reactive oxygen species. Furthermore, they showed that pre-incubation with either antioxidants or specific p38 MAPK inhibitors prevented the increase in APS IgG-mediated VCAM-1 expression.

Aronovich and co-workers (194) examined the effects of IgG from 7 APS patients on a rat cerebral microvascular EC line (brain vascular EC). They found that 4 samples increased ICAM-1 expression in brain vascular EC, which was blocked by a Ras
inhibitor farnesylthiosalicylate. These samples also caused phosphorylation of ERK, which was blocked by farnesylthiosalicylate. A lack of comparison with healthy control samples means it is uncertain whether stimulation of the Ras-ERK pathway is a truly specific effect of APS IgG in these cerebral cells.

1.5.2.3 Activation of the transcription factor NFκB

Several authors have shown that aPL stimulate activation of the NFκB pathway in EC. Meroni et al. (80) studied the effects of polyclonal and monoclonal human aPL on HUVEC. They compared monoclonal IgM anti-β2GPI antibody GRD15 with the control monoclonal IgM antibody TM1B9 (which does not bind β2GPI) and compared polyclonal affinity-purified anti-β2GPI from 2 patients with PAPS with polyclonal IgG from healthy controls. They found that, in comparison to the relevant controls, both the monoclonal and polyclonal human anti-β2GPI stimulated nuclear translocation of NFκB in HUVEC as well as expression of ICAM-1 and E-selectin and adhesion of a monocyte cell line (U937 cells) to these HUVEC. These effects were blocked by fluvastatin or simvastatin. In subsequent work (187), they showed that aPL-mediated activation of TLR4 in EC leads to activation of NFκB.

Espinola et al. (180) compared the effects of IgG taken from a single APS patient or healthy control IgG on NFκB transcriptional activation by transfecting cardiopulmonary aortic EC with the pNFκB-luciferase vector. Treatment of cardiopulmonary aortic EC cells with APS IgG increased transcriptional activation of NFκB by 135% whereas healthy control IgG only increased it by 14%. They also found that APS IgG but not healthy control IgG increased E-selectin expression on HUVEC by 400-fold. Recently, Ortona and co-workers (195) showed that affinity purified human IgG anti-vimentin/cardiolipin antibodies from patients with SN-APS stimulated NFκB phosphorylation in HUVEC, whereas healthy control IgG did not.

Inhibition of NFκB has been shown to reduce effects of APS IgG both in vitro and in vivo, although the evidence is limited and the degree of inhibition variable. Vega-Ostertag et al. (176) found that significant up-regulation of TF expression on cultured HUVEC induced by three APS IgG (tested from a cohort of 8 patients of whom 7 had VT and 3 PM events) was reduced by 10.2 – 98% in the presence of the NFκB inhibitor MG132. Further work by Montiel-Manzano et al. (196) has examined the effects of
MG132 upon aPL-induced thrombosis and EC activation in vivo in the murine pinch thrombosis model (188). Using IgG purified from a single patient with thrombotic APS they demonstrated that pre-treatment of mice with MG132 led to a significant reduction in both APS IgG induced thrombosis (by 93%) and white blood cell adhesion to EC in cremaster muscle. A potential limitation of these studies is their use of MG132 which is a proteasome inhibitor and not entirely specific for NFκB. Dunoyer-Geindre and co-workers (197) correlated APS IgG, murine monoclonal IgG anti-β2GPI, and rabbit IgG anti-β2GPI-induced NFκB activity and VCAM-1 expression on cultured HUVEC with a 1.5-fold reduction in rabbit IgG anti-β2GPI-induced VCAM-1 expression in the presence of a specific inhibitor of NFκB nuclear translocation.

The evidence discussed here indicates that aPL may activate EC through TLR2 or TLR4, following which the protein kinase p38 MAPK and the transcription factor NFκB may be activated. Although it is clearly evident that aPL activate EC, and data imply this activation occurs through a TLR dependent pathway, limited numbers of samples (both monoclonal and polyclonal) were used in all the studies described. One possible limitation of all these studies is the failure to compare effects of samples from patients with a history of thrombosis alone to samples from patients with a history PM alone upon EC activation. It may be that aPL that promote thrombosis alone use different signalling pathways to induce EC activation compared to aPL that promote PM alone. Next I will describe evidence identifying that aPL have effects on other thrombotic cell types; monocytes and platelets.

1.6 The role of other cell types in thrombotic APS

In addition to EC there is also a great deal of evidence indicating that aPL activate monocytes, and platelets resulting in thrombosis.

1.6.1 Effects of aPL on monocytes

The activation of monocytes by aPL has been extensively researched in the APS field, in particular aPL effects on monocyte TF expression and activity, the major initiator of blood coagulation. De Prost et al. (198) were the first to show that monocytes derived from 19 patients with SLE, 9 of which were LA positive, displayed increased procoagulant activity compared to healthy control monocytes and this activity was
attributed to a TF-like effect. Subsequently, increased TF expression, at both the mRNA and protein level, has been shown in monocytes from patients with APS (199, 200) and healthy monocytes exposed to both monoclonal and polyclonal aPL \textit{in vitro} (201, 202). Evidence that aPL increase TF activity in healthy monocytes treated with both monoclonal aPL (203) and aPL purified from APS patients (189, 204) has been more recently studied. It is important that aPL have been shown to increase monocyte TF activity in addition to TF expression as TF is known to be present in a non-coagulant “cryptic” form on the cell surface (205) and so it cannot be assumed that increased TF expression results in increased TF activity.

The upregulation of TF expression and activity in monocytes has been attributed to occur through TLR2 (189) and TLR4 (204) as well as the protein kinases ERK and p38 MAPK and the transcription factor NFκB (204, 206, 207). In addition to the evidence of signalling pathways being activated in aPL mediated monocyte TF expression, a number of other authors have identified that aPL mediate their effects on monocytes through TLR4 (203, 208), TLR8 (209, 210), p38 MAPK and NFκB (78, 208, 211, 212).

Lopez-Pedrera and colleagues (213) identified, by proteomic analysis of monocytes isolated from 51 patients with APS, the differential expression of several monocyte proteins between different clinical subgroups. They analysed monocytes from 32 patients fulfilling the APS classification criteria who had previous thrombotic events, 19 patients fulfilling the APS classification criteria with recurrent spontaneous abortions, 20 patients with thrombosis but no aPL and 15 healthy subjects. Expression of annexin I, annexin II (both involved in coagulation and inflammation), protein disulfide isomerase (which catalyses the oxidation, reduction and isomerisation of disulfide bonds), Nedd8 (involved in the proteolytic destruction of IκB), RhoA proteins (regulate the actions of activated macrophages); and Hsp60 (regulates transportation and refolding of proteins) were all significantly altered in APS patients with a previous history of thrombotic events compared to monocytes from all other groups tested. These proteins are all functionally related to processes associated with the induction of a pro-coagulant state, as well as autoimmune-related responses and the expression of each of these proteins was validated by western blot.

In addition to the evidence that exists that aPL cause increased expression of TF in monocytes through activation of the cell surface TLR, Cuadrado \textit{et al.} (211) have
investigated whether aPL can increase the expression of the proinflammatory cytokine VEGF which stimulates monocyte TF expression through its receptor Flt1. These authors have shown increased levels of VEGF and Flt1 in monocytes from 55 APS patients in comparison with healthy monocytes. The levels of VEGF and Flt1 were significantly higher in monocytes from 46 APS patients with thrombosis compared to monocytes from 9 APS patients without thrombosis. In fact these authors were the first to demonstrate that aPL from patients with different clinical manifestations of the APS may have differential effects on target cells. *In vitro* IgG purified from 7 of these APS patients increased healthy monocyte VEGF and Flt1 expression compared to IgG from a pool of 10 healthy controls and this was significantly inhibited by the p38 MAPK inhibitor SB203580, suggesting the involvement of p38 MAPK in aPL-induced VEGF and Flt1 upregulation.

The hypothesis that aPL from patients with different clinical manifestations of APS may have differential effects upon target cells has been directly studied in recent work carried out within our group. Lambrianides and co-workers (204) compared the effects of polyclonal IgG derived from different APS subsets and controls on the activation of signalling pathways and TF activity on the U937 human monocyte cell line and *ex vivo* healthy monocytes. TF activity in the monocyte cell line was only stimulated by IgG from thrombotic APS patients and not by IgG from APS patients who have only experienced obstetric complications or healthy control IgG. This TF activity effect was significantly reduced (by 45%) by a TLR4 antagonist and to a lesser extent (22.2%) by an anti-TLR2 antibody. Also, IgG from thrombotic APS patients and not IgG from PM APS patients or healthy controls stimulated p38 MAPK and NFκB phosphorylation in this cell line as well as healthy *ex vivo* monocytes. These data identify that IgG purified from patients with different manifestations of the APS have differential effects on the activation of signalling pathways and TF expression in monocytes.

There is evidence to show monocyte activation by aCL leads to up-regulation of the expression of protease-activated receptors (PARs) (214). PARs are G protein–coupled receptors activated by proteases through proteolytic cleavage and unmasking of an amino-terminal receptor sequence, which acts as a tethered ligand by binding to the second extracellular loop of the receptor to initiate transmembrane signalling. Four main members of the PAR family have been identified to date: PAR-1, PAR-3, and PAR-4, which are receptors for the serine protease thrombin, the main effector of the
coagulation cascade, and PAR-2, which is activated by trypsin, tryptase factor Xa, or factor VIIa (215). Lopez-Pedrera et al. (214) compared mRNA and protein expression of PAR-1, PAR-2, PAR-3 and PAR-4 in monocytes isolated from 62 PAPS patients (37 with a history of thrombosis only and 25 with a history of PM only). They identified a significantly higher mRNA and protein expression of PAR-1 and PAR-2, but not of PAR-3 or PAR-4 in the monocytes of these APS patients compared to monocytes from 20 healthy controls. Sub-group analysis identified that PAR-1 and PAR-2 mRNA and protein expression was significantly higher in APS patients with thrombosis compared to APS patients without thrombosis (PM only) and healthy controls. PAR-1 mRNA and protein expression was also increased in patients with a history of thrombosis but no APS (n=20) compared to healthy controls, suggesting that PAR-1 expression is increased in pro-thrombotic states but is not directly associated with APS. These authors also examined cell surface PAR-1 and PAR-2 expression in healthy monocytes treated with pooled IgG from 7 APS patients with a history of thrombosis and pooled IgG from 10 healthy controls. They found that both PAR-1 and PAR-2 cell surface expression was increased in monocytes treated with APS IgG compared to monocytes treated with healthy control IgG, illustrating that this increased PAR-1 and PAR-2 expression is an aPL IgG mediated effect.

1.6.2 Effects of aPL on platelets

Platelets are integral to regulate blood loss during injury. They continuously inspect the vessel wall for leakages and when the inner cells of the vessel wall, the endothelium, are injured, platelets respond by adhering to the exposed sub-endothelial structures. After the adherence of a first layer of platelets, newly arriving platelets interact with the already adhered platelets and aggregate together, forming a temporary platelet plug (216). Activation of platelets induces a pro-thrombotic state, as they increase their surface exposure of negative phospholipid thus acting as a platform for the assembly of the pro-thrombinase complex, used to generate thrombin in the coagulation cascade (217). Studies have shown that aPL from APS patients are able to bind to platelets and enhance platelet activation and aggregation in vitro as well as in vivo (218-221).

The activation of platelets cannot be measured from the blood of patients as platelets become activated during the collection of blood. The most reliable way of measuring in vivo platelet activation is to measure thromboxane metabolites in urine. Forastiero et al. (221) measured levels of 11-dehydro-thromboxane B2, a stable thromboxane metabolite,
in the urine of 19 patients with APS (comprised of a history of thrombosis in 12 and history of PM in 10) and 18 healthy control subjects. They identified that levels of 11-dehydro-thromboxane B₂ were significantly increased in the urine of patients with APS compared to healthy control subjects, indicating that circulating platelets in APS patients are more activated.

As has been discussed previously pathogenic aPL do not bind directly to phospholipid but to plasma proteins with affinity for phospholipid, such as β₂GPI. Studies have shown that plasma β₂GPI on its own does not bind to platelets; only β₂GPI in complex with antiβ₂GPI-antibody can bind to platelets and activate them (222, 223). Other in vivo studies have shown that the activation of platelets by the binding of β₂GPI antibody complexes is not due to an interaction of the antibodies with the Fc-receptor present on the platelet (148).

A group in the Netherlands investigated platelet membrane receptors that could bind β₂GPI-antibody complexes. Using co-immunoprecipitation they identified that these antibody complexes were able to bind to the low-density lipoprotein (LDL) receptor ApoER2’ (also known as LRP-8) and through this binding cause activation of platelets and the induction of thromboxane A₂ synthesis (224, 225).

Another receptor, the platelet membrane glycoprotein GPIIb/IIIa receptor has also been implicated in APS. Activation of this receptor mediates platelet aggregation and is considered a marker of platelet activation. Espinola et al. (226) used flow cytometry to show that aPL enhance the expression of platelet membrane glycoproteins, particularly GPIIb/IIIa and GPIIIa, when platelets were pre-treated with suboptimal doses of a thrombin receptor agonist peptide. Using the murine thrombosis model (described in section 1.4.3.1) Pierangeli and co-workers identified that infusion of an anti-GPIIb/GPIIIa antibody significantly reduced the ability of aPL to promote thrombosis; furthermore, compared to results obtained using wild type mice, a reduction in thrombus size was observed when aPL were used to treat mice deficient in GPIIb/GPIIIa (227).

A few studies have examined the signalling events involved in the increased platelet activation by aPL. Both p38 MAPK (228) and phosphatidylinositol-3-kinase (PI3K) (229) have been implicated in platelet activation by aPL. Vega-Ostertag and co-
workers (228) showed that after pretreatment with thrombin, IgG purified from 7 APS patients, but not IgG purified from 10 healthy controls, induced p38 MAPK phosphorylation in platelets, which was F(ab’)2 mediated. Platelet aggregation and thromboxane B2 production were both stimulated by IgG from APS patients but not IgG from healthy controls and the p38 inhibitor SB203580 inhibited these effects. Shi and co-workers (229) reported activation of the PI3K pathway via the GPIbα subunit of the platelet adhesion receptor GPIb-IX-V. They found that platelets exposed to a monoclonal anti-β2GPI antibody in the presence of β2GPI increased phosphorylation of Akt and GSK-3β compared with monoclonal anti-β2GPI antibody alone. This phosphorylation was reduced in platelets treated with a specific inhibitor of PI3K, the upstream activator of Akt and GSK-3β, as well as a monoclonal anti-GPIbα antibody. These findings are consistent with a role for both ApoER2’ and GPIbα in aPL mediated platelet activation as signalling via both receptors can lead to increased p38 MAPK and PI-3 kinase phosphorylation (230-233).

Having discussed the evidence relating to aPL effects of thrombotic cell types I will next discuss the effects of aPL on obstetric cells types, namely trophoblasts and endometrial cells.

1.7 The role of trophoblast cells in APS mediated PM

Women with APS are at high risk of complications throughout all stages of pregnancy from recurrent early pregnancy loss to severe late obstetric complications including pre-eclampsia, IUGR and prematurity. An important cell type that aPL have been shown to act upon and contribute to APS disease pathogenesis is the trophoblast. This section will outline the current understanding of how aPL effect trophoblast cells. In order to understand how aPL effect trophoblasts the role of trophoblasts in normal pregnancy will first be described.

1.7.1 Normal role of trophoblast cells in implantation and pregnancy

The birth of a healthy infant at the full term of pregnancy depends upon a co-ordinated series of events in the development of both embryo and placenta. Failure of one or more key component of placentation may result in a wide range of pregnancy
complications including first or second trimester foetal death or third trimester complications of pregnancy including pre-eclampsia and IUGR (234).

After fertilisation occurs in the fallopian tube, the embryo, encased in a protective coating known as the zona pellucida, passes through the fallopian tube developing from the fertilised ovum (zygote) to a mass of 12 to 16 cells (morula). Once in the uterine cavity the morula transitions to a blastocyst. The blastocyst is composed of a fluid filled cavity with an inner cell mass, surrounded by a trophoblast layer, the trophectoderm, which is destined to differentiate into the placenta (illustrated in Figure 1.4, section a). Within 72 hours of entering the uterine cavity the embryo hatches from the zona pellucida, thereby exposing its outer trophectoderm (235) (Figure 1.4, section b/c). Implantation occurs 6 to 7 days after fertilisation. Prior to implantation, the trophectoderm differentiates into two separate trophoblast subsets, the outer syncytiotrophoblast and the inner cytotrophoblast (Figure 1.4, section d). The syncytiotrophoblast of the blastocyst attach to and invade the uterine epithelium, creating a cavity into which the blastocyst may embed (Figure 1.4, section e/f). After implantation cytotrophoblast cells penetrate through the syncytiotrophoblast, enter the uterine stroma and form the extravillous cytotrophoblast (EVT) region.
**Figure 1.4: Stages of blastocyst implantation**

This figure outlines the stages of blastocyst implantation from release of the blastocyst from the zona pellucida (a) to invasion of the trophoblast into the uterine epithelium in order to implant the developing blastocyst (f).
EVT continue to invade maternal tissues throughout the first trimester with two main functions: to locate and surround the spiral arteries in the decidua to form a complex network with the uterine arterial circulation and to communicate with the mother via endocrine signals to promote blood flow to the implantation site and mediate the maternal adaptive response to pregnancy. In addition, the more proximal columns of cytotrophoblasts are penetrated by extra-embryonic mesoderm in which new fetoplacental blood vessels form by vasculogenesis to become chorionic villi that will separate maternal and foetal blood and regulate gaseous exchange (234).

The spiral arteries are ‘plugged’ until 12 weeks of gestation with endovascular trophoblast clumps at the site of implantation to prevent maternal blood flow into the trophoblastic villi (236, 237) (Figure 1.5 A). Thus, EVT invasion and villous formation occurs in a hypoxic environment to avoid oxidative stress. The nutritional needs of the developing embryo/foetus during the hypoxic first trimester are met by secretions from endometrial glands into the intervillous space (238). The ‘plugging’ of the spiral arteries drives vasculogenesis in the villi until the end of the first trimester, where the endovascular trophoblast clumps disappear and maternal blood enters the villi in order to provide oxygen to the placenta (234) (Figure 1.5 B). EVT invasion into the decidua and into the myometrium continues in the second trimester in order to form channels in the spiral arteries to allow further blood flow in the uterine tissue (234, 239).
**Figure 1.5: Role of EVT during normal pregnancy**

This figure illustrates EVT invading the spiral arteries at the site of implantation to maintain a hypoxic environment during early gestation (A). After 12 weeks the occlusions disappear and trophoblasts begin to line the spiral artery walls (B).
1.7.2 Effects of aPL on trophoblast cells

As aPL are strongly associated with thrombosis, PM in patients with APS was initially thought to arise from a thrombotic event occurring at the foetal-maternal interface. Early studies identifying placental infarction in aPL positive individuals supported this theory (240, 241). This perception was also sustained by the successful prevention of foetal loss with anticoagulants, especially heparin. Histological findings from several larger studies have now revealed that intravascular or intervillous blood clots are not more commonly found in miscarriage samples from APS patients with PM than in patients with PM who are aPL negative (242-244).

Sebire and colleagues (242) examined the products of conception obtained from women suffering first trimester miscarriage attending a recurrent miscarriage clinic. Products of conception were obtained from 31 patients with PAPS, 50 patients who were aPL negative and 34 patients with foetal chromosomal abnormality. In addition, products of conception from 20 control cases with no history of recurrent miscarriage who underwent elective terminations at 6-14 weeks gestation were also studied. Histological examination identified no difference in the frequency of intervillous thrombosis between any of these groups. In a subsequent study, Sebire and co-workers (243) examined mostly third trimester placentae from 121 patients who had experienced three or more consecutive pregnancy losses. Of the 121 patients assessed, 60 were APS patients and 61 were aPL negative thus lacking APS. These authors identified that pregnancy outcome was similar in both APS positive and negative groups regarding gestation at delivery, with the majority of pregnancies reaching full term, and antepartum obstetric complications. Several histological placental abnormalities were identified in both groups but most pregnancies were clinically uncomplicated with no significant placental abnormalities. In cases with pregnancy complications the placental pathology was primarily that of a utero-placental vasculopathy, such as placental infarction and preeclampsia, but there were no placental lesions or patterns of abnormalities specific to the APS patients. The prevalence of most histological abnormalities were identified to be similar to that found in an unselected obstetric population.

Van Horn et al. (244) examined the histological features of mostly second trimester decidual and placental tissues from 44 pregnancies in 26 women with APS and PM and 37 pregnancies in 21 women who were aPL negative but had APS-like clinical features.
Similar histological features were identified in both groups of patients out of 20 different histological parameters used to assess these specimens. When compared with 71 control placentae, from either elective pregnancy terminations or pregnancies complicated by idiopathic preterm delivery, tissue from both groups showed increased decidual necrosis, inflammation and vascular thrombosis as well as increased placental infarction, inter-villous thrombosis and fibrosis.

It is now understood that altered coagulation in the utero-placental circulation in either the first, second and/or third trimester is not specific to APS related PM. Therefore, the possible direct effects of aPL on obstetric cells, in particularly on trophoblasts have been investigated. Both murine and human monoclonal as well as polyclonal IgG antibodies from APS patients have been reported to bind to trophoblast monolayers in vitro (245-247). The exact mechanisms of aPL-mediated pregnancy failure however, remain poorly characterized. There is evidence for a direct in vitro aPL effect on the trophoblast as shown by a reduction of invasiveness, human chorionic gonadotrophin (hCG) release, proliferation and differentiation and increased apoptosis, summarised in the following section. These in vitro studies have investigated aPL effects on trophoblasts either by studying ex vivo trophoblasts isolated from the placenta of healthy pregnancies or a well characterised human first trimester extravillous trophoblast (HTR-8) cell line established by introducing the gene encoding simian virus 40 large T antigen into first trimester human trophoblasts in order for these cells to have an extended life-span. The HTR-8 cell line have shown to share a number of phenotypic similarities with their parental counterparts, whilst being able to be maintained longer in culture, for over 32 passages, making them a suitable cell line to study placental function (248)

1.7.2.1 Invasion and migration
An important feature of the trophoblast is its ability to invade and migrate. Implantation of the blastocyst into the uterine epithelium and development of a functional placenta are crucial events for the establishment of pregnancy. In normal placentation EVT of the placenta invade the uterine stroma and develop vascular connections between mother and foetus, without which complications would arise (234). In particular, defective trophoblast invasion is now well described not only in association with the development of later pregnancy complications such as IUGR and pre-eclampsia (249) but also in some cases of early pregnancy loss (242). Numerous
studies have shown that aPL reduced the ability of trophoblasts to invade and migrate (242, 246, 250-253).

Katsuragawa et al. (250) were the first to demonstrate, using an in vitro migration assay, that a monoclonal antibody to phosphatidylserine prevented the migration of first-trimester cytotrophoblasts isolated from products of conception obtained from 10 healthy women undergoing social abortions at 6-12 weeks of gestation. A migration assay allows the quantitation of trophoblast cells that migrate through a cell-permeable membrane, such as polyethylene terephthalate or collagen. Subsequently, Di Simone and colleagues (246, 254, 255) have carried out a number of studies investigating trophoblast migration. In their first study (254), using a migration assay, they showed that IgG purified from 2 patients with APS and a history of both PM and thrombosis significantly inhibited trophoblast migration (isolated from the placentae of healthy pregnancies after delivery at 36 weeks of gestation) compared to IgG purified from 2 control subjects with no history of pregnancy complication. This reduced migration was restored with treatment with LMWH. In a subsequent study Di Simone and co-workers (246) identified using both polyclonal IgG from two APS patients and two monoclonal antibodies that aPL can bind trophoblasts by reacting with β2GPI and/or with “pure” anionic phospholipid resulting in reduced trophoblast migration.

As discussed previously, Sebire et al. (242) examined histologically the products of conception obtained from women with and without PAPS suffering first trimester miscarriage attending a recurrent miscarriage clinic to identify a potential mechanism underlying the early pregnancy failures. Normal endovascular trophoblast invasion was identified significantly less frequently in patients with PAPS compared to patients without PAPS and controls. Of the 31 patients investigated with PAPS endovascular trophoblast invasion of decidual vessels was identified in only 24%. Whereas of 50 patients suffering first trimester miscarriage without PAPS endovascular trophoblast invasion of decidual vessels was identified in 61% and out of 20 control individuals, with no history of recurrent pregnancy loss who underwent termination of pregnancy for social reasons, endovascular trophoblast invasion of decidual vessels was identified in 75%. This study shows that there is a specific defect in decidual endovascular trophoblast invasion in PAPS patents suffering first trimester pregnancy loss as compared to patients without APS suffering first trimester pregnancy loss.
Mulla et al. (252) demonstrated, using an *in vitro* migration assay, that two murine anti-human β₂GPI monoclonal antibodies (ID2 and IIC5) limit the migration of the HTR-8 cell line when compared to an IgG control. In addition, they identified a mechanism for reduced HTR-8 cell migration by these aPL. They found that both ID2 and IIC5 reduced IL-6 production and STAT3 activity of cultured HTR-8 cells. Trophoblast cells constitutively express IL-6 throughout pregnancy, however these two murine anti-human β₂GPI monoclonal antibodies impaired IL-6 secretion by HTR-8 cells by 76.1% and 81.5% respectively and reduced IL-6 transcript levels by 29.4% and 28.7% respectively. They showed that an IL-6 receptor blocker inhibited HTR-8 cell migration, confirming that aPL limit trophoblast migration by down regulating trophoblast IL-6 production. Accumulation of secreted IL-6 in HTR-8 cells correlated with an increase in phosphorylated STAT3, without altering the expression levels of total STAT3, indicating activation of this signal transduction pathway. Moreover, it was shown that stimulation of trophoblast cells with human recombinant IL-6 up-regulated phosphorylated STAT3 levels, which was entirely negated upon co-treatment with an IL-6 receptor blocker. The two murine anti-human β₂GPI monoclonal antibodies reduced phosphorylated STAT3 levels without altering total STAT3 expression (252). The relevance however, of these experiments using murine monoclonal antibodies to human antigen requires verification with polyclonal IgG isolated from patients with obstetric APS.

Similarly, Jovanovic and co-workers (253) used both first trimester cytotrophoblasts isolated from the healthy placentae of social abortions at 6-12 weeks of gestation and HTR-8 cells to investigate trophoblast migration in cells treated with polyclonal IgG from patients with APS (clinical manifestations not stated) and raised aCL levels. Treatment with aPL isolated from APS patients result in inhibition of migration in cultured first trimester cytotrophoblasts by 84.4% and in HTR-8 cells by 39.8% when compared to cells treated with IgG from healthy controls. These authors proposed the following mechanism for reduced migration in trophoblasts treated with aPL. Evidence exists that for normal migration to occur an integrin switch and adherence to a physiological pattern of expression of adhesion molecules is critical. Therefore, employing both immunocytochemistry and ELISA they showed that the integrins relevant for cell adhesion and migration in HTR-8 cells, subunits α₁, α₅ and β₁ were inhibited when cells were cultured with IgG from APS patients, indicating that integrins are involved in the inhibition of trophoblast migration induced by aPL.
There is evidence both *in vivo* and *in vitro* using polyclonal IgG purified from patient with APS and monoclonal aPL supporting the idea that aPL inhibit trophoblast migration. The exact mechanism by which aPL inhibit trophoblast migration has yet to be fully characterised. One potential limitation of all the studies is the failure to compare effects of samples from patients with a history of either PM alone or thrombosis alone upon trophoblast migration. Given our findings in monocytes that only cells incubated with IgG from patients with thrombotic APS manifestations and not IgG from patients with PM increase TF activity (204), it may be that aPL that promote PM alone have effects upon trophoblast cells and are able to inhibit trophoblast migration but aPL that promote thrombosis alone are not.

1.7.2.2 Hormone secretion
The hormone hCG is produced by trophoblast cells of the implanted blastocyst, converting the corpus luteum of the menstrual cycle to the progesterone producing corpus luteum of pregnancy, which promotes decidualisation, protects pregnancy and supports the development of the embryo (256). It has been shown that aPL may alter the ability of trophoblasts to produce and secrete hCG (246, 250, 257-260), a hormone that maintains progesterone levels, crucial for sustaining normal pregnancy. Di Simone *et al.* (246) identified that polyclonal IgG from two APS patients (one with PM and VT and one with PM alone) significantly inhibit *in vitro* production of gonadotrophin-releasing hormone (GnRH)-induced hCG from cytotrophoblast monolayers prepared from term placentae of healthy women compared to control IgG from two aPL-positive women, each of whom have had two uncomplicated pregnancies. In a later paper by the same group (259) the importance of β2GPI in this aPL mediated effect was shown in experiments utilising a non-aPL-binding variant of this protein. Cytotrophoblast monolayers were incubated in the presence of polyclonal IgG from APS patients with either serum-free medium, human purified β2GPI or a β2GPI with a mutation introduced into the PL-binding domain (Domain V), which renders this protein unable to associate with trophoblast cells. As before, GnRH-induced hCG production was reduced in cells treated with polyclonal APS IgG in the presence of purified β2GPI. The ability however, of aPL to reduce GnRH-induced hCG levels was abrogated when cells were incubated with the β2GPI non-PL-binding domain mutant.
1.7.2.3 Proliferation, differentiation and apoptosis

Research has shown that aPL may effect trophoblast proliferation, differentiation (261, 262) and also cause apoptosis (260, 263, 264). The differences in trophoblast morphology and differentiation were examined in first trimester placental material in elective terminations from healthy aPL negative individuals and miscarriages from patients with APS in a study by Bose et al. (261). Normal features of trophoblast development during the first trimester, as seen by a rise in trophoblast area, increase in number of syncytiotrophoblast nuclei, increase in number of proliferating cytотrophoblast and decrease in cytотrophoblast: syncytiotrophoblast ratio, was seen in healthy tissue. In APS tissue however features of normal trophoblast development were absent or reversed, leading the authors to suggest that the presence of aPL alters the structure of the foetal-maternal barrier, increasing the risk for pregnancy complications. A limitation of this study however is that differences in trophoblast morphology and differentiation were compared in first trimester placental material from miscarriages to elective terminations from healthy aPL negative individuals. The study would have been more robust if first trimester placental material from spontaneous miscarriages of patients with and without aPL had been compared.

Quenby et al. (262) have shown that the presence of anti-β2-GPI antibodies results in failure of trophoblast differentiation and hence subsequent uteroplacental development. These authors demonstrate that EVT isolated from full term placentae of women with a normal pregnancy and delivery fuse together to form giant multinuclear cells when kept in culture. When these cells were incubated with murine monoclonal anti-β2-GPI antibodies an inhibition of cell fusion process and giant multinuclear cell formation occurred compared to untreated EVT. This effect was not seen when EVT were incubated with an irrelevant murine monoclonal antibody, showing this effect was specific to incubation with an anti-β2-GPI antibody.

A study by Schwartz et al. (260) showed placental growth was reduced and apoptosis was increased in cultured first trimester placental explants, removed from pregnancies terminated for psychosocial reasons, when cultured with serum from non-pregnant patients with PAPS (n=8) compared to cells cultured with serum from non-pregnant healthy controls (n=9). The rate of apoptotic cells was 80% higher in trophoblasts cultured in sera from women with PAPS compared to the rate of apoptotic cells in trophoblasts cultured in sera from non-pregnant healthy women. On the second, third
and fourth day of culturing trophoblast cells with the sera from PAPS patients and healthy controls a significant reduction in microscopic proliferation was seen in cells cultured with PAPS patient sera compared to healthy control sera.

Di Simone et al. (263) have examined the effect of aPL on Bcl2 (an anti-apoptotic protein) and Bax (which promotes cell death) in ex vivo trophoblast cells obtained from the placentae of healthy women after uncomplicated full term deliveries. These cells were cultured for up to 72 hours with either a human IgM monoclonal aPL isolated from an APS patient or a control monoclonal antibody. They found that aPL reduced the Bcl2/Bax ratio in cells cultured with monoclonal aPL compared to cells cultured with control monoclonal antibody but had no effect on cell survival. The authors suggest that the reduced Bcl2/Bax ratio may lead to cell apoptosis at a later stage but longer time course studies are required to answer this question.

A study by Mulla et al. (264) has also investigated the effects of two anti-β2GPI murine monoclonal antibodies, ID2 and IIC5, on HTR-8 cell apoptosis. Both antibodies reduced HTR-8 cell viability in a dose dependent manner. They confirmed that the decrease in HTR-8 cell viability in response to these anti-β2GPI antibodies was the result of apoptosis by showing a significant increase in activities of caspase-8, caspase-9 and caspase-3 at the same experimental time point. They also demonstrated that these monoclonal antibodies significantly increased HTR-8 cell cytokine and chemokine production of IL-8, IL-1β, monocyte chemotactic protein-1 (MCP-1) and growth related oncogene-alpha (GRO-α), and that this in turn compromised cell viability. Furthermore, HTR-8 cells treated with conditioned media, taken from trophoblasts treated with either ID2 or IIC5, induced more trophoblast cell death and significantly higher levels of active caspase-3 compared to HTR-8 cells treated with conditioned media from untreated cells (264).

1.7.3 Activation of trophoblast cell surface receptors and signalling pathways

Although there has been considerable research investigating the role of TLRs in aPL mediated activation of monocytes and EC, there has been relatively little research into the role of these cell surface receptors in aPL activation of trophoblasts. In a study described previously, Mulla et al. (264) have shown that disabling the TLR4/MyD88 pathway partially protects trophoblasts from cell death induced by anti-β2GPI antibodies. They transfected the HTR-8 cell line with MyD88 and TLR4 dominant
negative constructs, which compete with endogenous receptor for ligand binding but cannot transduce a signal thereby disabling these intracellular pathways, or with a control vector, which did not disable these intracellular pathways. These cells were then incubated with the murine anti-human β2GPI monoclonal antibodies ID2 and IIC5. At high concentrations (40µg/ml) these antibodies reduced HTR-8 cell viability whereas at lower concentrations of up to 20µg/ml they caused up-regulation of the inflammatory mediators IL-8, MCP-1, GRO-α and IL-1β. The aPL-induced expression of these inflammatory mediators was significantly reduced in trophoblast cells containing either MyD88 or TLR4 dominant negative constructs compared to cells transfected with control vector, supporting the hypothesis that these monoclonal aPL directly trigger a placental inflammatory response via the TLR4/MyD88 pathway. The authors went on to test polyclonal IgG purified from 18 patients with APS. These APS IgG treated cells showed increased expression of IL-8, GRO-α and IL-1β, but not MCP-1, in comparison to cells not treated with any IgG. However, since no cells were treated with control IgG from healthy subjects it is not possible to be sure that this effect is specific to APS IgG. A strength of this study was that samples from APS patients with VT alone (n=6), PM alone (n=6) and with both VT and PM (n=6) were tested in HTR-8 cells. A difference in IL-8 and GRO-α production was seen between HTR-8 cells treated with these APS subtypes with IgG from patients with VT and PM inducing the production of significantly more IL-8 compared to cells treated with IgG from APS patients with VT only. In addition, both IgG from patients with PM only and IgG from patients with VT and PM inducing the production of significantly more GRO-α in HTR-8 cells compared to cells treated with IgG from VT patient only. No difference in IL-1β production was seen in HTR-8 cells treated with the different APS subtypes. Unlike the monoclonal murine antibodies, the polyclonal samples were not tested in cells lacking MyD88 or TLR4 function, so that it was not possible to prove conclusively that these polyclonal IgG are acting via the TLR4 pathway. Overall, this study did show that monoclonal aPL directly trigger a placental inflammatory response via the TLR4/MyD88 pathway and that polyclonal IgG aPL from patients with all forms of APS triggers a similar response in these cells, which is therefore likely (though not certain) to operate via the same mechanism.

Further work by the same authors (252), again described previously, has investigated the underlying mechanism of reduced trophoblast migration by aPL. They examined
the inhibitory effects of the murine anti-human β2GPI monoclonal antibodies (ID2 and IIC5) on migration and IL-6 production of HTR-8 cells transfected with MyD88 and TLR4 dominant negative constructs to disable these intracellular pathways. They found that inhibition of the TLR4 and MyD88 pathways had no effect on the ability of these monoclonal antibodies to reduce IL-6 secretion. Others have also shown the importance of IL-6 in the regulation of trophoblast migration. Jovanovic and Vicovac (265) reported that IL-6 increased migration and the invasive potential of HTR-8 cells due to up-regulation of trophoblast integrins α1, α5 and β1. Dubrinsky et al. (266) have reported that inhibition of endogenous IL-6 in JEG-3 cells, a human placental choriocarcinoma cell line, inhibited migration and invasion. Champion et al. (267) however, have shown that IL-6 is expressed in early pregnancy but reported that neither exogenous nor endogenous IL-6 had an effect on the invasiveness of either HTR-8 cells or EVT isolated from first trimester (8-10 weeks) placental explants.

The work of Mulla et al. (252, 264) has helped us to gain a greater insight into how aPL, specifically two murine monoclonal anti-human β2GPI antibodies, trigger responses in trophoblast cells which in turn can lead to PM in the APS. Whether polyclonal antibodies however, isolated from patients with APS have the same effects on trophoblast cells, with regards to the signalling pathways they activate, remains unanswered.

A study by Redecha et al. (268) showed that PAR-2 is up-regulated in neutrophils from mice treated with IgG purified from APS patients (number of patients not stated) compared to mice treated with IgG purified from healthy controls. They also demonstrated in PAR-2 knock-out mice that the neutrophil activation, trophoblast injury and foetal death previously seen in wild-type mice after addition of APS IgG was negated, implicating this receptor in the pathogenesis of aPL-induced foetal injury.

Overall, the activation of signalling pathways by aPL in trophoblasts has not been extensively studied. Especially when you compare the evidence in trophoblasts to the evidence that exists for the activation of signalling pathways by aPL in EC and monocytes, which was discussed earlier. Studies investigating aPL activation of different cell surface receptors and intracellular signalling pathways need to be carried out so that we can better understand how aPL exert their effects on trophoblasts.
1.8 The role of the endometrium in obstetric APS

Both clinical and experiment evidence exist demonstrating that aPL have direct effects on endometrial cells that make up the inner membrane of the uterus, thereby impairing the process of implantation in the first trimester. Francis et al. (269) examined whether impaired endometrial differentiation before conception contributes to PM in patients with APS. They studied endometrial biopsies from 82 patients with recurrent early pregnancy loss, between cycle days 22 and 26, of whom 24 were aPL positive patients (and hence APS) and 58 were aPL negative. They used quantitative real-time polymerase chain reaction (qRT-PCR) analysis to determine the relative expression levels of transcripts that encode for three major endometrial differentiation markers associated with normal endometrial differentiation in healthy pregnancies: prolactin (PRL), TF and signal transducer and activator of transcription 5 (Stat5). All three of these endometrial differentiation markers transcript levels were significantly lower in endometrial biopsies from aPL positive patients compared to aPL negative patients. Mak and co-workers (270) have similarly shown in vitro two of these endometrial makers, PRL and Stat5 are significantly reduced in cultured human endometrial stroma cells incubated with a murine anti-β2GPI aPL. Therefore, aPL may target the endometrium prior to conception and impair endometrial differentiation, compromise implantation and cause PM in APS patients by reducing Stat5 and PRL expression (271).

Prostaglandins, secreted from endometrial cells, are also important for endometrial differentiation and the early phases of implantation. Pierro and co-workers (272) examined prostaglandin release from cultured decidual cells, obtained from patients undergoing operative laparoscopy for extrauterine pregnancies, treated with pooled aPL purified from patients who were strongly positive for IgG and IgM aCL. The aPL reduced prostaglandin release from these cells in a dose dependent manner and this effect was neutralised by pre-incubation of aPL with human secretory phospholipase A2. Secretory phospholipase A2 is secreted from endometrial cells and hydrolyses membrane phospholipid to initiate prostaglandin formation. Pierro and co-workers showed that aPL bind secretory phospholipase A2 thereby reducing prostaglandin synthesis and hypothesised that this process may lead to impaired implantation and maintenance of early pregnancy. The direct relevance of this study to APS related PM however, is limited due to the type of aPL and the target cells used. In particular, there
is no evidence in the article that the aPL were taken from patient with a history of PM and the decidual cells used were from patients with ectopic pregnancies and so may not share all of the morphological and functional characteristics of endometrial cells.

A fully active complement system is needed to protect both mother and developing foetus from infectious and other toxic agents. The activation of complement has been implicated in aPL mediated PM. Complement regulatory proteins, including decay acceleration factor (DAF), prevent uncontrolled complement activation in successful pregnancies. Francis et al. (269), whose work was described previously, demonstrated reduced mRNA and protein expression of DAF in endometrial biopsies from 24 aPL positive recurrent miscarriage patients compared to 58 aPL negative recurrent miscarriage patients. Animal studies have also implicated complement in aPL mediated effects on endometrial cells. Holers et al. (167) injected pregnant BALB/c mice with IgG purified from 1 APS patient with a history of thrombosis and high aPL titres and identified that these mice experienced increased foetal reabsorption with decidual deposition of IgG and C3 when compared to mice injected with IgG purified from a healthy control.

There is extensive evidence identifying that aPL have direct effects of different target cells, from increasing the expression and activity of TF on monocytes to decreasing the migration and invasion ability of trophoblasts. It is unclear however, whether aPL that result in VT alone or PM alone have different mechanisms of action upon these target cells. For instance, do aPL from patients with PM alone primarily act upon trophoblast and endometrial cells whilst aPL from patients with VT alone act solely upon EC, monocytes and platelets to promote a pro-coagulant state. The majority of studies investigating the effects of aPL on these target cells have not distinguished clearly the effects of these subgroups of aPL.
1.9 Aims of PhD

The primary objective of my PhD was to compare the effects of IgG purified from aPL positive patients with different clinical manifestations of the APS on the cellular activation of a pregnancy related cell type; trophoblast cells and a thrombotic related cell type; EC.

There are many studies investigating the role of different signalling pathways in aPL mediated activation of thrombotic cell types. On the whole a comparatively limited number of pathways have been investigated and TLR in particular have conflicting evidence as to which is involved. There is evidence of patients with thrombotic APS having differential effects on signalling pathways in monocytes but there are limited similar studies on PM relevant cells or in EC. The intracellular signalling pathways activated in pregnancy related cell types associated with APS and whether these are differentially activated by aPL in patients with PM alone compared to those who only experience VT is poorly characterised. My hypothesis is that aPL from patients with VT alone are likely to have different properties than aPL derived from patients with PM alone, which has been recently confirmed by work at UCL using a human monocyte cell line (204).

The aim of my PhD was to compare the effects of aPL from patients with different clinical manifestations of the APS on the activation of TLR2 and TLR4, TLR adaptor proteins and their related signalling molecules in a trophoblast cell line (HTR-8) and in HUVEC. As well as this I compare the effects of aPL from patients with different clinical manifestations of the APS on the activation of cytokines in both these cell types, the effect of these aPL on trophoblast migration and the expression of CAMS on HUVEC. Time course experiments were carried out in order to examine the effects of aPL on these outcome measures over time so as to gain a greater understanding of how aPL cause cellular injury in the APS.
Chapter II

Materials and Methods
Materials and Methods

2.1 Patients and controls

Serum samples were obtained by informed consent from patients under my supervisors and Dr Hannah Cohen’s care at University College London Hospital, London, UK and through collaboration with Professor Silvia Pierangeli at University of Texas Medical Branch, Galveston, USA, and Professor Pier Luigi Meroni at University of Milan, Milan, Italy. All patients fulfilled APS classification criteria (described in Introduction 1.2.1) (32). Serum samples were collected from patients with different clinical manifestations of the APS: patients with VT only (VT+/PM-) and patients with PM only (VT-/PM+). Control serum samples were collected from individuals who fulfilled the American College of Rheumatology classification criteria for SLE (273) who were aPL positive but did not have APS (aPL+/APS-) and healthy individuals who were aPL and APS negative (Healthy control). Table 2.1 illustrates the clinical and laboratory data of the patients and controls selected for use in this study.
### Table 2.1: Clinical and laboratory features of patients and controls

<table>
<thead>
<tr>
<th></th>
<th>VT+/PM- (n=9)</th>
<th>VT-/PM+ (n=7)</th>
<th>aPL+/APS- (n=7)</th>
<th>Healthy Controls (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (Mean ± SEM)</strong></td>
<td>53.3 ± 5.9</td>
<td>43.4 ± 1.9</td>
<td>39.1 ± 4.9</td>
<td>37.6 ± 3</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td>6 F / 3 M</td>
<td>7 F</td>
<td>7 F</td>
<td>7 F / 3 M</td>
</tr>
<tr>
<td><strong>PAPS</strong></td>
<td>6 (66.6%)</td>
<td>6 (85.7%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>SLE</strong></td>
<td>3 (33.3%)</td>
<td>1 (14.3%)</td>
<td>7 (100%)</td>
<td>0</td>
</tr>
<tr>
<td><strong>No. pregnancies</strong></td>
<td>4</td>
<td>24</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td><strong>Live births</strong></td>
<td>4</td>
<td>16</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total APS-related PM</strong></td>
<td>0</td>
<td>7 (6 ST-PL, 1 TT-PL)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Arterial thrombosis</strong></td>
<td>5 (3 CVA, 2 TIA)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Venous thrombosis</strong></td>
<td>5 (4 DVT, 4 PE)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>LA-positive</strong></td>
<td>8</td>
<td>6</td>
<td>4</td>
<td>NT</td>
</tr>
<tr>
<td><strong>Serum aCL (Mean GPLU ± SEM)</strong></td>
<td>144.3 ± 23.4</td>
<td>120.9 ± 14.6</td>
<td>79.4 ± 17.8</td>
<td>6.9 ± 1.4</td>
</tr>
<tr>
<td><strong>Serum anti-β2GPI (Mean SU ± SEM)</strong></td>
<td>83.3 ± 17.3</td>
<td>84.4 ± 28.9</td>
<td>34.3 ± 11.3</td>
<td>3.1 ± 1.5</td>
</tr>
</tbody>
</table>

Clinical and laboratory features of patients and controls used. Abbreviations: aCL, anti-cardiolipin antibodies; anti-β2GPI, anti-β2-glycoprotein-I antibodies; CVA, cerebrovascular accident; DVT, deep vein thrombosis; F, female; GPLU, IgG phospholipid units; LA, lupus anticoagulant; M, male; NT, not tested; PAPS, primary antiphospholipid syndrome; PM, pregnancy morbidity; PE, pulmonary embolus; CVA, cerebrovascular accident; SEM, standard error of the mean; SLE, systemic lupus erythematosus; ST-PL, second trimester pregnancy loss; SU, standard units; TIA, transient ischemic attack; TT-PL, third trimester pregnancy loss.
2.2 Purification and immunological characterisation of IgG

2.2.1 Purification and concentration of IgG

IgG was purified from serum by protein G sepharose affinity chromatography (Thermo Scientific). In summary, the column was washed with 5 mL of binding buffer (0.1 M sodium phosphate pH 7.2). Patient or control serum was diluted 1:2 with binding buffer, filter sterilised and placed in the column, so that serum IgG binds to the protein G within the matrix of the column. The column was then washed with 10 mL of binding buffer and the IgG eluted into 3 mL elution buffer (0.1 M glycine pH 2-3). Glycine in the elution buffer lowers the pH to detach the IgG from the column. The eluted fraction was then neutralised by adding 300 µL 1 M Tris pH 8-9. Finally, the columns were washed with 5 mL of elution buffer and 5 mL of phosphate buffered saline (PBS) and then stored in 5 mL of storage solution (0.02% sodium azide in PBS).

IgG from the collected fraction was concentrated using an Amicon ultra centrifugal filter (Millipore) with a molecular weight cut off of 30 KDa. The eluted fraction from IgG purification was placed in the filter, which was spun at 7,500 g for 20 minutes. This process retains IgG (of molecular weight 150 KDa), which was then washed twice with 2 mL PBS and centrifuged at 7,500 g for 20 minutes, before being transferred to clean microcentrifuge tubes and made up to a total volume of 1 mL with PBS. The concentration of purified and concentrated IgG was determined using the Nanodrop ND-1000 Spectrophotometer (LabTech International).

2.2.2 Endotoxin removal

Patient and control IgG samples were passed through Detoxi-Gel™ Endotoxin removing columns (Thermo Scientific) to remove endotoxin. The column contains immobilised polymixin B that binds the lipid A portion of bacterial lipopolysaccharide and thus removes it from contaminated samples. Endotoxin removal was performed under sterile conditions as follows. Each column was sequentially washed with 5 mL sodium deoxycholate, followed by 5 mL water and then 5 mL PBS. The purified IgG sample was added to the column and the flow through discarded. Following which the IgG was eluted from the column by the addition of 1 mL of PBS and stored at -20°C. The column was then washed with 1% sodium deoxycholate, then water and finally 25% ethanol in which it was stored at 4°C.

The IgG samples were confirmed to be endotoxin free (<0.25 endotoxin units (EU)/ml)
Chapter II: Materials and Methods

by the *Limulus* Amoebocyte Lysate assay. Endotoxin standard (Sigma) was reconstituted in endotoxin free water to obtain a stock concentration of 4000 EU/mL. This stock was then diluted to make a range of standard concentrations, from 400 EU/mL to 0.015 EU/mL in sterile capped polystyrene tubes. Next, E-Toxate (Sigma) was reconstituted in 5 mL endotoxin free water. The E-Toxate will react with endotoxin present in a sample, forming a gel. 100 µL of purified IgG at 500 µg/mL, as well as 100 µL PBS, endotoxin free water and the endotoxin standard serial dilutions (400 EU/mL to 0.015 EU/mL) were placed in non-siliconised glass tubes. Next, 100 µL of E-Toxate was added to each tube and the tubes were incubated at 37°C for one hour. After incubation the tubes were inverted once to determine whether a gel was formed. In the presence of a gel a sample was considered as endotoxin positive to a threshold determined by the endotoxin standard, whilst absence of a gel confirmed that the IgG sample was free of endotoxin. The assay was able to detect levels of endotoxin greater or equal to 0.25 EU/ml, as determined by the endotoxin standard. Endotoxin removal was repeated on those samples where a gel had formed until they were endotoxin negative.

2.2.3 Detection of aCL antibodies

The aCL activity of purified IgG and also the aCL activity of patient and control serum before IgG purification was confirmed by ELISA. A 96 well Polysorb plate (Nunc, Thermo Scientific) was marked vertically to divide it in to a test half and control half. Wells in the test half were coated with 50 µL of 50 µg/mL CL (Sigma) (diluted in ethanol) and wells in the control half of the plate were coated with 50 µL of ethanol alone. Plates were left uncovered and incubated overnight at 4°C. The following day plates were washed twice with PBS and then blocked with 100 µL of 10% FCS/PBS at room temperature for 1 hour to block non-specific binding of Ig to the plastic. Plates were then washed twice with PBS. Next, either purified IgG or patient or control serum was added to the plate. Purified IgG was serially diluted 1:2 with 10% FCS/PBS down 4 wells on both the test half and control half of the plate starting at 500 µg/mL with a final volume of 50 µL per well. In the case of patient and control serum, serum was diluted 1:50 with 10% FCS/PBS and 50 µL of each sample was added in triplicate on both the test half and control half of the plate. 50 µL of a standard containing polyclonal IgG of known CL binding activity defined as GPLU (Louisville APL Diagnostics, Inc. Texas, USA) was added to each plate, on both the test and control half. Plates were incubated
for 90 minutes at room temperature before being washed three times with PBS. Goat anti-human IgG alkaline phosphatase conjugate (Sigma) was diluted 1:1000 in 10% FCS/PBS and 50 µL was added to each well of the plate. Plates were incubated for 1 hour at room temperature before being washed three times with PBS. Finally 50 µL of diethanolamine (DEA) buffer, diluted as follows: 1 mL buffer in 4 mL water plus 1 p-nitrophenylphosphate (p-NPP) tablet (KPL), was added to each well of the plate. Plates were incubated for 30 minutes at room temperature before being read on a TECAN GENios Microplate reader at 405 nm. Background binding to control wells lacking CL was subtracted from binding to CL in corresponding test wells and then expressed relative to the standard as GPLU.

2.2.4 Detection of anti-β2GPI antibodies

The anti-β2GPI activity of purified IgG and the anti-β2GPI activity of patient and control serum before IgG purification was also confirmed by ELISA. A maxisorb plate (Nunc, Thermo Scientific) was marked vertically to divide it in two halves: the test half and the control half. Wells in the test half of the plate were coated with 50 µL of 4 µg/mL β2GPI (Louisville APL Diagnostics, Inc. Texas, USA) diluted in PBS and wells in the control half of the plate were coated with 50 µL of PBS alone. Plates were incubated overnight at 4°C before being washed twice with PBS/0.1% Tween. Plates were blocked with 150 µL of 0.5% gelatin in PBS and incubated for 1 hour at 37°C. Following this step plates were washed three times with PBS/0.1% Tween. Next, either purified IgG or patient or control serum was added to the plate. Purified IgG was serially diluted with 1% bovine serum albumin (BSA)/PBS 1:2 down 4 wells on both the test half and control half of the plate starting at 500 µg/mL with a final volume of 50 µL per well. In the case of patient and control serum, serum was diluted 1:50 with 1% BSA/PBS and 50 µL of each sample was added in triplicate on both the test half and control half of the plate. 50 µL of an in house standard diluted at 1:400 was also used and serially diluted (1:2) down 6 wells on both the test half and control half of the plate. The in house standard is a patient with positive aPL but no APS. 100 standard units (SU) of the in house standard is equivalent to 125 ng/mL HCAL (IgG Sapporo standard-Centre for Disease Control, USA). Plates were incubated for 1 hour at room temperature and then washed three times with PBS/0.1% Tween. Goat anti-human IgG alkaline phosphatase conjugate (Sigma) was diluted 1:1000 in 1% BSA/PBS and 50 µL was added to each well of the plate. Plates were incubated for 1 hour at room temperature before being washed three times with PBS.
temperature before being washed three times with PBS/0.1% Tween. Finally 50 µL of DEA buffer, diluted as for aCL ELISA was added to each well of the plate and incubated for 30 minutes at room temperature before being read on a TECAN GENios Microplate reader at 405 nm. Background binding to control wells lacking β2GPI was subtracted from binding to β2GPI in corresponding test wells and then expressed relative to the in-house standard as SU.

2.3 Cell culture

2.3.1 HTR-8

The HTR-8 cell line immortalised by SV40, were a kind gift from Dr Charles Graham (Queens University, Kingston, ON, Canada). The HTR-8 cell line was established by introducing the gene encoding simian virus 40 large T antigen into first trimester human trophoblasts (248). HTR-8 cells were grown and maintained in RPMI 1640 (Gibco), supplemented with 10% FCS (PAA Laboratories) and 100 units/mL penicillin 100 µg/mL streptomycin (pen/strep) (Gibco) at 37°C/5% CO₂.

2.3.2 HUVEC

Umbilical cords were obtained by informed consent from women with healthy pregnancies undergoing elective cesarean sections at University College London Hospital. Cords were collected in RPMI supplemented with pen/strep and 1.25 µg/mL Amphotericin B (Gibco) and kept at 4°C until cells were extracted later that day. HUVEC were extracted from umbilical cords under sterile conditions as follows. Cords were first washed in pre-warmed RPMI (containing pen/strep and amphotericin B) and any excess blood was drained from the cord, before one end of the cord was clamped shut. At the other end of the cord, the umbilical vein was cannulated and the cord was washed through twice with RPMI (containing pen/strep and amphotericin B). After every wash the clamp was taken from the other end of the cord and the cord drained of media. After washing the cord was clamped shut again at one end. EC were dissociated from the vessel wall by passing pre-warmed 0.1% collagenase type II (Gibco) (dissolved in RPMI and filter-sterilised before use) through the cannula into the vein, clamping the cord shut on both ends and incubating the cord for 10 minutes in a sterile 37°C incubator. The cord was then unclamped on one end and cells and collagenase-containing medium were drained from the cord and collected into a centrifuge tube. The vein was next flushed with RPMI containing 10% FCS (plus pen/strep and amphotericin B) to inactivate the collagenase and collected into the
cell/collagenase mixture. Harvested cells were spun at 150 g for 5 minutes at room temperature and the cell pellet was re-suspended in pre-warmed MCDB131 media (Gibco) containing 10% FCS, pen/strep, amphotericin B, 2 mM L-glutamine (Gibco) and 6 µg/mL EC growth supplement (Upstate). HUVEC were seeded on 25 cm² tissue culture (TC) flasks and allowed to grow to 90% confluence at 37°C/5% CO₂; these cells were considered to be at passage 0 (P-0). Once confluent cells were split into a 75 cm² TC flask and grown and maintained in this size flask. Experiments were carried out using 80% confluent cells at passages between P-2 to P-3.

2.4. In Vitro exposure of HTR-8 and HUVEC to patient and control IgG

2.4.1 HTR-8

The night before each experiment HTR-8 cells were plated out in 6 well TC plates at 4 x 10⁵ cells per well in 2 mL of RPMI, containing 2% FCS and pen/strep. On the day of the experiment medium was changed back to 10% FCS levels. In each experiment cells were incubated with 100 µg/mL of patient (VT+/PM- or VT-/PM+) or control (aPL+/APS- or healthy control) purified IgG or 10 ng/mL TNF-α (Sigma) or 10 µg/mL LPS (Sigma) for 15 minutes to 72 hours. In some experiments HTR-8 cells were pretreated for 1 hour with either 10 µg/mL of the inhibiting polyclonal anti-human TLR2 antibody (InvivoGen) or 1 µM of the TLR4 inhibitor CLI-095 (InvivoGen) or 1 µg/mL of the TLR4 antagonist Ultra Pure Rhodobacter sphaeroides LPS (InvivoGen). After incubation with IgG cells were processed and either, protein extracted for western blot; RNA extracted for qRT-PCR; cells stained for flow cytometry analysis; or supernatant used in an ELISA.

2.4.2 HUVEC

The night before each experiment HUVEC were plated out in 12 well TC plates at 1 x 10⁵ cells per well in 1 mL of MCDB131 media containing 2% FCS, pen/strep, amphotericin B, L-glutamine and EC growth supplement. On the day of the experiment medium was changed back to 10% FCS levels. In each experiment cells were incubated with 200 µg/mL of patient (VT+/PM- or VT-/PM+) or healthy control purified IgG or 10 µg/mL LPS for 15 minutes to 72 hours. After incubation with IgG cells were processed and either, protein extracted for western blot; RNA extracted for qRT-PCR; cells stained for flow cytometry analysis; or supernatant used in a Milliplex.
2.5 Measurement of IgG induced cell activation by western blot

2.5.1 Protein extraction

Cell extracts were prepared as follows. The 6 well TC plates were placed on ice and each well was washed with ice cold PBS. Lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA 1% NP-40, 0.1% SDS, 0.5% Na-Deoxycholate, 10 mM NaF, 1 mM Na3VO4, and complete mini protease inhibitor cocktail tablets [Roche]) was then added to each well for 10 minutes on ice. Cells were removed by scraping from each well and transferred to ice cold microcentrifuge tubes, following which cells were lysed by passage through a 26 gauge needle five times and then placed on ice for a further 15 minutes. Next lysates were spun at 16,000 g at 4°C for 5 minutes to pellet and remove cell debris. Cell lysate supernatants were then transferred to microcentrifuge tubes and stored at -20°C for future analysis.

2.5.2 Determination of protein concentration

Protein concentration of the cell lysate was determined using the bicinchoninic acid (BCA) protein Assay (Thermo Scientific). Protein standards were made by diluting a 2 mg/mL Albumin Standard to eight concentrations ranging between 2,000 µg/mL to 25 µg/mL. Working reagent was then prepared by mixing 50 parts of BCA reagent A (containing sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1 M sodium hydroxide) with 1 part BCA reagent B (containing 4% cupric sulphate). 10 µL of the standard was pipetted into wells of a maxisorp plate along with 10 µL of cell lysate supernatants. Next 200 µL of working reagent was added to each well and the plate was incubated at 37°C for 30 minutes. Next the maxisorp plate was read on a TECAN GENios Microplate reader at 560 nm. The protein concentrations of the cell lysate supernatants were determined from the Albumin Standard.

2.5.3 Western blotting for the analysis of signalling pathways

20 µg cell lysate and 8 µL 4x Laemmli buffer (0.25 M Tris pH 6.8, 5% SDS, 40% Glycerol, 0.05% Bromphenol blue and 1% β-Mercaptoethanol) was made up with water to a final volume of 30 µL. Samples were heated to 100°C for 5 minutes to denature the proteins, and then centrifuged at 16,000 g for 3 minutes.

Samples were run on a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions to separate proteins on the
basis of their molecular weight. Gel casting glass plates were assembled onto a setting rig. A resolving gel was made (Table 2.2) and 7 mL poured between the glass plates. The resolving gel was topped up with water saturated butanol and left to set for 20 minutes following which the water saturated butanol was removed by washing with water. Next around 3 mL of stacking gel (Table 2.2) was added on top of the resolving gel and a gel comb inserted to form wells. This gel was then left to set for 20 minutes.

<table>
<thead>
<tr>
<th></th>
<th>Resolving gel (10%)</th>
<th>Stacking gel (5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>5.9 mL</td>
<td>5.5 mL</td>
</tr>
<tr>
<td>30% Acrylamide mix</td>
<td>5 mL</td>
<td>1.3 mL</td>
</tr>
<tr>
<td>Tris</td>
<td>3.8 mL</td>
<td>1 mL</td>
</tr>
<tr>
<td></td>
<td>1.5 M Tris pH 8.8</td>
<td>1 M Tris pH 6.8</td>
</tr>
<tr>
<td>10% Sodium dodecyl sulphate (SDS)</td>
<td>150 μL</td>
<td>80 μL</td>
</tr>
<tr>
<td>10% Ammonium Persulphate</td>
<td>150 μL</td>
<td>80 μL</td>
</tr>
<tr>
<td>Tetramethylethlenediamine (TEMED)</td>
<td>11 μL</td>
<td>11 μL</td>
</tr>
</tbody>
</table>

*Table 2.2: SDS-PAGE components*

Components used to make up the resolving and stacking gels.

Once the stacking gel had set the gel comb was removed and the glass slides containing the gel were placed in the running rig, which contained running buffer (192 mM glycine, 25 mM Tris, 0.1% SDS). Next 10 μL of prestained broad range protein marker (7-175 kDa) ladder (New England BioLabs) was added to the first well of the gel and samples containing 20 μg of protein were added to the remaining wells. Then 150 volts was applied to the gel for approximately 1 hour 30 minutes or until the dye had run to the bottom of the gel.

The resolved proteins were then transferred to a nitrocellulose membrane (Amersham Biosciences) as follows. The gel was carefully removed and placed in a transfer cassette next to nitrocellulose membrane and sandwiched between filter paper and sponges all pre-soaked in transfer buffer (192 mM glycine, 25 mM Tris, 0.1% SDS and 20% Ethanol). The cassette was then placed in an electrophoresis rig containing transfer buffer and 100 volts applied for 1 hour.
Following protein transfer the nitrocellulose membranes were removed from the transfer cassette and blocked with 5% BSA (Sigma) diluted in Tris buffered saline 0.1% tween (TBS-T) for 1 hour on a shaker, to prevent non-specific binding when the primary antibody is added.

Membranes were incubated overnight at 4°C with different dilutions of rabbit or mouse monoclonal or polyclonal anti-human antibodies (Table 2.3). Following overnight incubation with primary antibodies membranes were washed three times with TBS-T. Next membranes were incubated for 1 hour in 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-mouse IgG (Dako) diluted in 5% BSA TBS-T. After incubation with the secondary antibody membranes were washed again three times with TBS-T. Protein bands were visualised by chemiluminescence (GE Healthcare) developed on to photographic film and their intensity quantified by densitometric analysis (QuantityOne software, Biorad, USA). Results were expressed as a ratio of relative expression of phosphorylated protein to total protein.

Phosphorylated and total protein for the same signalling protein were analysed on the same membrane. After incubation and detection of the phosphorylated protein membranes were washed for 5 minutes with TBS-T then the anti-phosphorylated protein antibody was removed by washing twice with 0.2 M sodium hydroxide for 5 minutes. Finally the membrane was washed again with TBS-T before being blocked for 30 minutes in 5% BSA TBS-T. After removal of the antibody specific for phosphorylated protein membranes were incubated overnight with antibody to the total protein and the process was repeated.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution with 5% BSA TBS-T</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-hTLR4</td>
<td>1 µg/mL</td>
<td>R&amp;D systems</td>
</tr>
<tr>
<td>anti-GAPDH</td>
<td>1:500</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>anti-phosphorylated p38 MAPK (Thr&lt;sup&gt;180&lt;/sup&gt;/Tyr&lt;sup&gt;182&lt;/sup&gt;)</td>
<td>1:1000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-total p38 MAPK</td>
<td>1:1000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-phosphorylated NFkB p65 (Ser&lt;sup&gt;536&lt;/sup&gt;)</td>
<td>1:500</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-total NFkB p65</td>
<td>1:500</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-phosphorylated p44/42 MAPK (Erk1/2) (Thr202/Tyr204)</td>
<td>1:2000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-total Erk 1</td>
<td>1:2000</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>anti-phosphorylated Akt (Ser473)</td>
<td>1:2000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-total Akt</td>
<td>1:2000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-phosphorylated IRF3</td>
<td>1:1000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>anti-total IRF3</td>
<td>1:1000</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Murine anti-D1 of β&lt;sub&gt;2&lt;/sub&gt;GPI: mAb16</td>
<td>1:500</td>
<td>A kind gift from Dr. Mike Iverson and Dr. Matt Linnik, La Jolla Pharmaceuticals, CA, USA</td>
</tr>
</tbody>
</table>

**Table 2.3: Antibodies**

List of antibodies and the dilutions to which the nitrocellulose membrane was incubated with.
2.6 Measurement of IgG induced cell activation by quantitative real time polymerase chain reaction

2.6.1 RNA extraction

Cells were washed with 1 mL diethlypyrocarbonate (DEPC) treated PBS (DEPC inactivates RNase enzymes, thus protecting RNA from degradation) before 500 µL of TRIzol (Invitrogen) per well was added and incubated for 5 minutes at room temperature. TRIzol is a monophasic solution of phenol and guanidine isothiocyanate, which maintains the integrity of RNA whilst breaking down cells and cellular components. Next, 100 µL chloroform was added, mixed and incubated for 3 minutes at room temperature before centrifugation at 14,000 g for 15 minutes at 4°C to separate RNA. The clear upper aqueous phase containing the RNA was removed and mixed with 250 µL isopropanol per sample, and left for 5 minutes at room temperature. Samples were then centrifuged at 14,000 g for 10 minutes at 4°C following which the supernatant was removed and 1 mL 80% ethanol added per sample. Samples were mixed before being centrifuged at 5,000 g for 5 minutes at 4°C, following which the supernatant was removed and the pellet air-dried. Next, 20 µL of DEPC treated water and 1 µL RNasin (Promega) was added to each sample. RNA concentration was measured on the Nanodrop ND-1000 Spectrophotometer and samples diluted to 1 µg/µL in DEPC treated water.

2.6.2 Complementary DNA synthesis

0.5 µL random primers (Promega), 1 µg RNA and 1 µL dNTP mix (Promega) were added to a sterile microcentrifuge tube and made up to a total volume of 12 µL with DEPC treated water. This mixture was heated to 65°C for 5 minutes than chilled on ice. 4 µL of 5x first-strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂) and 2 µL 0.1 M Dithiothreitol (DTT) was added to the sample and incubated at room temperature for 2 minutes. Finally 1 µL of SuperScript II Reverse Transcriptase (Invitrogen) was added to the sample, which was incubated at 42°C for 50 minutes. The reaction was then stopped by heating the sample to 70°C for 15 minutes. The complementary (c)DNA was diluted 1:10 with DEPC treated water.

2.6.3 Primer design and optimisation

Primer pairs where either designed using Primer-BLAST (NCBI) or obtained from Applied Biosystems as TaqMan probes. Where possible, primer pairs and TaqMan

Chapter II: Materials and Methods
probes were designed or selected to span exon junctions to avoid amplification of contaminating genomic DNA and to a maximum amplicon size of around 100 base pairs. Dissociation curve analysis was performed on those primers designed using Primer-BLAST to ensure the amplification of specific products and the absence of primer dimers. The sequences of the primers obtained from Primer-BLAST and used for quantitative real time PCR amplification are described in appendix 1, as well as the product codes for the Taq-man probes obtained from Applied Biosystems.

2.6.4 Quantitative real time polymerase chain reaction
cDNA was quantitated using SYBER Green (Invitrogen) or TaqMan (Applied Biosystems) in 20 µL reactions in a 96 well plate. For SYBER Green each reaction contained: 10 µL Platinum SYBR Green master mix (Invitrogen), 5 µL DEPC treated water, 2 µL of a 5 µM forward and reverse primer working solution and 3 µL cDNA. For TaqMan each reaction contained: 10 µL TaqMan master mix (Applied Biosystems), 1 µL TaqMan probe (Applied Biosystems) and 9 µL cDNA. Samples were run on a DNA Engine Opticon continuous fluorescence detector (MJ Research) under the following conditions, for SYBER Green reactions: Initial denaturation: 95°C for 3 minutes, followed by 45 cycles of: 95°C for 30 seconds, 64°C for 30 seconds and 72°C for 30 seconds and for TaqMan reactions: Initial denaturation: 95°C for 10 minutes, followed by 41 cycles of: 95°C for 15 seconds, 60°C for 1 minute. A melt curve was performed from 65°C to 95°C, read every 0.3°C and held for 1 second between reads, in order to ensure only one primer product was made.

Gene expression was determined relative to the housekeeping gene gapdh (glyceraldehyde 3-phosphate dehydrogenase) mRNA using the comparative cycle threshold (Ct) method. In brief, the Ct of the test primer products were normalised to the Ct of the gapdh primer products (ΔCt), following this ΔΔCt was calculated. These ΔΔCt values were used to calculate the fold change (ΔΔCt2) between the test and the gapdh genes. Results were expressed as fold change relative to untreated cells. Calculations were performed in Microsoft Excel following equations provided by Applied Biosystems.
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2.7 Measurement of IgG induced cytokine secretion in cell supernatant

2.7.1 Enzyme Linked Immunosorbent Assay

A number of different commercially available ELISA kits were used in order to detect the presence of cytokines secreted into the supernatant of HTR-8 cells after the addition of patient and control IgG.

2.7.1.1 IL-8 ELISA

An IL-8 sandwich ELISA kit (BD Biosciences) was used to detect the presence of IL-8 in HTR-8 cell supernatants. The kit includes a 96 well plate pre-coated with a monoclonal antibody specific for IL-8. 100 µL of standards or supernatant were added to the wells in duplicate, the plate was covered with a plate sealer and incubated for 2 hours at room temperature. Any IL-8 present in the supernatant binds to the immobilised antibody on the ELISA plate. The wells were washed 5 times with wash buffer and blotted dry. Following washing, streptavidin-horseradish peroxidase conjugate mixed with biotinylated anti-human IL-8 antibody was added to each well, producing an antibody-antigen-antibody “sandwich”. The ELISA plate was incubated for 1 hour at room temperature following which the wash step was repeated 7 times and the plate blotted dry. Next, TMB substrate solution was added, which produces a blue colour in direct proportion to the amount of IL-8 present in the supernatant or standard. After 30 minutes incubation with TMB substrate solution in the dark, stop solution was added and absorbance read at 450 nm on a TECAN GENios Microplate reader. The mean absorbance for each set of duplicate standards and supernatants were calculated and the mean zero standard absorbance was subtracted from each sample. The standard curve was plotted using PRISM software on a log-log graph and the unknown supernatant IL-8 concentrations were extrapolated.

2.7.1.2 IFN-α ELISA

An IFN-α sandwich ELISA kit (PBL Interferon Source) was used to detect the presence of IFN-α in the HTR-8 cell supernatants. The kit includes a 96 well plate pre-coated with a monoclonal antibody specific for IFN-α. 50 µL of standards or supernatants were added to the wells in duplicate, the plate was covered with a plate sealer and incubated for 1 hour at room temperature. The wells were washed once with wash buffer and blotted dry. Next, each well was incubated with an anti-human IFN-α horseradish peroxidase conjugated secondary antibody for 1 hour at room temperature then washed 3 times and blotted dry. Then 50 µL of horseradish peroxidase solution
was added to each well for 1 hour at room temperature, after which the plate was washed four times. Next 100 µL TMB substrate solution was added to each well for 15 minutes in the dark, following which 100 µL of stop solution was added and absorbances read at 450 nm on a TECAN GENios Microplate reader. The mean absorbance for each set of duplicate standards and supernatants was calculated and the mean zero standard absorbance was subtracted from each sample. The standard curve was plotted using PRISM software on a log-log graph and the unknown supernatant IFN-α concentrations were extrapolated.

2.7.1.3 IL-1β ELISA
An IL-1β sandwich ELISA kit (R&D Systems) was used to detect the presence of IL-1β in the HTR-8 cell supernatants. The kit includes a 96 well plate pre-coated with a monoclonal antibody specific for IL-1β. 200 µL of standards or supernatants were added to the wells in duplicate, the plate was covered with a plate sealer and incubated for 2 hours at room temperature. The wells were washed 3 times with wash buffer and blotted dry. Next 200 µL of an anti-human IL-1β horseradish peroxidase conjugated secondary antibody was added to each well. The ELISA plate was incubated for 1 hour at room temperature then washed 3 times. After washing 200 µL of substrate solution was added for 20 minutes in the dark, before 50 µL of stop solution was added. The microwell absorbances were read at 450 nm on a TECAN GENios Microplate reader. The mean absorbance for each set of duplicate standards and supernatants was calculated and the mean zero standard absorbance was subtracted from each sample. The standard curve was plotted using PRISM software on a log-log graph and the unknown supernatant IL-1β concentrations were extrapolated.

2.7.1.4 IL-6 ELISA
An IL-6 sandwich ELISA kit (R&D Systems) was used to detect the presence of IL-6 in the HTR-8 cell supernatants. A mouse anti-human IL-6 capture antibody, diluted to a working concentration of 2 µg/mL in PBS, was added to all wells of a 96 well maxisorb plate (final volume 50 µL per well). The plate was covered with a plate sealer and incubated overnight at room temperature. The following day the plate was washed a total of 3 times with PBS 0.05% Tween and then blotted dry. The plate was blocked for 1 hour at room temperature with 150 µL of 1% BSA diluted in PBS, after which the plate was washed. Next, 50 µL of standards or supernatants were added to the wells in duplicate, the plate was covered with a plate sealer and incubated for 2 hours at room
temperature. Following which 50 µL of biotinylated goat anti-human IL-6 diluted to 50 ng/mL with 1% BSA in PBS was added to each well and the plate was covered and incubated for 2 hours at room temperature. The wash step was then repeated and 50 µL streptavidin-HRP was added to each well and incubated for 20 minutes. The plate was washed for the final time and then 100 µL of substrate solution added for 20 minutes in the dark before 50 µL of stop solution was added. The microwell absorbances were read at 450 nm on a TECAN GENios Microplate reader. The mean absorbance for each set of duplicate standards and supernatants was calculated and the mean zero standard absorbance was subtracted from each sample. The standard curve was plotted using PRISM software on a log-log graph and the unknown supernatant IL-6 concentrations were extrapolated.

2.7.2 Bead-based Milliplex immunoassay
A Milliplex human cytokine magnetic bead panel kit (Millipore) was used to simultaneously detect the presence of TNF-α, IL-8, IL-6 and IL-1β in the cell culture supernatant of HUVEC after the addition of patient and control IgG. This Milliplex technology enables multiplex immunoassays in which one antibody to a specific cytokine is attached to a set of beads with the same colour, and the second antibody to the cytokine is attached to a fluorescent reporter dye label. The use of different coloured beads enables the simultaneous multiplex detection of many other cytokines in the same sample. A dual detection flow cytometer is used to sort out the different assays by bead colours in one channel and determine the cytokine concentration by measuring the reporter dye fluorescence in another channel.

To begin, 200 µL of wash buffer was added to each well of a 96 well plate provided in the kit. The plate was sealed and mixed on a plate shaker for 10 minutes at room temperature. The wash buffer was then decanted and the plate blotted dry. Next, 25 µL of either a standard (as provided in the kit) or cell culture supernatant samples were added to wells. In addition 25 µL of assay buffer was added to wells containing sample and 25 µL of cell culture media was added to the wells containing standard. 25 µL of premixed beads were then added to all wells and the plate was sealed and placed on a plate shaker overnight at 4°C. After overnight incubation, the plate was washed twice by adding 200 µL wash buffer, placing in a hand-held magnet for 1 minute, decanting contents, and then blotting dry. Next, 25 µL detection antibodies were added to each well, the plate sealed and placed on a plate shaker for 1 hour at room temperature,
following which 25 µL of streptavidin-Phycoerythrin was added to each well, the plate sealed and placed on a plate shaker for 30 minutes at room temperature. Following this incubation the plate was washed twice as above and then 150 µL sheath fluid was added to each well. The plate was read on a Luminex 200 (Biorad) and data was analysed using xPONENT software.

2.8 Measurement of IgG induced cell activation by flow cytometry
Flow cytometry was used to detect TLR4 expression on HTR-8 cells and E-selectin and VCAM-1 expression on HUVEC.

2.8.1 Staining of TLR4 on HTR-8 cells
HTR-8 cells were first washed twice with PBS. In order to detach the cells from the bottom of the TC plate without disrupting the cell surface 500 µL of detachment buffer (1 mM EDTA, 0.1% sodium azide diluted in PBS) was added to each well and the cells were left on ice for 15 minutes. Detached cells were transferred to microcentrifuge tubes and spun at 150 g for 5 minutes. The cell pellet was then re-suspended in 100 µL PBS before being transferred to a 96 well round bottom plate. The cells were then washed once with PBS and once with FACS buffer (PBS, 1% BSA, 0.1% sodium azide). This process was done by centrifuging the plate at 500 g for 4 minutes, discarding the supernatant from the wells and re-suspending the cell pellet in 200 µL of either PBS or FACS buffer. After the last wash the cell pellet was re-suspended in 50 µL of TLR4 antibody (mouse anti-human CD284:FITC, Serotec), diluted 1:100 in FACS buffer, or isotype control (FITC IgG2a, BD biosciences), diluted 1:50 in FACS buffer. The cells were incubated in darkness for 30 minutes at 4°C. The cells were then washed twice with FACS buffer and fixed by re-suspending them in 200 µL of FACS buffer containing 2% paraformaldehyde (PFA) and placed in small FACS tubes. The number of HTR-8 cells positive for TLR4 was measured on a LSRFortessa (BD biosciences) and results analysed using FlowJo software (TreeStar).

2.8.2 Staining of E-selectin and VCAM-1 on HUVEC
After the experimental time point had finished HUVEC were washed with HBSS (Gibco) and detached by adding 400 µL trypsin (Gibco). Detached cells were then transferred to microcentrifuge tubes and spun at 150 g for 5 minutes and the cell pellet re-suspended in 100 µL PBS before being transferred to a 96 well round bottom plate. The wells of the plate were washed once with PBS and once with FACS buffer, by
centrifuging the plate at 500 g for 4 minutes, discarding the supernatant from the wells and re-suspending the cell pellet in 200 µL of either PBS or FACS buffer. After the last wash the cell pellet was re-suspended in 50 µL of antibody (PE mouse anti-human CD62E [E-selectin], BD Pharmingen, or PE mouse anti-human CD106 [VCAM-1], BD Pharmingen), diluted 1:100 in FACS buffer, or isotype control (PE mouse IgG1κ isotype control, BD Pharmingen), diluted 1:50 in FACS buffer. The cells were incubated in darkness for 30 minutes at 4°C. The cells were then washed twice with FACS buffer before being re-suspended in 200 µL of FACS buffer containing 1% PFA and placed in small FACS tubes. The number of cells positive for E-selectin or VCAM-1 was measured on an LSRFortessa and results analysed using FlowJo software.

2.9 Measurement of IgG induced HTR-8 cell activation by migration assay

A migration assay was performed in order to assess the ability of HTR-8 cells to migrate through a polymerised collagen layer, thereby sticking to a polycarbonate membrane underneath this collagen layer (Figure 2.1), in the presence of patient and control IgG. Eighteen to twenty-four hours prior to commencing the migration assay HTR-8 cells at 80% confluence were placed in serum free RPMI containing pen/strep. Following incubation in serum free media HTR-8 cells were harvested by washing twice with 10 mL HBSS and then 2 mL of trypsin was added to detach cells from the plate. Cells were then harvested into a centrifuge tube in serum free RPMI to a total volume of 10 mL in order to neutralise the trypsin. Cells were centrifuged at 150 g for 5 minutes and the cell pellet re-suspended in 5 mL of serum free RPMI. A cell count was then performed using a haemocytometer and cells re-suspended, in serum free RPMI, at 5 x 10⁵ cells per mL.

The plates and reagents of a QCM 24-well collagen-based cell invasion assay (Chemicon International) were brought to room temperature. The collagen layer of the invasion chamber insert was rehydrated with the addition of 300 µL of pre-warmed serum free RPMI for 30 minutes. After rehydration 250 µL of the RPMI was carefully removed from the insert, so as not to disturb the collagen-coated membrane, and each insert was placed inside a well of a 24 well TC plate (see Figure 2.1). Next, 250 µL of the 5 x 10⁵ cells per mL HTR-8 cell suspension was added to the insert (final cell concentration per invasion chamber equal to 1.25 x 10⁵ cells) and 500 µL of serum free media was added to the well of the TC plate, outside the insert. IgG from each of the patient or control groups were added to separate invasion chambers. There was also a
migration control where no patient or control IgG was added. The plate was covered and incubated for 48 hours at 37°C/5% CO₂.

Figure 2.1: Collagen based cell invasion assay chamber
Schematic illustration of the QCM collagen-based cell invasion assay in a 24 well TC plate.

After 48 hours, the invasion chamber insert was removed from the TC well and the non-invading cells/media from the top of the insert was removed using a pipette. The invasion chamber insert was placed in a clean well of a 24 well TC plate containing 400 µL of cell stain, in order to stain invading cells that had passed through the collagen layer and stuck to the polycarbonate membrane, and incubated for 20 minutes at room temperature. The invasion chamber insert was then dipped several times into a beaker of water in order to rinse off the cell stain. Whilst the insert was still moist, a cotton-tipped swab was used to remove non-invading cells from the interior of the insert and the inserts were left to air dry. The stained invasion chamber insert was transferred to a clean well of a 24 well TC plate containing 200 µL of extraction buffer for 15 minutes at room temperature. The stain was extracted from the underside of the insert by gently tilting the insert back and forth several times during the incubation. After 15 minutes the invasion chamber insert was removed from the well and 100 µL of the dye mixture was transferred to a 96-well maxisorp plate and the optical density of the wells was read on a TECAN GENios Microplate reader at 560 nm. The percentage of cells migrated was worked out relative to the migration control well where no patient or control IgG was added which was considered to have 100% migration.

2.10 Statistics
For each outcome the experiments were repeated at least three times independently and data are expressed as mean ± the standard error of the mean (SEM) of these triplicates.
The experimental negative control, untreated cells and the experimental positive controls, LPS and/or TNF-α, were used to identify whether the experiment undertaken had worked and so were removed from any statistical analysis. Statistical comparisons were undertaken on data obtained from cells treated with VT+/PM- IgG, VT-/PM+ IgG, aPL+/APS- IgG and healthy control IgG. Data was tested for normality using the Kolmogorov-Smirnov test (when >5 data points) or by plotting data on a normality plot. All data was identified to be normally distributed therefore, statistical analysis was undertaken using one-way analysis of variance (ANOVA) and assessed for overall statistically significance at the 5% level (p<0.05). The unpaired t test was used to make direct comparisons on the four variables, that is cells treated with VT-/PM+ IgG, VT+/PM- IgG, aPL+/APS- IgG and healthy control IgG. The paired t test was used to make direct comparisons of cells treated with IgG ± a signalling inhibitor. Data analysis was performed using the GraphPad Prism software program (GraphPad Software, San Diego, CA).
Chapter III

Effects of IgG derived from patients with different clinical manifestations of the APS on trophoblast signalling pathways
Chapter III: Effects of APS IgG on trophoblast signalling pathways

**Effects of IgG derived from patients with different clinical manifestations of the APS on trophoblast signalling pathways**

Work in this chapter investigates aPL mediated activation of trophoblast cells. Experiments were carried out to compare the effects of aPL from APS patients with thrombotic APS (VT+/PM-) and aPL from APS patients with obstetric APS (VT-/PM+) on cell surface receptors and signalling pathways in trophoblasts. Diverse experimental evidence exists implicating the activation of various different cell surface receptors and intracellular signalling pathways by aPL. Little is known however, regarding the cell surface receptors and intracellular pathways activated by aPL in trophoblast cells and whether these pathways are differentially activated by IgG purified from APS patients with purely thrombotic disease compared to IgG purified from APS patients who have experienced only obstetric APS.

TLR2 and TLR4 have been implicated in numerous studies in aPL activation of EC and monocytes, however little is known about the activation of these TLR in trophoblasts. Therefore, the aim of this work was to investigate TLR2 and TLR4 and their related signalling pathways activated in HTR-8 trophoblast cells by IgG purified from either VT+/PM- or VT-/PM+ APS patients.

Another aim of these experiments was to identify if aPL activate TLR4 in HTR-8 cells via a MyD88 dependent or independent signalling pathway. This aim was achieved by investigating mRNA expression of the TLR adaptor proteins MyD88, TRIF and TRAM and also signalling molecules downstream to either the MyD88 dependent or independent pathways. In the case of the MyD88 dependent pathway the phosphorylation of the signalling molecules p38 MAPK, ERK and NFκB in HTR-8 cells by patient and control IgG was investigated. In the case of the MyD88 independent pathway, that is the TRIF/TRAM pathway the mRNA and protein expression of the signalling molecule IRF-3 as well as the chemokine RANTES and the cytokine IFN-α was investigated. The final aim of the work described in this chapter was to investigate the mRNA expression of PARs by aPL, as PAR signalling has also been implicated in aPL activation of different cell types. Time course experiments were carried out in order to examine the temporal effects of aPL on these outcome measures.
3.1 Patient and control IgG selection and characteristics

Purified IgG from 20 female subjects were selected for use in experiments in this chapter from my cohort of 33 subjects as outlined in section 2.1 of Materials and Methods. Ten samples were selected from patients with APS divided equally into 5 patients with VT only (VT+/PM-) and 5 patients with PM only (VT-/PM+). Of the remaining 10 subjects selected 5 were aPL positive but lacked APS (aPL+/APS-) and 5 were healthy controls. These patient and control samples are referred to as cohort 1 and a single sample of purified IgG from each of the 4 groups (VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls) was produced by pooling IgG from the 5 subjects in that group.

Of the 5 VT+/PM- patients, 3 had a history of arterial thrombosis and 2 had a history of venous thrombosis. Two of the VT+/PM- patients had 2 previous live births each and none of them had experienced any pregnancy complications. Of the VT-/PM+ patients selected, 1 patient had suffered a third trimester pregnancy loss and 4 patients a second trimester pregnancy loss. The aPL+/APS- group all had SLE and 3 individuals had experienced 4 live births between them with no pregnancy complications. In the healthy control group 1 individual had 3 live births and no pregnancy complications.

The ability of purified and endotoxin free IgG to bind CL and $\beta_2$GPI at the experimental concentration (100 $\mu$g/mL) was then determined (Figure 3.1). Purified IgG aCL levels range from 12.7 GPLU to above the cut-off of this assay of 96 GPLU in the VT+/PM- group, from 23.7 GPLU to 86.3 GPLU in the VT-/PM+ group and from 6.8 GPLU to above the cut-off of 96 GPLU in the aPL+/APS- group (Figure 3.1 a). Purified IgG anti-$\beta_2$GPI levels range from 0 SU to 75.7 SU in the VT+/PM- group, from 0 SU to 46 SU in the VT-/PM+ group and from 6.2 SU to 37.8 SU in the aPL+/APS- group (Figure 3.1 b). Healthy controls had no aCL or anti-$\beta_2$GPI activity.
Figure 3.1: IgG aCL and anti-β₂GPI levels for cohort 1

All purified IgG was tested at 100 µg/mL for binding to aCL (a) and anti-β₂GPI (b). IgG samples that have aCL activity above the cut-off of this assay are plotted as the upper limit of the assay (96 GPLU).
3.2 Effects of IgG derived from patients with different clinical manifestations of the APS on TLR2 and TLR4 mRNA and protein expression in HTR-8 cells

HTR-8 cells were treated with 100 µg/mL pooled patient (VT+/PM- or VT-/PM+) or pooled control (aPL+/APS- or healthy control) IgG for 2, 6, and 24 hours following which RNA was extracted from the cells, cDNA synthesised and TLR2 and TLR4 mRNA expression measured by qRT-PCR. Figure 3.2 shows that HTR-8 cells treated with IgG purified from VT-/PM+ APS patients induced the expression of higher amounts of TLR4 mRNA compared to HTR-8 cells treated with IgG purified from VT+/PM- patients. At 24 hours (Figure 3.2 f) a 1.92-fold increase in TLR4 mRNA was seen in VT-/PM+ treated cells compared to cells treated with healthy control IgG, this difference however was not significant. At this same time point there was only a 1.08-fold increase in TLR4 mRNA expression in HTR-8 cells treated with VT+/PM- IgG compared to cells treated with healthy control IgG. This trend, although not as convincing, was also seen for TLR2 mRNA expression at 24 hours (Figure 3.2 c) where VT-/PM+ treated cells induce a 1.9-fold increase in TLR2 mRNA compared to healthy control IgG and in VT+/PM- treated cells there was a 1.5-fold increase in TLR2 mRNA compared to healthy control IgG. None of these differences however reached statistical significance. Mean transcript levels over time, as plotted in Figure 3.2 g and h, show that TLR4 transcript levels are higher than TLR2 transcript levels at 24 hours in VT-/PM+ treated cells.
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Figure 3.2: Effects of patient and control IgG on HTR-8 cell mRNA expression of TLR2 and TLR4

HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows qRT-PCR analysis of TLR2 and TLR4 mRNA expression at 2 hours (a & d), 6 hours (b & e) and 24 hours (c & f). The mean ± SEM of quantitative analysis from four independent experiments is plotted. Graphs g and h portray mean TLR2 and TLR4 transcript expression across the 3 time points. No IgG data comparisons show statistical significance using parametric statistical analysis.
Expression of the cell surface receptor TLR4 was also studied at the protein level by immunoblot. As Figure 3.3 shows, both the positive controls LPS and TNF-α did not increase TLR4 protein expression above that of the untreated control at 2 and 24 hours making it difficult to interpret these data. At 2 hours (Figure 3.3 a) there was no difference in TLR4 protein expression between patient or control groups. At 6 hours (Figure 3.3 b) HTR-8 cells treated with the positive controls LPS and TNF-α did increase TLR4 protein expression above that of the untreated control. At this time point HTR-8 cells treated with APS IgG from both VT+/PM- and VT-/PM+ patients increased TLR4 protein expression 3-fold compared to cells treated with healthy control IgG; however, this difference was not significant. At 24 hours (Figure 3.3 c) HTR-8 cells treated with patient and control IgG did not increase TLR4 protein expression above that of the untreated control and there was no difference in TLR4 protein levels between patient and control IgG treated cells.

An alternative method of measuring TLR4 protein expression was also studied by flow cytometry. Figure 3.4 shows that treatment with any of the patient or control IgG or the positive controls TNF-α and LPS increased the percentage of cells that tested positive for TLR4. For example, at 24 hours (Figure 3.4 e) there was 26.8% TLR4 positivity in untreated cells compared with approximately 60% TLR4 positivity in cells treated with patient or control IgG or the positive controls. There were no differences in TLR4 expression between HTR-8 cells treated with patient or control IgG at any of the time points investigated. This finding was also true for the MFI (mean fluorescent intensity). The MFI was lower in untreated cells (MFI of 199 at 24 hours) than in cells treated with patient or control IgG or the positive controls (MFI of around 233 at 24 hours); however, there was no difference in the MFI of TLR4 between those HTR-8 cells treated with patient or control IgG.
Figure 3.3: Effects of patient and control IgG on TLR4 protein production in HTR-8 cells
HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows western blot analysis of TLR4 phosphorylation at 2 hours (a), 6 hours (b) and 24 hours (c). The mean ± SEM of quantitative analysis from four independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
Figure 3.4: Effects of patient and control IgG on HTR-8 cell TLR4 expression
HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows percentage cell or mean fluorescent intensity (MFI) of TLR4 expression measured by flow cytometry at 2 hours (a & b), 6 hours (c & d) and 24 hours (e & f). The mean ± SEM of quantitative analysis from three independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
3.3 Effects of IgG derived from patients with different clinical manifestations of the APS on TLR4 MyD88 dependent signalling pathways in HTR-8 cells

I next examined the activation of the TLR4 MyD88 dependent signalling pathway by aPL from patients with different clinical manifestations of the APS. Firstly mRNA expression of MyD88 in HTR-8 cells after treatment with pooled patient or control IgG was investigated. As shown in Figure 3.5, HTR-8 cell mRNA expression of MyD88 was not increased following addition of either patient or control IgG at 2, 6 or 24 hours. TNF-α increased mRNA expression of MyD88 at 6 and 24 hours compared to cells treated with healthy control IgG. LPS however, did not increase MyD88 mRNA expression.

In addition to investigating MyD88 mRNA production, I examined the phosphorylation of TLR4 MyD88 dependent signalling molecules p38 MAPK and p65 NFκB. Figure 3.6 shows that neither IgG from VT+/PM- APS patients nor IgG from VT-/PM+ APS patients increased p38 MAPK or NFκB p65 phosphorylation at 15 minutes and 2 hours. All groups tested (VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls) demonstrated relative expression levels of phosphorylated to total p38 MAPK and NFκB p65 comparable to the untreated control sample at these time points. By 6 hours all patient and control IgG groups tested increased p38 MAPK or NFκB p65 phosphorylation comparable to TNF-α.

The phosphorylation of other TLR4/MyD88 dependent pathways Erk and also Akt, which is part of the TLR2 dependent pathway, were analysed. Akt was investigated to examine the possibility that aPL may signal through a TLR2 dependent pathway. Figure 3.7 shows that the relative phosphorylation of Akt and Erk was not increased beyond levels seen in untreated HTR-8 cells by any patient or control IgG or TNF-α at any of the time points tested. In fact the high levels of phosphorylated Erk and Akt shown in the untreated sample suggest that these proteins had already reached maximal phosphorylation before the addition of patient or control IgG.
Figure 3.5: Effects of patient and control IgG on HTR-8 cell mRNA expression of MyD88
HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows qRT-PCR analysis of MyD88 mRNA expression at 2 hours (a), 6 hours (b) and 24 hours (c). The mean ± SEM of quantitative analysis from four independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
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Figure 3.6: Effects of patient and control IgG on the phosphorylation of p38 MAPK and NFκB p65 in HTR-8 cells

HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls as well as the positive control TNF-α (10 ng/mL). Graph shows western blot analysis of p38 MAPK and NFκB p65 relative expression at 15 minutes (a & b), 2 hours (c & d) and 6 hours (e & f). The mean ± SEM of quantitative analysis from four independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
Figure 3.7: Effects of patient and control IgG on the phosphorylation of Akt and Erk in HTR-8 cells

HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls as well as the positive control TNF-α (10 ng/mL). Graph shows western blot analysis of Akt and Erk relative expression at 15 minutes (a & b), 2 hours (c & d) and 6 hours (e & f). The mean ± SEM of quantitative analysis from four independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
3.4 Effects of IgG derived from patients with different clinical manifestations of the APS on TLR4 non-MyD88 dependent signalling pathways in HTR-8 cells

I next set out to investigate whether IgG from APS patients activated HTR-8 cells via the TRIF/TRAM pathway, also known as the non-MyD88 dependent pathway. The mRNA expression of TRAM and TRIF in HTR-8 cells incubated with patient and control IgG were measured by qRT-PCR. Figure 3.8 shows that at all the time points tested TRAM mRNA levels did not increase above that of the untreated control after treatment with either patient or control IgG (Figure 3.8 a-c). This finding is also true for TRIF mRNA levels at the 2 hour time point (Figure 3.8 d). At 6 hours however (Figure 3.8 e), there was a 3.4-fold increase in TRIF mRNA expression in HTR-8 cells treated with IgG from VT-/PM+ patients compared with cells treated with healthy control IgG. Interestingly, this increase in TRIF mRNA expression disappeared by 24 hours (Figure 3.8 f) where TRIF mRNA expression was similar in HTR-8 cells treated with either patient or control IgG.
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Figure 3.8: Effects of patient and control IgG on HTR-8 cell mRNA expression of TRAM and TRIF

HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows qRT-PCR analysis of TRAM and TRIF mRNA expression at 2 hours (a & d), 6 hours (b & e) and 24 hours (c & f). The mean ± SEM of quantitative analysis from four independent experiments is plotted. Graphs g and h portray mean TLR2 and TLR4 transcript expression across the 3 time points. No IgG data comparisons show statistical significance using parametric statistical analysis.
In further experiments I examined whether other signalling pathways downstream to TRIF may be activated. One of these signalling molecules, IRF-3, was examined at both the mRNA and protein level. Figure 3.9 shows that at all time points tested there was no increase in the mRNA expression of IRF-3 in HTR-8 cells treated with either of the patient or control IgG. The effects of pooled patient and control IgG on the phosphorylation of IRF-3 in HTR-8 cells were investigated by immunoblot (Figure 3.10). Similar to the findings of IRF-3 mRNA expression Figure 3.10 shows there was no difference in the phosphorylation of IRF-3 in HTR-8 cells treated with either patient or control IgG. In both the mRNA and protein data the positive controls LPS and TNF-α did not increase IRF-3 expression above that of the untreated control making it difficult to interpret these data; therefore, I cannot conclude that APS IgG does not increase IRF-3 expression in HTR-8 cells at either the mRNA or protein level.

RANTES, also known as chemokine (C-C motif) ligand 5 (CCL5) is a chemokine that can be produced as a direct result of activation of the TLR4 non-MyD88 dependent signalling pathway; therefore, I chose to study this chemokine as another parameter to determine if APS patient IgG activate HTR-8 cells through the TLR4 non-MyD88 dependent pathway. The mRNA expression of RANTES after treatment with pooled patient and control IgG was investigated (Figure 3.11). HTR-8 cells treated with the positive controls TNF-α and LPS increased RANTES mRNA expression compared to the untreated sample at all four time points tested. In contrast, HTR-8 cells treated with patient or control IgG did not increase RANTES mRNA expression above that of the healthy control at any time points.
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Figure 3.9: Effects of patient and control IgG on HTR-8 cell mRNA expression of IRF-3

HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows qRT-PCR analysis of IRF-3 mRNA expression at 2 hours (a), 6 hours (b) and 24 hours (c). The mean ± SEM of quantitative analysis from four independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
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Figure 3.10: Effects of patient and control IgG on the phosphorylation of IRF-3 in HTR-8 cells

HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows western blot analysis of IRF-3 relative expression at 2 hours (a), 6 hours (b) and 24 hours (c). The mean ± SEM of quantitative analysis from four independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
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Figure 3.11: Effects of patient and control IgG on HTR-8 cell mRNA expression of RANTES
HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows qRT-PCR analysis of RANTES mRNA expression at 2 hours (a), 6 hours (b) and 24 hours (c). The mean ± SEM of quantitative analysis from four independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
Similar to RANTES, the cytokine IFN-α is produced as a direct result of activation of the TLR4 non-MyD88 dependent signalling pathway. In order to ascertain if the trend in increased TRIF transcript levels in HTR-8 cells treated with IgG from VT-/PM+ APS patients results in the differential production of this cytokine, the production of IFN-α by HTR-8 cells treated with pooled patient or control IgG was investigated using a IFN-α ELISA kit. Unfortunately, as Figure 3.12 shows, both the positive control LPS and TNF-α did not increase IFN-α production at 72 hours above that of the untreated control making it difficult to conclude much from these data. There was no difference however in IFN-α production in HTR-8 cell treated with patient or control IgG (concentration of IFN-α remains around 3 - 3.5 pg/mL).
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Figure 3.12: Effects of patient and control IgG on HTR-8 cell production of IFN-α

HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows IFN-α expression measured in the supernatant by ELISA at 72 hours. The mean ± SEM of quantitative analysis from three independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
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3.5 Can the increased TRIF mRNA expression identified in HTR-8 cells treated with VT-/PM+ IgG be attributed to activation of TLR4?

Given that TRIF and TRAM are adaptor proteins for both TLR3 and TLR4, I investigated whether the increased expression of TRIF mRNA at 6 hours in HTR-8 cells treated with VT-/PM+ IgG was due to the activation of TLR3. Figure 3.12 demonstrates that TLR3 mRNA expression was not significantly increased in HTR-8 cells treated with patient IgG compared to control IgG at 2, 6 and 24 hours.

Next I investigated whether pre-treating cells with an inhibitor of TLR4 would inhibit the effects of VT-/PM+ IgG on TRIF mRNA expression, using CLI-095, a cyclohexene derivative that specifically suppresses TLR4 activation by blocking signalling mediated by the intracellular domain of TLR4. Figure 3.14 shows that pre-treatment with the TLR4 inhibitor CLI-095 inhibited VT-/PM+ IgG induced TRIF mRNA expression in HTR-8 cells. Treatment of HTR-8 cells with VT-/PM+ IgG (but none of the other patient or control IgG) led to an increase in TRIF mRNA expression; however, this effect was completely abrogated with the addition of the TLR4 inhibitor CLI-095 (p=0.039).

A TLR2 neutralising antibody was also used to determine the specificity of the TLR4 inhibition of TRIF mRNA expression in cells treated with VT-/PM+ IgG. Figure 3.15 shows that pre-treatment with the TLR2 neutralising antibody inhibited VT-/PM+ IgG induced TRIF mRNA expression in HTR-8 cells (p=0.026). Pre-treatment with the TLR2 neutralising antibody did not inhibit VT-/PM+ induced TRIF mRNA expression to the same extent as the TLR4 inhibitor, suggesting that either there is some TLR2 mediated effect or the TLR2 neutralising antibody is not specific.
Figure 3.13: Effects of patient and control IgG on HTR-8 cell mRNA expression of TLR3

HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows qRT-PCR analysis of TLR3 mRNA expression at 2 hours (a), 6 hours (b) and 24 hours (c). The mean ± SEM of quantitative analysis from four independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
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Figure 3.14: Effects of patient and control IgG on HTR-8 cell mRNA expression of TRIF with or without TLR4 inhibitor CLI-095

Results of qRT-PCR analysis of HTR-8 cell TRIF mRNA expression at 6 hours following treatment with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL) with or without prior pre-treatment with the TLR4 inhibitor CLI-095. Graph shows mean ± SEM of quantitative analysis from three independent experiments (p=0.039).
Figure 3.15: Effects of TLR4 and TLR2 inhibitors on TRIF mRNA expression in HTR-8 cells treated with patient and control IgG

Results of qRT-PCR analysis of HTR-8 cell TRIF mRNA expression at 6 hours following treatment with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL) with or without prior pre-treatment with the TLR4 inhibitor CLI-095 or a TLR2 neutralising antibody. Graph shows mean ± SEM of quantitative analysis from three independent experiments (p=0.026).
3.6 Effects of IgG derived from patients with different clinical manifestations of the APS on PAR signalling in HTR-8 cells

I investigated if PAR mRNA expression was affected in HTR-8 cells treated with patient IgG compared to cells treated with control IgG. Figure 3.16 shows at 2 hours PAR-1 mRNA levels were significantly increased in HTR-8 cells treated with IgG from both VT+/PM- and VT-/PM+ compared to those cells treated with control IgG with a 3.75- and 3.25-fold increase respectively in PAR-1 mRNA expression compared to cells treated with healthy control IgG. At 6 hours (Figure 3.16 b) this pattern of PAR-1 mRNA expression continued; however, HTR-8 cells treated with aPL+/APS- IgG also increased PAR-1 mRNA expression compared to HTR-8 cells treated with healthy control IgG. By 24 hours (Figure 3.16 c) PAR-1 mRNA expression in HTR-8 cells treated with both patient and control IgG were similar to that of the untreated control. PAR-2 mRNA expression showed a similar trend to PAR-1 mRNA expression at 2 hours (Figure 3.16 d), in that HTR-8 cells treated with VT-/PM+ and VT+/PM- IgG had increased expression of PAR-2 mRNA compared to HTR-8 cells treated with the two control groups. Due to the large error bars however, this result was not significant. At 6 and 24 hours (Figure 3.16 e and f) PAR-2 mRNA expression in HTR-8 cells treated with patient and control IgG remained at similar levels with no significant difference between cells treated with patient or control IgG. As can be seen in the time course curves (Figure 3.16 g and h) HTR-8 cells treated with patient IgG increased PAR-1 and PAR-2 mRNA expression to the greatest extent above that of cells treated with control IgG at the earliest time point, 2 hours, with levels of PAR-2 mRNA expression higher than PAR-1 mRNA expression.
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Figure 3.16: Effects of patient and control IgG on HTR-8 cell mRNA expression of PAR-1 and PAR-2

HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows qRT-PCR analysis of PAR-1 and PAR-2 mRNA expression at 2 hours (a & d), 6 hours (b & e) and 24 hours (c & f). The mean ± SEM of quantitative analysis from four independent experiments is plotted. Graphs g and h portray TLR2 and TLR4 transcript expression across the 3 time points. Significant differences were determined by one-way ANOVA and unpaired t test (*p<0.05, **p<0.01).

2 hours

6 hours

24 hours

Time course curve

Effect of patient and control IgG on HTR-8 cell mRNA expression of PAR-1 and PAR-2
### 3.7 Discussion:
The principal finding of this chapter is that treatment with IgG from VT-/PM+ patients increased TLR4 and TRIF mRNA expression in HTR-8 cells to a greater extent than treatment with IgG from VT+/PM- patients or either of the control IgG groups. I was able to show that this increased TLR4 and TRIF mRNA expression was linked as mRNA expression of TLR3, the only other TLR that is capable of activating the adaptor protein TRIF, was not increased in HTR-8 cells treated with VT-/PM+ IgG. Furthermore, pre-treatment of HTR-8 cells with a TLR4 inhibitor completely abrogated TRIF mRNA expression in VT-/PM+ treated cells.

Various groups have shown that trophoblast cells express functional TLR4 (274). Using dominant negative mutants to inhibit TLR4 and MyD88 signalling Mulla et al. (264) have shown an inflammatory response triggered by anti-β2GPI antibodies, leading to the up-regulation of IL-8, MCP-1, GRO-α and IL-1β, which was dependent upon activation of the TLR4/MyD88 pathway. In a later study however, when investigating aPL effects on HTR-8 cell migration Mulla et al. concluded that the down regulation of IL-6, which caused a reduced HTR-8 cell migration in cells treated with two murine anti-human β2GPI monoclonal antibodies, occurred independently of both TLR4 and its adaptor protein MyD88 (252). I am inclined to believe from my studies and the work of Mulla and colleagues that the signalling pathway activated depends upon the effects the aPL are eliciting on trophoblast cells. It may be that aPL trigger a TLR4/MyD88 pathway to induce the production of inflammatory mediators but trigger the activation of a different pathway to inhibit trophoblast migration.

In my work, investigation of downstream signalling molecules to the TLR4-TRIF signalling cascade was unable to identify the activation of other signalling molecules by IgG purified from APS patients with a history of PM. The signalling molecules investigated were by no means exhaustive. A TLR adaptor protein not investigated was SARM. This adaptor protein can inhibit TRIF mediated activation. One explanation for my findings could be that although aPL from APS patients with a history of PM increased TLR4 and TRIF mRNA expression, it could be that SARM is opposing this increased expression. This explanation may be the reason why I did not see an effect on downstream signalling molecules. It would be worth investigating SARM expression in VT-/PM+ treated HTR-8 cells to see if this is the case.
I studied the protein kinase p38 MAPK as part of the TLR4 MyD88 dependent signalling cascade in HTR-8 cells because it has been studied widely in aPL activation of monocytes (78, 204, 206, 207, 211) and EC (171, 176, 192, 193). Work of colleagues here at UCL have shown a differential effect of APS IgG with IgG from VT+/PM- patients but not IgG from VT-/PM+ patients causing phosphorylation of p38 MAPK in monocytes, leading to up-regulation of TF (204). To my knowledge my work is the first to investigate the activation of p38 MAPK in trophoblast cells by aPL from patients with different clinical manifestations of the APS. In contrast to findings in monocytes and EC, I did not find an increase in the phosphorylation of p38 MAPK in HTR-8 cells treated with IgG from either VT+/PM- or VT-/PM+ APS patients. It is important to note that the VT+/PM- patient IgG used in this study are a selection of the patients used in the study by Lambrianides et al. (204) that showed that VT+/PM- IgG and not VT-/PM+ IgG caused phosphorylation of p38 MAPK in monocytes. Therefore, these VT+/PM- IgG have differential effects in monocytes compared to trophoblasts, that is VT+/PM- IgG increase the phosphorylation of p38 MAPK in monocytes but have no effect on the phosphorylation of p38 MAPK in trophoblast cells.

I identified a significant difference in PAR-1 mRNA expression in HTR-8 cells treated with APS IgG (both VT+/PM- and VT-/PM+) compared to HTR-8 cells treated with control IgG (both aPL+/APS- and healthy control) and a trend for increased PAR-2 mRNA expression in HTR-8 cells treated with APS IgG compared to control IgG. The activation of PARs has been examined within the APS field (214, 268). Lopez-Pedrera and co-workers (214) identified an increase in PAR-1 and PAR-2 mRNA and cell surface expression in monocytes of 62 patients with APS, consisting of 37 patients with previous thrombotic events and 25 patients with recurrent pregnancy losses, compared to monocytes from 20 healthy controls. They also identified PAR-1 and PAR-2 cell surface expression was higher in monocytes from patients with thrombosis only compared to monocytes from patients with no thrombosis (that is they only had recurrent miscarriage). I did not see this same subgroup effect in HTR-8 cells. That is there was no difference in PAR-1 or PAR-2 mRNA expression between HTR-8 cells treated with VT+/PM- or VT-/PM+ IgG. The key differences between work carried out in this chapter and that of Lopez-Pedrera and co-workers is that Lopez-Pedrera identified differential expression of protein at the cell surface, whereas I investigated mRNA expression. Also, Lopez-Pedrera and co-workers investigated PAR expression in a thrombotic cell type, monocytes, taken from APS patients whereas I investigated
PAR expression in an obstetric cell type, trophoblasts, investigating the effects of IgG isolated from thrombotic and non-thrombotic APS patients on a healthy cell line. Overall, the work in this chapter and the work of Lopez-Pedrera and co-workers identify an importance of PARs in aPL activation of both a thrombotic and obstetric cell type.

A limitation of some of my experiments is that the positive controls LPS or TNF-α did not always increase expression levels above that of the untreated control. This makes it difficult to fully interpret the data, especially when the patient and control IgG also did not have an effect. Another limitation of these experiments, which does not enable me to draw a firm conclusion, is that although I did identify a trend that HTR-8 cells treated with IgG from VT-/PM+ increased TLR4 and TRIF mRNA expression above that seen in VT+/PM- treated cells and cells treated with control IgG these results are not statistically significant.

In summary, I have identified a trend that IgG purified from VT-/PM+ patients activate HTR-8 cell mRNA expression of the cell surface receptor TLR4 and its adaptor protein TRIF. Work carried out in this chapter has identified that IgG purified from VT+/PM-patients have differential effects on monocytes than on trophoblast cells in regards to the phosphorylation of p38 MAPK. I have also shown that IgG from APS patients, whether from patients with thrombotic or obstetric disease, significantly activate PAR-1 mRNA expression in HTR-8 cells. This same trend was identified in PAR-2 mRNA expression in HTR-8 cells. The next chapter will investigate outcome measures potentially affected by aPL activation of these signalling pathways in trophoblasts.
Chapter IV

Effects of IgG derived from patients with different clinical manifestations of the APS on trophoblast cytokine production and migration
Effects of IgG derived from patients with different clinical manifestations of the APS on trophoblast cytokine production and migration

Having established the signalling effects of aPL from patients with different clinical manifestations of the APS on the activation of HTR-8 cells, I next sought to measure the effects of these aPL on downstream outcomes. Activation of trophoblasts by aPL has previously been shown to effect trophoblast expression of certain cytokines and chemokines. The first aim of the work in this chapter was to examine the expression of IL-8, IL-1β, and MCP-1 in HTR-8 cells treated with aPL. I wanted to investigate if these molecules are differentially activated by IgG purified from APS patients with purely thrombotic disease (VT+/PM-) compared to IgG purified from APS patients who have experienced obstetric APS (VT-/PM+) only.

Another aim of this section of my work was to investigate the effects of APS IgG upon HTR-8 cell migration and the effects of APS IgG on HTR-8 cell migration in the presence of TLR4 inhibitors. In addition, effects of aPL on the production of the cytokine IL-6 and the phosphorylation of Stat3, which have been shown to regulate the ability of trophoblast cells to migrate, was also studied.

4.1 Patient and control IgG selection and characteristics

IgG was purified from a different cohort of 20 patients in this section, referred to as cohort 2. In addition to cohort 2, some experiments in this chapter use IgG samples from cohort 1 (described in Chapter 3). The cohort used for each experiment is depicted in the legend of each figure. Of the 20 subjects in cohort 2, 10 had APS: 5 with VT only (VT+/PM-) and 5 with PM only (VT-/PM+). The other 10 subjects selected were control subjects divided between 5 individuals that have aPL but do not have APS (aPL+/APS-) and 5 healthy controls. HTR-8 cells were exposed to a single sample of purified IgG for each of the 4 groups (VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls) produced by pooling IgG from the 5 subjects in that group.

Of the 5 VT+/PM- patients 3 were male and the other 2 female. Two had a history of arterial thrombosis, 2 had a history of venous thrombosis and 1 had experienced both. Of the VT-/PM+ patients selected in this cohort 1 patient suffered a third trimester
pregnancy loss and 4 patients experienced a second trimester pregnancy loss. The aPL+/APS- group all had SLE, were all female and 3 individuals had 5 live births between them with no pregnancy complications. The healthy control group were all female and 1 individual had 3 live births and no pregnancy complications.

The ability of purified and endotoxin free IgG to bind CL and β2GPI at experimental concentration (100 µg/mL) was then determined (Figure 4.1). Purified IgG aCL levels ranged from 34.7 GPLU to above the cut-off of this assay of 96 GPLU in the VT+/PM- group, from 11.5 GPLU to 88.6 GPLU in the VT-/PM+ group and from 14.2 GPLU to 52.02 GPLU in the aPL+/APS- group. Purified IgG anti-β2GPI activity ranged from 10.1 SU to above the cut-off of this assay of 100 SU in the VT+/PM- group, from 24.2 SU to above the cut-off of this assay of 100 SU in the VT-/PM+ group and from 10.07 to above the cut-off of this assay of 100 SU in the aPL+/APS- group. Healthy controls have no aCL or anti-β2GPI activity.
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Figure 4.1: IgG aCL and anti-β₂GPI levels for cohort 2
All purified IgG was tested at 100 µg/mL for binding to aCL (a) and anti-β₂GPI (b). IgG samples that have aCL or anti-β₂GPI activity above the cut-off of these assay are plotted as the upper limit of the assay (96 GPLU and 100 SU respectively).
4.2 Effects of IgG derived from patients with different clinical manifestations of the APS on the production of cytokines and chemokines in HTR-8 cells

4.2.1 IL-8

Both qRT-PCR and ELISA was used to examine HTR-8 cell expression of IL-8 following incubation with patient and control IgG. As Figure 4.2 shows IL-8 mRNA expression was increased in HTR-8 cells treated with the positive controls TNF-α (at 2 and 6 hours) and LPS (at all time points). In HTR-8 cells treated with patient and control IgG there was no increase in IL-8 transcript levels at any time point tested.

I then measured the effect of patient and control IgG on IL-8 production in cell culture supernatants. Figure 4.3 shows that IL-8 is constitutively expressed (around 50 pg/mL) at all time points tested (2, 6 and 72 hours) in untreated HTR-8 cells. The positive control TNF-α increased expression of IL-8 at 2, 6 and 72 hours. HTR-8 cells treated with patient and control IgG however did not increase expression of IL-8 above basal untreated levels at any of the time points tested.
Figure 4.2: Effects of patient and control IgG on HTR-8 cell mRNA expression of IL-8
HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls (cohort 1) as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows qRT-PCR analysis of IL-8 mRNA expression at 2 hours (a), 6 hours (b) and 24 hours (c). The mean ± SEM of quantitative analysis from four independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
Figure 4.3: Effects of patient and control IgG on HTR-8 cell IL-8 expression
HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls (cohort 1) as well as the positive control TNF-α (10 ng/mL). Graph shows IL-8 expression measured in the supernatant by ELISA at 2 hours (a), 6 hours (b) and 72 hours (c). The mean ± SEM of the duplicate analysis of a single experiment is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
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One possibility for the lack of increased IL-8 expression in HTR-8 cells exposed to APS IgG could be because experiments were undertaken in reduced serum conditions where cells were cultured in 2% FCS and so perhaps a limiting supply of β2GPI. Therefore, I carried out further experiments in increased serum conditions whereby cells were cultured in 10% FCS and also in the presence of healthy control serum as an alternative source of β2GPI, for 72 hours. As Figure 4.4 shows, although an increase in serum conditions (both FCS and healthy serum) did increase relative expression levels of IL-8, there was still no difference in the expression of IL-8 in those HTR-8 cells treated with patient IgG compared to those treated with control IgG. In particular, neither patient nor control IgG activated the mRNA expression or production of IL-8 in HTR-8 cells in different serum conditions.

4.2.2 IL-1β

Next, I investigated the effects of patient and control IgG on the mRNA and protein expression of IL-1β, a cytokine known to induce trophoblast cell death. Figure 4.5 shows that HTR-8 cells treated with the positive controls TNF-α and LPS showed increased mRNA expression of IL-1β at all the time points tested. At the earlier time points of 2 and 6 hours HTR-8 cells treated with both patient and control IgG did not increase IL-1β mRNA expression above that of the untreated control. At 24 hours however (Figure 4.5 c), HTR-8 cells treated with IgG from the patient group VT-/PM+ showed a 1.9-fold increase in IL-1β mRNA expression compared to HTR-8 cells treated with healthy control IgG. This difference however was not statistically significant.

Given the almost two-fold increase in IL-1β mRNA expression in HTR-8 cells treated with VT-/PM+ IgG compared to HTR-8 cells treated with healthy control IgG at 24 hours, I chose to investigate this response at the protein level. As shown in Figure 4.6 however, neither HTR-8 cells treated with the positive controls TNF-α nor LPS or HTR-8 cells treated with patient or control IgG produced detectable levels of IL-1β in cell culture supernatants after 24 or 72 hours of culture.
Figure 4.4: Effects of different serum conditions on the expression of IL-8 in HTR-8 cells treated with patient and control IgG

HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls (cohort 1) as well as the positive control TNF-α (10 ng/mL) for 72 hours in different serum conditions. Graph shows IL-8 expression measured in the supernatant by ELISA. The mean ± SEM of the duplicate analysis of a single experiment is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
Figure 4.5: Effects of patient and control IgG on HTR-8 cell mRNA expression of IL-1β
HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls (cohort 1) as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows qRT-PCR analysis of IL-1β mRNA expression at 2 hours (a), 6 hours (b) and 24 hours (c). The mean ± SEM of quantitative analysis from four independent experiments is plotted. Graph d portrays mean IL-1β transcript expression across the 3 time points. No IgG data comparisons show statistical significance using parametric statistical analysis.
**Figure 4.6: Effects of patient and control IgG on HTR-8 cell IL-1β expression**

HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls (cohort 1) as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows IL-1β expression measured in the supernatant by ELISA at 24 hours (a) and 72 hours (b). The mean ± SEM of the duplicate analysis of a single experiment is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
4.2.3 MCP-1

The mRNA expression of the chemokine MCP-1 was investigated in HTR-8 cells treated with patient and control IgG. Figure 4.7 shows HTR-8 cells treated with TNF-α increased MCP-1 mRNA expression at the earlier time points of 2 and 6 hours. This increase in MCP-1 mRNA expression in TNF-α treated cells decreased by 24 hours. In contrast, HTR-8 cells treated with LPS increased MCP-1 mRNA expression at the later time point of 24 hours. There was no difference however in MCP-1 mRNA expression between cells treated with patient and control IgG at any of the time points investigated.
**Figure 4.7: Effects of patient and control IgG on HTR-8 cell mRNA expression of MCP-1**

HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls (cohort 1) as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows qRT-PCR analysis of MCP-1 mRNA expression at 2 hours (a), 6 hours (b) and 24 hours (c). The mean ± SEM of quantitative analysis from four independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
4.3 Effects of IgG derived from patients with different clinical manifestations of the APS on HTR-8 cell migration

4.3.1 Migration

I next tested the effect of patient and control IgG on the ability of HTR-8 cells to migrate. Given that the migration assay was to be undertaken in serum free conditions I first confirmed that there was β2GPI in the migration assay system by carrying out an immunoblot probing for β2GPI in both the IgG added to the migration assay and in the cell culture supernatant of the assay after the migration assay was finished. Figure 4.8 confirms that β2GPI was present in the migration assay despite the use of serum free media, as β2GPI was detected in both the patient and control IgG (Figure 4.8 a) and in the top and bottom chambers of the migration assay (Figure 4.8 b).

As can be seen in Figure 4.9 HTR-8 cells treated with IgG from VT-/PM+ patients reduced the ability of these cells to migrate by 22.4% compared to untreated controls. HTR-8 cells treated with IgG from VT+/PM- patients however, did not have any effect of HTR-8 cell migration and migration levels remained at 105.5% compared to untreated controls. Using the unpaired t test statistical significance was seen between migration in HTR-8 cells treated with VT+/PM- IgG and VT-/PM+ IgG (p<0.001) and between HTR-8 cells treated with VT-/PM+ IgG and healthy control IgG (p<0.05).
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Figure 4.8: Investigating the presence of $\beta_2$GPI in the migration assay system
Immunoblot to detect the presence of $\beta_2$GPI in (a) patient and control IgG or (b) supernatant taken from the top and bottom wells of the migration assay.

Figure 4.9: Effects of patient and control IgG on HTR-8 cell migration
The ability of HTR-8 cells to migrate after treatment with 100 μg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls (Cohort 2) was measured using a transwell migration assay after 48 hours. Untreated cell migration was set at 100% and the relative migration of HTR-8 cells exposed to IgG from patient and control groups was analysed from this. Graph shows mean ± SEM of quantitative analysis from six independent experiments. Statistical significance shown using the unpaired t test (*p<0.05, ***p<0.001)
4.3.2 Can IL-6 and Stat3 be linked to HTR-8 cell migration

Trophoblast migration has been linked to IL-6 production and Stat3 phosphorylation. It has been shown that aPL limit trophoblast migration by reducing IL-6 production and Stat3 activity (252). Therefore, I treated HTR-8 cells with 100 µg/mL of patient and control IgG (cohort 2) for 2, 6 and 24 hours and measured IL-6 mRNA expression by qRT-PCR (Figure 4.10). HTR-8 cells treated with TNF-α increased IL-6 mRNA expression to the greatest extent at the earlier time points of 2 and 6 hours, whereas HTR-8 cells treated with LPS increased IL-6 mRNA expression to the greatest extent at the later time point tested of 24 hours. There was no change in IL-6 mRNA expression at 2, 6 and 24 hours from that of the untreated control in HTR-8 cells treated with patient or control IgG.

I then examined the baseline secretion of IL-6 over time in untreated HTR-8 cells and the production of IL-6 in HTR-8 cells treated with patient and control IgG (cohort 2). Figure 4.11 a shows untreated HTR-8 cells constitutively secreted IL-6, with levels accumulating in the cell culture supernatants over time. Similar to my IL-6 mRNA expression findings, HTR-8 cells treated with TNF-α and LPS increased IL-6 secretion into the cell culture supernatant. There was no significant effect, at both time points tested, on IL-6 production in HTR-8 cells treated with patient or control IgG (Figure 4.11 b and c).

The effects of patient and control IgG (cohort 2) on the phosphorylation of Stat3 in HTR-8 cells were measured by immunoblot. Figure 4.12 indicates that polyclonal IgG purified from either VT+/PM- or VT-/PM+ APS patients or control IgG had no effect on the phosphorylation of Stat3 at 48 hours.
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Figure 4.10: Effects of patient and control IgG on HTR-8 cell mRNA expression of IL-6
HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls (cohort 2) as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows qRT-PCR analysis of IL-6 mRNA expression at 2 hours (a), 6 hours (b) and 24 hours (c). The mean ± SEM of quantitative analysis from four independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
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Figure 4.11: Effects of patient and control IgG on HTR-8 cell IL-6 expression

HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls (cohort 2) as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows IL-6 expression measured in the supernatant by ELISA in untreated cells at different time points (a), or after treatment with patient and control IgG at 48 hours (b) and 72 hours (c). The mean ± SEM of quantitative analysis from three independent experiment is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
Figure 4.12: Effects of patient and control IgG on the phosphorylation of Stat3 in HTR-8 cells
HTR-8 cells were treated with 100 µg/mL pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls (cohort 2) as well as the positive controls TNF-α (10 ng/mL) and LPS (10 µg/mL). Graph shows western blot analysis of Stat3 relative expression at 48 hours. The mean ± SEM of quantitative analysis from three independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
4.3.3 The effect of inhibiting TLR4 on the migration of HTR-8 cells treated with IgG derived from patients with different clinical manifestations of the APS

To establish if VT-/PM+ IgG inhibits HTR-8 cell migration via a TLR4 dependent signalling pathway I investigated whether treating HTR-8 cells with a TLR4 inhibitor blocked the ability of VT-/PM+ IgG to inhibit HTR-8 cell migration. HTR-8 cells were pre-treated with the TLR4 inhibitor CLI-095, or the TLR4 antagonist *Rhodobacter sphaeroides* LPS prior to incubation with either patient or control IgG in the transwell migration assay for 48 hours (Figure 4.13). Pre-treatment of HTR-8 cells with either of the TLR4 inhibitors resulted in restoration of the ability of HTR-8 cells treated with IgG from VT-/PM+ patients to migrate. In HTR-8 cells treated with VT-/PM+ IgG the ability of these cells to migrate, as measured by the transwell migration assay, was reduced by 22.4% compared to untreated controls. Pre-treatment with the TLR4 inhibitors CLI-095 and *Rhodobacter sphaeroides* LPS resulted in migration levels of HTR-8 cells treated with VT-/PM+ IgG of 136.8% and 120.4% respectively. This result was shown to be statistically significant using the one-way ANOVA test (p=0.0234) and paired student t test between HTR-8 cells treated with VT-/PM+ IgG and VT-/PM+ IgG plus the TLR4 inhibitor CLI-095 (p<0.05).
Figure 4.13: Effects of patient and control IgG on HTR-8 cell migration in the presence of TLR4 inhibitors

The ability of HTR-8 cells to migrate after treatment with pooled IgG from VT+/PM-, VT-/PM+, aPL+/APS- and healthy controls (Cohort 2) in the presence of the TLR4 inhibitor CLI-095 and the TLR4 antagonist Rhodobacter sphaeroides LPS, was measured using a transwell migration assay after 48 hours. Untreated cell migration was set at 100% and the relative migration of the patient and control groups in the presence and absence of inhibitor/antagonist was analysed from this. Graph shows mean ± SEM of quantitative analysis from three independent experiments in the presence of inhibitor/antagonist. Significance determined using one-way ANOVA (p=0.0234) and the paired t test (* = p<0.05)
4.4 Discussion

The primary aim of this chapter was to investigate outcome measures in aPL activation of HTR-8 cells downstream of signalling pathways investigated in the previous chapter. The key finding of this chapter was that HTR-8 cell migration was reduced in cells treated with IgG from patients with VT-/PM+ but not in cells treated with IgG from patients with VT+/PM-, or either of the control groups. The inhibition of migration in HTR-8 cells treated with VT-/PM+ IgG was restored in cells pre-treated with a TLR4 inhibitor. The migration ability of trophoblast cells after incubation with IgG from APS patients is well presented in the literature (246, 250-253), my study however is the first to directly compare the effects of IgG from patients with different clinical manifestations of the APS on the ability of HTR-8 cells to migrate and identified that only aPL from obstetric APS patients and not aPL from thrombotic APS patients inhibit HTR-8 cell migration.

EVT cell invasion is highly regulated and disruption of its tight control is associated with various pregnancy complications such as early and late miscarriage (275), pre-eclampsia (276) and foetal growth restriction (277). Despite its importance for successful pregnancy the factors that control trophoblast cell invasion are poorly understood (267). I examined IL-6 production and Stat3 phosphorylation as a mechanism for reduced HTR-8 cell migration in VT-/PM+ treated HTR-8 cells. Recent studies provide some evidence that the cytokine IL-6 may play a role in EVT invasion (265, 266). Specifically in the field of APS, a study by Mulla et al. (252) demonstrated that two murine anti-human β2GPI monoclonal antibodies limit HTR-8 cell migration by impairing HTR-8 cell constitutive IL-6 secretion and reducing Stat3 phosphorylation. In contrast, I found that the mRNA and protein expression of IL-6 was unchanged in HTR-8 cells treated with patient or control IgG. Therefore, this cytokine is unlikely to have had any effect on the reduction of trophoblast migration seen in HTR-8 cells treated with VT-/PM+ IgG. Furthermore, I did not find any difference in the phosphorylation of Stat3 in HTR-8 cells treated with patient and control IgG. My results are supported by recent data published by Champion et al. (267) who found that IL-6 is expressed in early pregnancy, but reported that neither exogenous nor endogenous IL-6 had an effect on the invasiveness of either HTR-8 cells or EVT isolated from first trimester (8-10 weeks) placental explants.
I investigated HTR-8 cell migration in cells treated with patient and control IgG following pre-treatment with TLR4 inhibitors. I found that both pre-treatment with the TLR4 inhibitor CLI-095, and the TLR4 antagonist *Rhodobacter sphaeroides* LPS restored trophoblast migration in HTR-8 cells treated with VT-/PM+ IgG to levels seen in cells treated with control IgG. These results suggest that regulation of trophoblast migration operates through activation of TLR4. This data contrasts with the results of Mulla *et al.* (252) who reported that the down regulation of IL-6 which caused a reduced trophoblast migration in HTR-8 cells treated with two murine anti-human β2GPI monoclonal antibodies occurred independently of both TLR4 and its adaptor protein MyD88. The key difference between my experiments and those carried out by Mulla and co-workers (252) is that I used polyclonal IgG purified from patients with the APS whereas Mulla and co-workers used murine anti-human β2GPI monoclonal antibodies. My work eliminates cross-species reactivity as I am using human aPL on a human cell line whereas Mulla and co-workers are using murine monoclonal antibodies on a human cell line.

Both clinical observations (244, 278) and experimental findings (252, 264) suggest that pregnancy failure in APS patients may involve an inflammatory response at the maternal-foetal interface. Therefore, I examined the activation of IL-8, IL-1β, and MCP-1 in HTR-8 cells treated with IgG from APS patients with either thrombotic or obstetric APS. IL-8 is an inflammatory mediator that is constitutively expressed by first trimester trophoblasts and HTR-8 cells (279). Mulla *et al.* (264) have previously shown that IgG purified from patients with VT only, PM only and patients who have experienced both VT and PM up-regulate HTR-8 cell IL-8 expression compared to levels seen in untreated cells. The authors also report evidence of differential effects of IgG from APS clinical subgroups, with a significant increase in IL-8 expression identified in cells treated with IgG from patients who have experienced both VT and PM compared to patients who have only experience PM. A limitation to their study is that they did not use any healthy control or any other control IgG for comparison to show this was not a non-specific effect of IgG.

The experiments carried out in this chapter sought to examine IL-8 expression following aPL activation of HTR-8 cells by both qRT-PCR and ELISA. Neither at the mRNA nor protein level was IL-8 increased above that seen in the untreated sample in HTR-8 cells treated with IgG from patients or controls. Even when I sought to change
the serum levels in the culture medium to the levels used by Mulla et al. (264) there was no increase in IL-8 expression from HTR-8 cells treated with patient or control IgG compared to the untreated sample and certainly no difference seen between patient groups as reported by Mulla et al. (264). Another key difference between experiments carried out in this chapter and the experiments carried out by Mulla et al. (264) was that I used an IgG concentration of 100 µg/mL to treat HTR-8 cells whereas Mulla and co-workers treated HTR-8 cells with far less IgG, at 12.5 µg/mL. In addition to experiments carried out utilising patient derived aPL, Mulla et al. (264) also carried out similar experiments using two murine anti-β2GPI monoclonal antibodies. In these studies the authors did use a control murine IgG and were able to show that this control IgG did not increase IL-8 expression to the increased levels seen when HTR-8 cells were treated with two murine anti-β2GPI monoclonal antibodies.

IL-1β, a cytokine known to induce trophoblast cell death (280), was also investigated. Mulla et al. (264) showed that aPL from patients with VT only, PM only and both VT and PM up-regulate HTR-8 IL-1β expression when compared to untreated cells. Similar to IL-8, I wanted to investigate whether I could replicate these results this time using healthy control IgG as a control. The experiments carried out in this chapter sought to examine IL-1β expression following aPL activation of HTR-8 cells by both qRT-PCR and ELISA. Results of qRT-PCR at 24 hours showed that HTR-8 cells treated with IgG from the patient group VT-/PM+ increased IL-1β mRNA expression compared to control groups and the VT+/PM- patient group (data not significant, one-way ANOVA p=0.53). This finding was an interesting trend seen in the VT-/PM+ treated cells; however, when expression of IL-1β in cell culture supernatants was analysed relatively little IL-1β was produced in both untreated cells and cells treated with patient and control IgG. In fact relative levels of IL-1β shown in experiments by Mulla et al. (264) are similar to that seen in these experiments (around 6 pg/mL), but unlike Mulla and co-workers I did not identify a difference between the untreated HTR-8 cells and cells treated with APS IgG. Even though Mulla et al. (264) identified a difference in IL-1β levels between untreated controls and HTR-8 cells treated with APS IgG the relative levels of IL-1β are so low (6 pg/mL) I believe they may not be biologically significant.
Mulla et al. (264) also investigated the activation of the chemokine MCP-1. Both Mulla and co-workers and data in this chapter identified that IgG purified from APS patients had no effect on the mRNA expression (studied in this chapter) or protein expression (studied by Mulla et al. (264)) of MCP-1 in HTR-8 cells when compared to untreated controls.

In results from both this and the previous chapter I have shown that aPL from patients with obstetric APS have differential effects on a cell type related to pregnancy loss, HTR-8 cells, compared to aPL from patients with only thrombotic APS. The next chapter will investigate whether the reverse is true for a cell type more relevant to VT. That is whether IgG purified from APS patients with only thrombotic manifestations have differential effects on activation of signalling pathways, cytokine production or expression of CAMS in HUVEC compared to IgG purified from APS patients with only obstetric manifestations.
Chapter V

Effects of IgG derived from patients with different clinical manifestations of the APS on EC signalling pathways, cytokine production and expression of CAMS
Chapter V: Effects of APS IgG on EC

Effects of IgG derived from patients with different clinical manifestations of the APS on EC signalling pathways, cytokine production and expression of CAMS

The activation of EC by aPL has been widely studied within the APS field. The potential involvement however of only a select number of signalling molecules have been studied in aPL mediated activation of EC. Furthermore, these experiments have largely utilised polyclonal IgG isolated from patients with a history of thrombosis only and have not examined the effect of IgG from patients with a history of PM only on this thrombotic cell type. The aim of this chapter was to investigate whether certain signalling molecules are differentially activated in HUVEC by IgG purified from APS patients with purely thrombotic disease (VT+/PM-) compared to IgG purified from APS patients who have experienced only obstetric APS (VT-/PM+). In particular, the mRNA expression of TLR2 and TLR4 was investigated as well as the adaptor proteins MyD88 and TRIF. Downstream of the MyD88 dependent signalling pathway, p38 MAPK and NFκB phosphorylation was also investigated.

Once the effects of aPL on HUVEC signalling were established the next aim of this chapter was to investigate the effect of aPL on downstream outcome measures. The production of the cytokines TNF-α, IL-8, IL-6 and IL-1β as well as VCAM-1 and E-selectin expression on HUVEC following treatment with IgG from patients with different manifestations of the APS was investigated.

5.1 Patient and control IgG selection and characteristics

Patients and healthy control IgG from cohort 2 were used in these experiments (described in chapter IV). The exceptions being that the control group aPL+/APS- was omitted from these studies and HUVEC were treated with IgG at a concentration of 200 ug/mL. The ability of purified and endotoxin free IgG to bind CL and β2GPI at experimental concentration (200 µg/mL) was determined (Figure 5.1). Purified IgG aCL levels ranged from 47 GPLU to above the cut-off of this assay of 96 GPLU in the VT+/PM- group, from 19.8 GPLU to above the cut-off of this assay of 96 GPLU in the VT-/PM+ group and from 0 GPLU to 3.8 GPLU in the healthy control group. Purified IgG anti-β2GPI activity ranged from 20.2 SU to above the cut-off of this assay of 100.
SU in the VT+/PM- group, from 71.8 SU to above the cut-off of this assay of 100 SU in the VT-/PM+ group and from 0 SU to 4.5 SU in the healthy control group.
Figure 5.1: IgG aCL and anti-β2GPI levels of cohort 2 at experimental concentration of 200 µg/mL

All purified IgG was tested at 200 µg/mL for binding to aCL (a) and anti-β2GPI (b). IgG samples that have aCL or anti-β2GPI activity above the cut-off of these assay are plotted as the upper limit of the assay (96 GPLU and 100 SU respectively).
5.2 Activation of HUVEC cell surface receptors and intracellular signalling pathways by IgG derived from patients with different clinical manifestations of the APS

Similar to experiments carried out in trophoblasts, I examined aPL activation of TLRs and downstream signalling molecules in HUVEC to investigate whether IgG from different subsets of patients with APS (VT+/PM- or VT-/PM+) activate these signalling receptors and molecules differentially.

Primarily, the mRNA expression of TLR2 and TLR4 in HUVEC after treatment with 200 µg/mL pooled patient and control IgG was investigated by qRT-PCR. Figure 5.2 shows that HUVEC treated with LPS increased TLR2 mRNA expression at all time points tested and TLR4 mRNA expression at 6 and 24 hours. At 2 hours (Figure 5.2 a) HUVEC treated with VT-/PM+ IgG showed the highest increase, 1.3-fold, in both TLR2 and TLR4 mRNA expression compared to HUVEC treated with healthy control IgG, this difference was no longer seen at 6 and 24 hours. At 6 and 24 hours (Figure 5.2 e & f) HUVEC treated with healthy control IgG showed the highest increase in TLR4 mRNA expression compared to the two patient groups. Overall however there was no significant difference at any time point tested in TLR2 or TLR4 mRNA expression between HUVEC treated with patient or control IgG.

The mRNA expression in HUVEC of the TLR4 adapter proteins MyD88 and TRIF after treatment with 200 µg/mL pooled patient and control IgG was analysed. As Figure 5.3 shows HUVEC treated with LPS increased MyD88 mRNA expression at 6 and 24 hours but not at 2 hours. In contrast HUVEC treated with LPS increased TRIF mRNA expression at 2 hours and 24 hours. HUVEC treated with APS IgG did not increase MyD88 mRNA expression above that of cells treated with healthy control IgG at any of the time points tested. Similarly, at both 6 and 24 hours (Figure 5.3 e and f) TRIF mRNA expression did not increase in HUVEC treated with APS IgG above that of HUVEC treated with healthy control IgG. At 2 hours HUVEC treated with APS IgG increased TRIF mRNA expression above that seen in HUVEC treated with healthy control IgG, with VT-/PM+ IgG increasing TRIF mRNA to the greatest extent.
Figure 5.2: Effects of patient and control IgG on HUVEC mRNA expression of TLR2 and TLR4

HUVEC were treated with 200 µg/mL pooled IgG from VT+/PM-, VT-/PM+ and healthy controls as well as the positive control LPS (10 µg/mL). Graph shows qRT-PCR analysis of TLR2 and TLR4 mRNA expression at 2 hours (a & d), 6 hours (b & e) and 24 hours (c & f). The mean ± SEM of quantitative analysis from three independent experiments is plotted. Graphs g and h portray mean TLR2 and TLR4 transcript expression across the 3 time points. No IgG data comparisons show statistical significance using parametric statistical analysis.
Figure 5.3: Effects of patient and control IgG on HUVEC mRNA expression of MyD88 and TRIF

HUVEC were treated with 200 µg/mL pooled IgG from VT+/PM-, VT-/PM+ and healthy controls as well as the positive control LPS (10 µg/mL). Graph shows qRT-PCR analysis of MyD88 and TRIF mRNA expression at 2 hours (a & d), 6 hours (b & e) and 24 hours (c & f). The mean ± SEM of quantitative analysis from three independent experiments is plotted. Graphs g and h portray mean MyD88 and TRIF transcript expression across the 3 time points. No IgG data comparisons show statistical significance using parametric statistical analysis.
I then examined the phosphorylation of TLR4 MyD88 dependent signalling molecules p38 MAPK and p65 NFκB in HUVEC treated with 200 µg/mL of patient and control IgG by immunoblot. As Figure 5.4 shows, at 15 minutes HUVEC treated with VT+/PM- IgG showed a significant 3.2-fold increase in the phosphorylation of p38 MAPK compared to healthy control IgG, whereas HUVEC treated with VT-/PM+ IgG showed only a 1.5-fold increase in the phosphorylation of p38 MAPK compared to healthy control IgG. This difference in phosphorylation of p38 MAPK was no longer seen at 45 minutes. At 2 hours the relative expression of phospho-p38/p38 in HUVEC treated with patient and control IgG was no different to the positive control LPS. At 6 and 24 hours this response disappeared and relative expression of phospho-p38/p38 in HUVEC treated with patient and control IgG remained similar to that of untreated HUVEC.

The relative expression of NFκB p65, shown in Figure 5.5, in HUVEC treated with patient and control IgG was no different to untreated HUVEC at all time points tested. The positive control LPS phosphorylated NFκB p65 to the greatest extent at the earlier time points tested of 15 and 45 minutes and then again at 24 hours.
Figure 5.4: Effects of patient and control IgG on the phosphorylation of p38 MAPK in HUVEC

HUVEC were treated with 200 µg/mL pooled IgG from VT+/PM-, VT-/PM+ and healthy controls as well as the positive control LPS (10 µg/mL). Graph shows western blot analysis of p38 MAPK relative expression at 15 minutes (a), 45 minutes (b), 2 hours (c), 6 hours (d), 24 hours (e) and the mean time point curve (f). The mean ± SEM of quantitative analysis from three independent experiments is plotted. Significant differences were determined by one-way ANOVA and unpaired t test (*=P<0.05).
Figure 5.5: Effects of patient and control IgG on the phosphorylation of p65NFκB in HUVEC

HUVEC were treated with 200 µg/mL pooled IgG from VT+/PM−, VT−/PM+ and healthy controls as well as the positive control LPS (10 µg/mL). Graph shows western blot analysis of NFκB p65 relative expression at 15 minutes (a), 45 minutes (b), 2 hours (c), 6 hours (d), 24 hours (e) and the mean time point curve (f). The mean ± SEM of quantitative analysis from three independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
5.3 Expression of cytokines in HUVEC following treatment with IgG from patients with different clinical manifestations of the APS

In this section I investigated the mRNA and protein expression of a number of different cytokines including TNF-α, IL-8, IL-6 and IL-1β after HUVEC had been treated with patient or control IgG.

5.3.1 TNF-α

Firstly, I examined the mRNA expression of the cytokine TNF-α in HUVEC after treatment with 200 µg/mL patient and control IgG. As Figure 5.6 shows, HUVEC treated with LPS increased TNF-α mRNA expression above that seen in the untreated controls at all time points tested. The highest fold change in LPS treated HUVEC was seen at 2 hours with a 55.9-fold change in TNF-α mRNA expression compared to the control gene GAPDH. In comparison, patient and control IgG had very little effect on TNF-α mRNA expression. At 2 hours there was a significant 3.2-fold difference in TNF-α mRNA expression between HUVEC treated with IgG from VT+/PM- patients compared to IgG from healthy controls (p<0.05 using unpaired t test). At all other time points there was no significant difference seen in TNF-α mRNA expression between patient and control groups.

The production of TNF-α by HUVEC into the supernatant after treatment with 200 µg/mL pooled patient and control IgG was also assessed using a multiplex cytokine magnetic bead ELISA kit. As Figure 5.7 shows levels of TNF-α produced by HUVEC treated with patient or healthy control IgG was similar at both time points tested, although at 72 hours there was a trend towards increased TNF-α levels, with a 2-fold difference in TNF-α produced in HUVEC treated with VT-/PM+ IgG compared to healthy control IgG, although this difference failed to reach statistical significance.
Figure 5.6: Effects of patient and control IgG on HUVEC mRNA expression of TNF-α

HUVEC were treated with 200 µg/mL pooled IgG from VT+/PM-, VT-/PM+ and healthy controls as well as the positive control LPS (10 µg/mL). Graph shows qRT-PCR analysis of TNF-α mRNA expression at 1 hour (a), 2 hours (b), 6 hours (c) and 24 hours (d). The mean ± SEM of quantitative analysis from three independent experiments is plotted. Significance determined using one-way ANOVA (2 hours p=0.0146), and unpaired t test (* = p<0.05).
Figure 5.7: Effects of patient and control IgG on HUVEC TNF-α expression
HUVEC were treated with 200 µg/mL pooled IgG from VT+/PM-, VT-/PM+ and healthy controls as well as the positive control LPS (10 µg/mL). Graph shows TNF-α expression measured in the supernatant at 24 hours (a) and 72 hours (b). The mean ± SEM of quantitative analysis from three independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
5.3.2 IL-8

The mRNA expression of the cytokine IL-8 was assessed in HUVEC after treatment with 200 µg/mL of pooled patient or control IgG. Figure 5.8 shows that HUVEC treated with LPS increased IL-8 mRNA expression above that of the untreated control at all time points tested. There was no significant difference however in IL-8 mRNA expression between HUVEC treated with patient and control IgG at any time points tested.

The production of IL-8 by HUVEC into the supernatant after treatment with 200 µg/mL pooled patient and control IgG was also assessed. As Figure 5.9 shows, at both time points tested, HUVEC treated with patient and control IgG produced similar levels of IL-8 as cells treated with LPS, with no difference in cytokine levels seen between patient and control groups.
Figure 5.8: Effects of patient and control IgG on HUVEC mRNA expression of IL-8

HUVEC were treated with 200 µg/mL pooled IgG from VT+/PM-, VT-/PM+ and healthy controls as well as the positive control LPS (10 µg/mL). Graph shows qRT-PCR analysis of IL-8 mRNA expression at 1 hour (a), 2 hours (b), 6 hours (c) and 24 hours (d). The mean ± SEM of quantitative analysis from three independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
Figure 5.9: Effects of patient and control IgG on HUVEC IL-8 expression

HUVEC were treated with 200 µg/mL pooled IgG from VT+/PM-, VT-/PM+ and healthy controls as well as the positive control LPS (10 µg/mL). Graph shows IL-8 expression measured in the supernatant at 24 hours (a) and 72 hours (b). The mean ± SEM of quantitative analysis from three independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
5.3.3 IL-6

The mRNA expression of IL-6 was assessed after treatment with 200 µg/mL of pooled patient and control IgG. Figure 5.10 shows that at all time points tested, HUVEC treated with the positive control LPS increased IL-6 mRNA expression. The greatest fold change in LPS treated cells was seen at 2 hours with a 13.1-fold increase in IL-6 mRNA compared to the control gene GAPDH. There was no significant difference in IL-6 mRNA expression between HUVEC treated with patient and control IgG at any time points tested.

The production of IL-6 by HUVEC into the supernatant after treatment with 200 µg/mL pooled patient and control IgG was also assessed. As can be seen in Figure 5.11 the production of IL-6 in HUVEC treated with patient and control IgG increased above untreated control levels from 430 pg/mL at 24 hours to 1900 pg/mL at 72 hours. There was no significant difference however in IL-6 production between patient and control groups at either time point tested.
Figure 5.10: Effects of patient and control IgG on HUVEC mRNA expression of IL-6

HUVEC were treated with 200 µg/mL pooled IgG from VT+/PM-, VT-/PM+ and healthy controls as well as the positive control LPS (10 µg/mL). Graph shows qRT-PCR analysis of IL-6 mRNA expression at 1 hour (a), 2 hours (b), 6 hours (c) and 24 hours (d). The mean ± SEM of quantitative analysis from three independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
**Figure 5.11: Effects of patient and control IgG on HUVEC IL-6 expression**

HUVEC were treated with 200 µg/mL pooled IgG from VT+/PM-, VT-/PM+ and healthy controls as well as the positive control LPS (10 µg/mL). Graph shows IL-6 expression measured in the supernatant at 24 hours (a) and 72 hours (b). The mean ± SEM of quantitative analysis from three independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
5.3.4 IL-1β

The final cytokine investigated was IL-1β. HUVEC mRNA expression of IL-1β was assessed after treatment with 200 µg/mL pooled patient and control IgG. As Figure 5.12 shows, similar to all other cytokines tested, HUVEC treated with LPS increased IL-1β mRNA expression at all time points tested, with the greatest fold increase (39.3-fold) seen at 2 hours. At all time points tested patient and control IgG had very little effect on IL-1β mRNA expression, with no significant difference in IL-1β mRNA expression seen between the groups.

IL-1β production by HUVEC into the supernatant after treatment with 200 µg/mL pooled patient and control IgG was also assessed. IL-1β levels in HUVEC treated with LPS as well as patient and control IgG fell below the detection limit of the Luminex assay (<0.125 pg/mL) and so could not be analysed.
Figure 5.12: Effects of patient and control IgG on HUVEC mRNA expression of IL-1β

HUVEC were treated with 200 µg/mL pooled IgG from VT+/PM-, VT-/PM+ and healthy controls as well as the positive control LPS (10 µg/mL). Graph shows qRT-PCR analysis of IL-1β mRNA expression at 1 hour (a), 2 hours (b), 6 hours (c) and 24 hours (d). The mean ± SEM of quantitative analysis from three independent experiments is plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
5.4 Expression of HUVEC CAMS following treatment with IgG from patients with different clinical manifestations of the APS

HUVEC expression of the CAMS VCAM-1 and E-selectin were examined by flow cytometry following treatment with 200 µg/mL pooled patient or control IgG (Figure 5.13). In order to compare data between HUVEC the percentage of cells and percentage MFI of cells expressing VCAM-1 and E-selectin were calculated as a percentage of the untreated control (set at 0%). LPS treatment of HUVEC for 6 hours increased VCAM-1 expression by 29.6% compared to untreated controls, with MFI increasing by 94% compared to untreated controls. LPS treatment of HUVEC for 4 hours increased expression of E-selectin by 30.6% compared to untreated controls, with MFI increasing by 53.3% compared to untreated controls. HUVEC treated with either patient or healthy control IgG did not increase the percentage of cells or the percentage MFI of cells expressing either VCAM-1 or E-selectin on the cell surface above the levels seen in untreated HUVEC.
Figure 5.13: Effects of patient and control IgG on HUVEC extracellular VCAM-1 and E-selectin production

HUVEC were treated with 200 µg/mL pooled IgG from VT+/PM-, VT-/PM+ and healthy controls as well as the positive control LPS (10 µg/mL). Results of flow cytometry analysis of HUVEC VCAM-1 (a & b) and E-selectin (c & d) expression. Graphs show percentage cell and percentage MFI calculated from untreated control. The mean ± SEM of quantitative analysis from three (VCAM-1) or four (E-selectin) independent experiments are plotted. No IgG data comparisons show statistical significance using parametric statistical analysis.
5.5 Discussion

The phosphorylation of the protein kinase p38 MAPK has been studied extensively in the APS field. With regards to EC the phosphorylation of p38 MAPK by APS IgG has been examined by a number of different authors (171, 176, 192, 193). Similar to others, I found that pooled IgG from VT+/PM- APS patients induced a 3.2-fold increase in p38 MAPK phosphorylation in HUVEC compared to healthy control IgG. Vega-Ostertag and co-workers (176) showed that treating HUVEC with 6 APS IgG (of 8 tested) induced a 5.3- to 9-fold increase in p38 MAPK phosphorylation compared to healthy control IgG. One key difference between my work and the work carried out by Vega-Ostertag et al. (176) is that I also treated HUVEC with APS IgG from patients who have only ever experienced PM (VT-/PM+) and was able to demonstrate that these IgG did not have the same effect on p38 MAPK phosphorylation in HUVEC as IgG from VT+/PM- patients. My results have shown that aPL from APS patients who have only experienced VT have differential effects on the activation of HUVEC as do aPL from APS patients who have only experienced PM.

Despite the above finding, I was unable to show that the downstream transcription factor NFκB was activated the same way in HUVEC by VT+/PM- IgG. I examined the phosphorylation of NFκB p65 by western blot, the same method as was used to analyse the phosphorylation of p38 MAPK. Examining the literature a number of different authors have shown that APS IgG cause phosphorylation of the transcription factor NFκB in EC (80, 176, 180, 187, 195-197). The key differences between these studies and the experiments carried out in this chapter examining the phosphorylation of NFκB in HUVEC by APS IgG is the methodology used. Meroni and co-workers (80) showed that monoclonal and polyclonal human anti-β2GPI (but not control antibodies) stimulated nuclear translocation of NFκB. In subsequent work by this group (187), they showed that aPL-mediated activation of TLR4 in EC leads to activation of NFκB. Espinola and co-workers (180) identified that APS IgG increased transcriptional activation of NFκB in cardiopulmonary aortic endothelial cells. Inhibition of NFκB has been shown to reduce in vitro (176, 197) and in vivo (196) effects of APS IgG on EC. The only study to look at the phosphorylation of NFκB by western blot is the work by Ortona and co-workers (195) who showed that affinity purified human IgG antivimentin/cardiolipin antibodies from patients with SN-APS stimulated NFκB phosphorylation in HUVEC, whereas healthy control IgG did not.
In this chapter I examined TLR2 and TLR4 mRNA expression in HUVEC treated with pooled IgG from VT+/PM-, VT-/PM+ and healthy control IgG. I did not identify any differences in the mRNA expression of either of these TLRs in HUVEC. There is evidence however to suggest both TLR2 and TLR4 may be implicated in aPL activation of EC (188-190). The experimental methods used in my studies and the studies in the literature are different and may explain why different conclusions were drawn. None of the studies looking into TLR2 or TLR4 activation in HUVEC by APS IgG investigated mRNA expression. Current published studies examined the activation of either TLR2 and TLR4 by APS IgG in HUVEC whereby experiments were carried out either in vitro (189) or in vivo (188) in cells where these pathways had been disabled.

Furthermore, I was unable to find a significant difference in the mRNA expression of the TLR adaptor proteins MyD88 and TRIF in HUVEC treated with pooled IgG from VT+/PM-, VT-/PM+ or healthy control IgG, which correlate with my TLR findings. Raschi et al. (187) are the only authors to have examined the activation of the TLR adaptor protein MyD88 in HUVEC after treatment with APS IgG. Employing measures that inhibit TLR pathways they found that a human monoclonal IgM aPL anti-β2GPI and affinity purified polyclonal anti-β2GPI IgG isolated from 3 patients with APS activated intracellular signalling pathways in an EC cell line; immortalised human microvascular ECs, in a manner comparable to that induced by LPS or IL-1, whereas control antibodies did not. However, these effects were abrogated in cells that had the MyD88 or TNF receptor-associated factor pathways knocked out. Their findings implicated the involvement of the TLRs in aPL mediated cell activation, suggesting that anti-β2GPI antibodies activate EC through either direct or indirect involvement of one or more TLRs. Similar to published data implicating TLR2 and TLR4 in aPL activation of EC, Raschi et al. (187) utilised different methods to what I did to demonstrate the importance of MyD88 in aPL activation of EC. This may go some way to explain differences in the significance of MyD88 in aPL activation of EC reported by Raschi and co-workers (187) to those reported in this work.

In this chapter I investigated the mRNA and protein expression of the cytokines TNF-α, IL-8, IL-6 and IL-1β in HUVEC following treatment with APS and control IgG. A number of authors have investigated the expression of these cytokines by APS IgG in HUVEC. Vega-Ostertag et al. (176) treated HUVEC with IgG from 2 APS patients both with a history of VT and PM and found that they increased both IL-6 and IL-8
production in HUVEC compared to a healthy control IgG. Clemens and co-workers (281) demonstrated that HUVEC treated with a human monoclonal aPL IgG antibody (HL-5B) increased IL-8 mRNA expression compared to a control IgG. Examining gene expression in HUVEC treated with 4 PAPS-derived anti-β₂GPI antibodies Hamid and co-workers (282) identified up regulation of a number of cytokines and chemokines genes, including IL-6 and IL-1β (up-regulated by 2.5- and 2.4-fold respectively). Further analysis identified that IL-8 production was higher in HUVEC treated with 4 PAPS-derived anti-β₂GPI antibodies compared to 4 healthy control IgG. Although I did identify an increase in TNF-α mRNA expression in HUVEC treated with VT+/PM- IgG at 2 hours compared to HUVEC treated with healthy control IgG, at all other time points and for all other cytokines tested I did not identify any difference in the mRNA or protein expression of these cytokines in HUVEC treated with APS IgG compared to healthy control IgG.

A number of authors have identified that aPL activate EC in vitro and in vivo by demonstrating enhanced expression of the CAMS VCAM-1 and E-selectin (152-154, 177, 179, 180). I examined the cell surface expression of VCAM-1 and E-selectin in HUVEC treated with APS and healthy control IgG; however, I did not identify any difference in the expression of either of these CAMS in cells treated with APS IgG compared to healthy control IgG. Pierangeli et al. (152) in an in vitro study identified that HUVEC monolayers exposed to IgG purified from 6 aPL positive patients (3 with a history of thrombosis only, 1 with PM only, 1 with both thrombosis and PM and 1 with no APS clinical events) displayed a significant 2.3- to 4.4-fold increase in VCAM-1 expression for 5 IgG patient samples tested. Only 1 of these IgG samples however, from a patient with a history of both thrombosis and PM, significantly increased the expression of E-selectin by 3.8-fold compared to IgG purified from healthy controls. The work of Pierangeli and co-workers (152) identified that some, but not all APS IgG cause an increase in VCAM-1 and E-selectin expression on HUVEC. An explanation for why I did not identify an increase in the expression of these CAMS on HUVEC may be because I used a pool of IgG from 5 different patients and so the effect of was masked

In summary, although I have been unable to implicate the involvement of upstream TLR signalling pathways in HUVEC treated with IgG purified from APS patients, I have been able to show that only IgG purified from patients with thrombotic APS
phosphorylate the protein kinase p38 MAPK in HUVEC, which is not the case for IgG purified from patients with obstetric APS or healthy control IgG. These results support previous findings in monocytes (204), another cell type relevant to VT.
Chapter VI

Overall findings and future experiments
Overall findings and future experiments

6.1 Overall findings

The underlying premise of all the experiments described in this thesis was that the biological effects exerted by aPL upon different cell types depend both on the nature of the cells and the nature of the aPL added. Whereas it is well known that the binding properties of aPL affect their biological properties, I was testing something different; the effect of aPL derived from patients with different clinical manifestations of the APS. In particular, I compared IgG derived from patients with pure thrombotic APS and those derived from patients with pure obstetric APS. Even though the IgG from these different clinical groups had similar binding properties, they showed clear differences in their effects in biological assays.

Work carried out in HUVEC identified that only IgG derived from patients with thrombotic APS phosphorylated p38 MAPK. Identifying similar findings to previous work carried out at UCL in another thrombotic cell type, monocytes (204). When investigating p38 MAPK phosphorylation in HTR-8 cells however, IgG derived from patients with thrombotic APS did not trigger the phosphorylation p38 MAPK in this pregnancy related cell type. In fact, IgG derived from patients with thrombotic APS had very little effect in HTR-8 cells except for up-regulating the mRNA expression of PAR-1 and PAR-2, something that IgG derived from patients with obstetric APS also did. This work clearly identifies that IgG derived from patients with thrombotic APS have different effects on thrombotic compared to obstetric cell types.

In contrast, IgG derived from patients with obstetric APS did not stimulate the phosphorylation of p38 MAPK in either HUVEC or HTR-8 cells. It was identified however, that IgG derived from patients with obstetric APS have a potentially important biological effect on HTR-8 cells as they inhibited the migration ability of these cells, a crucial property of trophoblasts required for the development of a successful pregnancy. I found some clues to the mechanism of this biological effect. It is probably TLR4 dependent as I identified that the inhibited migration seen in HTR-8 cells treated with IgG derived from patients with obstetric APS was restored after pre-treatment with a TLR4 inhibitor. This hypothesis also fits in with the ability of these IgG derived from patients with obstetric APS to up-regulate the mRNA expression of TLR4 and its adaptor protein TRIF. Despite multiple lines of investigation however, I could not
discern a clear intracellular pathway linking TLR4 to migration. One important hypothesis that I tested was the possible involvement of IL-6 in inhibiting HTR-8 migration; however, I could not detect any effect of either IgG derived from obstetric or thrombotic APS patients on IL-6 production in HTR-8 cells.

Overall, this thesis has identified that aPL from patients who present with different clinical manifestations of the APS may have differential effects on thrombotic and pregnancy related cell types.

6.2 Future experiments

In order to take these findings forward and elucidate the signalling pathways and cytokines involved in aPL activation of trophoblasts and EC I would consider the following points. To improve my experiments I would increase the number of patient IgG tested and would test individual APS patient IgG samples. Unfortunately, due to the limited number of patient serum samples available to me I was unable to do this and so I chose to treat cells with a pool of IgG, testing a pool from 5 individuals in each patient or control group. Close examination of experiments similar to mine in the literature, something I carried out in a published systematic review (283), enabled me to identify that this limitation is common to other studies, resulting in the use of small numbers of polyclonal IgG from APS patients in other cell signalling studies. Also, due to the limited number of patients’ serum to purify IgG from it proved unfeasible to obtain patient IgG samples from the time soon after an APS related event. Many of the samples acquired were from patients who had long since had an APS related clinical event. If resources were available to me I would ideally increase the number of patients tested in each patient group and I would purify IgG from serum obtained close to an APS related clinical event.

Future experiments would include trying to identify a mechanism for the inhibition of trophoblast migration seen in HTR-8 cells treated with IgG derived from patients with obstetric APS. Transforming growth factor betas (TGFβ) are members of a large superfamily of cytokines. Several studies have suggested that the different TGFβ isoforms may regulate trophoblast invasion (284-287). I could investigate the expression of TGFβ in HTR-8 cells treated with patient and control IgG to identify if the expression of TGFβ is inhibited in HTR-8 cells treated with IgG derived from
patients with obstetric APS and thus resulting in an inhibition of migration in cells treated with IgG from these patients.

In future experiments I would also utilise additional different experimental methods to determine the signalling pathways activated by aPL in HTR-8 cells and HUVEC. To understand the TLRs and their adaptor proteins which aPL target it would be interesting to undertake *in vivo* studies in mice where these TLR and their adaptor proteins have been knocked-out, either individually or in combination. A well characterised murine model such as the Pierangeli thrombosis model (147) and a pregnancy model similar to that used by Blank and colleagues (165) utilising TLR or TLR adaptor protein knockout mice would identify if aPL interact with TLR and their adaptor proteins in order to cause thrombosis or PM respectively. This would continue similar work already carried out by Pierangeli *et al.* (188) where using two different types of mice; one type (C3H/HeJ) with a mutation in the TLR4 gene such that it was unable to respond to LPS and the other type (C3H/HeN) with no TLR4 mutation, hence was LPS-responsive, these authors demonstrated that TLR4 is involved in the pathogenesis of aPL-induced thrombosis and aPL activation of EC.

Despite disagreements concerning the intensity and duration, there is currently treatment available to patients with both thrombotic and obstetric APS, which go some way in preventing recurrent disease in the majority of patients. We cannot be content with these treatments however for a number of reasons. For instance, for the treatment of thrombotic APS, warfarin requires frequent laboratory monitoring and subjects a patient to an increase risk of bleeding. In the case of heparin for the treatment of obstetric APS we currently do not understand the full mechanisms by which this drug works in preventing recurrent pregnancy loss and despite treatment the incidence of severe late pregnancy complications remain high. It is therefore important to continue researching how aPL elicit their effects on different cell types so that we can gain a greater understanding of how aPL work and possibly in the future improve treatment regimes or the treatment available for patients with the APS. The work produced in this thesis will hopefully add to the continuing expanse of knowledge in this field.
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Appendix 1

Sequence of the primer pairs designed using Primer-BLAST:

GAPDH Fw: 5’ GAG TCA ACG GAT TTG GTC GT 3’
GAPDH Rv: 5’ TTG ATT TTG GAG GGA TCT CG 3’
TRAM Fw: 5’ AGG AAA GCA GGA GGG AGC 3’
TRAM Rv: 5’ AAG GCA TTG ATG GTT TGG AG 3’
TRIF Fw: 5’ CCA CCA TCC TCG GCT TTG C 3’
TRIF Rv: 5’ GGA GTG GCG TCT GGT CTT TGA 3’
PAR1 Fw: 5’ CCA CCT TAG ATC CCC GGT CAT 3’
PAR 1 Rv: 5’ GTG GGA GGC TGA CTA CAA ACA 3’
PAR 2 Fw: 5’ CAG TGG CAC CAT CCA AGG AA 3’
PAR 2 Rv: 5’ CAG GGC CAT GCC GTT ACT T 3’

TaqMan probes obtained from Applied Biosystems:

GAPDH: Hs02758991_g1
TLR2: Hs01014511_m1
TLR3: Hs01531078_m1
TLR4: Hs00152939_m1
MyD88: Hs01573837_g1
IRF3: Hs01547283_m1
TNF: Hs00174128_m1
RANTES: Hs00174575_m1
MCP-1: Hs00234140_m1
IL-8: Hs00174103_m1
IL-1β: Hs01555410_m1
IL-6: Hs00985639_m1