Identification of Anti-Beta$_2$ Glycoprotein I Auto-antibody Regulated Gene Targets in the Primary Antiphospholipid Syndrome Using Gene Microarray Analysis

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Abstract

Anti-Beta$_2$-Glycoprotein I antibodies (anti-β_2GPI) are strongly associated with thrombosis in patients with primary antiphospholipid syndrome (PAPS). Anti-β_2GPI activate endothelial cells (EC) resulting in a pro-thrombotic and pro-inflammatory phenotype. In order to characterise EC gene regulation in response to anti-β_2GPI, early global gene expression was assessed in human umbilical vein endothelial cells (HUVEC) in response to affinity purified anti-β_2GPI. Sera were collected from patients with PAPS and IgG was purified using HiTrap Protein G Sepharose columns. Polyclonal anti-β_2GPI were prepared by passing patient IgG through NHS activated sepharose coupled to human β_2GPI. Anti-β_2GPI preparations were characterized by confirming their β_2GPI co-factor dependence, binding to β_2GPI and ability to induce leukocyte adhesion molecule expression and IL-8 production *in vitro*. Two microarray experiments tested differential global gene expression in 6 individual HUVEC donors in response to 5 different PAPS polyclonal anti-β_2GPI (50 μg/ml) compared to 5 normal control IgG (50 μg/ml) after 4 hours incubation . Total HUVEC RNA was extracted and cRNA was prepared and hybridised to Affymetrix HG-133A (Exp.1) and HG-133A_2 (Exp.2) gene chips. Data were analyzed using a combination of the MAS 5.0 (Affymetrix) and GeneSpring (Agilent) software programmes. Significant change in gene expression was defined as greater than two fold increase or decrease in expression ($p<0.05$).

Novel genes not previously associated with PAPS were induced including chemokines CCL20, CXCL3, CX3CL1, CXCL5, CXCL2 and CXCL1, the receptors Tenascin C, OLR1, IL-18 receptor 1 and growth factors, CSF2, CSF3, IL-6, IL1β and FGF18. Downregulated genes were transcription factors/signaling molecules including ID2. Microarray results were confirmed for selected genes (CSF3, CX3CL1, FGF18, ID2, SOD2, Tenascin C) using quantitative real-time RT-PCR analysis. This study revealed a complex anti-β_2GPI-regulated gene expression profile in HUVEC *in vitro*. The novel chemokines and pro-inflammatory cytokines identified in this study may contribute to the vasculopathy associated with PAPS.

Dedication

This thesis is dedicated to my friend Lou Ann, whose selfless courage, infinite capacity to hope, determination, and love inspire me each day.

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Declaration

I declare that I have personally prepared this report and that the work described is my own unless otherwise stated. All sources of information are acknowledged by means of reference.

Colleen Hamid

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Abbreviations

ACL	anticardiolipin antibody
ADP	adenosine diphosphate
AECA	anti-endothelial cell antibody
ALL	acute lymphocytic leukemia
ANCA	antineutrophil cytoplasmic antibody
ANOVA	analysis of variance
APL	antiphospholipid antibody
APS	antiphospholipid syndrome
aPTT	activated partial thromboplastin time
B_2-GPI	$Beta_2$ Glycoprotein I
CnA	calcineurin
CCL20	macrophage inflammatory protein-3α
CD	cluster of differentiation number
cDNA	complementary DNA
CMV	cytomegalovirus
cRNA	complementary RNA
CX3CL1	fractalkine
DIC	disseminated intravascular coagulation
DNA	deoxyribonucleic acid
dRVTT	dilute Russel's viper venom time
DSCR1	Down Syndrome Critical Region 1
DVT	deep vein thrombosis
EBV	Epstein Barr virus
EC	endothelial cell(s)
ELISA	enzyme linked immuno-assay
EST	expressed sequence tag
ET-1	endothelin 1
FCS	Foetal calf serum
FGF	fibroblast growth factor
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor

GO	gene ontology
GPL	IgG aCL affinity purified aPL
hCG	human chorionic gonadotrophin
HIT	heparin induced thrombocytopenia
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HUVEC	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule 1
ID2	Inhibitor of DNA binding 2
Ig	immunoglobulin
IL	interleukin
INR	international normalized ratio
IRAK	Interleukin-1 receptor associated kinase
IUGR	intra-uterine growth restriction
LA	lupus anticoagulant
LAC	lupus anticoagulant activity
LPS	Lipopolysaccharide
MIAME	minimum information about microarray experiment
mRNA	messenger ribonucleic acid
MYD88	myeloid differentiation protein
NCBI	National Centre for Biotechnology Information
NF-κB	nuclear factor-κB
NHS	normal human serum
NOS	nitric oxide synthase
OLR1	oxidized low density lipoprotein receptor
OR	odds ratio
OxLDL	oxidized low density lipoprotein
p38MAPK	p38 MAP kinase
PAPS	primary antiphospholipid syndrome
PBMC	peripheral blood mononuclear cell
PCA	procoagulant activity
PE	pulmonary embolism
PET	pre-eclampsia

PF4	platelet factor 4
PL	phospholipid
qRT-PCR	quantitative reverse transcription polymerase chain reaction
SLE	Systemic Lupus Erythematosus
SOD2	Superoxide dimutase 2
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TLR	toll like receptor
TNC	tenascin C
TNF	tissue necrosis factor
TRAF	TNF-receptor associated factor
TXB_2	thromboxane B_2
VCAM-1	vascular cell adhesion molecule 1
VDRL	Venereal Disease Research Laboratory
VEGF	vascular endothelial cell growth factor
VTE	venous thromboembolism

Chapter 1

General Introduction

1.1 History of APS

In 1906, A.P. vonWasserman developed the first serologic test for a complement-fixing antibody in the sera of patients with syphilis. Antibodies in the sera of such patients reacted with extracts of syphilitic tissues but were also found to interact with normal human and animal tissues. The antigen was later identified in 1941 by Pangborn as a mitochondrial phospholipid called cardiolipin (diphosphatidylglycerol)(Levine, Branch, & Rauch 2002; Hanly 2003) and the antigen used in the test was an extract from bovine hearts. In an effort to control the infection, the Wasserman test facilitated the widespread screening of the population using serologic tests for syphilis. These tests became the basis of the Venereal Disease Research Laboratory (VDRL) test for syphilis in the 1930s, a rapid serum flocculation procedure similar to that still in use for monitoring of treatment rather than diagnosis (Levine, Branch, & Rauch 2002; Hanly 2003).

Widespread use of the VDRL test included screening of military personnel at the onset of World War II and premarital testing required by State Boards of Health identified numbers of individuals who tested positive in the VDRL test but had no history of sexually transmitted disease. It was found that many patients with systemic lupus erythematosus (SLE) had a positive VDRL test but no clinical or serological evidence of infection with syphilis (Triplett 2000). As the result of the high rate of false positives, the more specific Treponema Pallidum Immobilization (TPI) diagnostic test for syphilis was introduced in the late 1940s. TPI confirmed that the VDRL test was detecting antibodies secondary to infections other that syphilis. In 1952, Moore and Mohr divided biologic false positive serologic test results for syphilis into two groups, acute (transient) or chronic. Transient false positives could follow acute viral infections and vaccination but persistent positive results over a period of 6 months or greater were consistent with autoimmune disorders such as SLE, Sjogren's syndrome and rheumatoid arthritis (Hanly 2003). They also made the association between false positivity and increased incidence of thrombosis and thrombocytopenia in young women. Conley and Hartmann were the first to describe a circulating anticoagulant in the plasma of two patients with SLE (Conley & Hartmann, 1952). The patients' plasma demonstrated a prolongation of the whole blood clotting time and prothrombin time and no coagulation factor deficiency then measurable. This was

the first reference to the "lupus anticoagulant" (LA) and its association with prolongation of phospholipid dependent coagulation tests. In 1957 Laurell and Nilsson (1957) demonstrated that the cardiolipin in the Kahn flocculation test could adsorb out LA activity suggesting that LA activity was due to an anti-phospholipid antibody. Feinstein and Rapaport applied the term lupus LA to these antibodies twenty years later because LA's were originally found in patients with SLE (Feinstein & Rapaport 1972).

Preliminary work by Hughes *et al.* investigating CNS Lupus in patients with "Jamaican neuropathy" identified numerous patients who showed false positive VDRL tests (Wilson & Hughes, 1975). This finding led to subsequent extensive investigation of antibodies to phospholipids in these patients. In this way the strong link between LA and increased incidence of thrombosis was recognized. Investigation of increasing numbers of patients revealed numerous instances of anti-DNA negative or atypical SLE patients associated with thrombosis, cerebral disease, and/or recurrent miscarriages. In 1983, Harris and Hughes (with colleagues Asherson, Gharavi) at the Hammersmith Hospital (London, U.K.) developed a solid phase radioimmunoassay for the detection of anticardiolipin antibodies (aCL). This assay was approximately 400 times more sensitive than the conventional VDRL test (Harris et al., 1983). Using this assay, they reported correlation between the presence of aCL and the incidence of both arterial and venous thrombosis, recurrent pregnancy loss and thrombocytopenia. The development of an enzyme-linked immunosorbent assay (ELISA) for aCL determination and quantitation followed in 1984 (Koike et al., 1984). The proposal of the "anticardiolipin" or "antiphospholipid syndrome" (APS) as a new clinical entity in 1985 was facilitated by subsequent clinical and epidemiological studies utilizing the new assay (Hughes 1985).

1.2 Clinical features of APS

Primary antiphospholipid syndrome (PAPS) occurs in patients when clinical evidence of another autoimmune disease is absent, and accounts for about 50 percent of cases of APS. It is characterized by recurrent arterial and/or venous thrombosis, recurrent pregnancy loss and thrombocytopenia in association with

the presence of antiphospholipid antibodies (aPL). APS, in association with another autoimmune disease such as SLE, is referred to as secondary APS.
Since APS was first described in 1983 and recognized as an entity apart from the LA associated phenomenon, the spectrum of associated clinical manifestations has grown considerably. In addition to thrombosis, recurrent fetal loss and thrombocytopenia, they encompass neurological disorders (epilepsy, atypical migraine, chorea, transverse myelopathy, dementia), obstetric complications (fetal distress, preeclampsia and pregnancy induced hypertension), valvular heart disease and cutaneous features (livedo reticularis, leg ulcers, gangrene skin ulcers) (Koike et al., 1984; Hojnik et al., 1996; Durrani, Gordon, & Murray 2002). Accelerated atherosclerosis is also associated with APS (Harats et al., 1999; Tak & Firestein 2001; Cervera et al., 2002).

aPL have been associated with both valvular and ischaemic heart disease. Up to 4 percent of patients with APS have at least one heart valve abnormality (Vianna et al., 1994). PAPS is associated with a high incidence of cardiac valvular disease (35-60% of patients) (Badui et al., 1995; Garcia-Torres et al.,1996; Espanola-Zavaleta et al., 1999; Turiel et al., 2000). Pathological findings in PAPS include mitral and/or aortic valve lesions and/or thickening, intravascular or coronary thrombosis or myocardial infarction. A significant correlation between aPL titre**,** degree of valvular thickening and thrombotic events has been identified (Turiel et al., 2000). Other pathological findings include fibrin deposits and vascular proliferation in the absence of inflammation (Garcia-Torres et al., 1996). Using immunohistochemical methods, sub-endothelial deposits of immunoglobulins containing aPL are consistently present in cardiac valves of patients with APS (Hojnik et al., 1996). APL have been associated with an increased risk of cardiovascular events. Hamsten (1986) noted that 21 percent of men who had myocardial infarcts under the age of 45 had elevated levels of aPL, and a higher incidence of another cardiovascular event in the next five years compared to patients with no antibodies. A prospective study of middle-aged men identified increased aPL level was an independent risk factor for myocardial infarction (Vaarala et al., 1995). A prospective study of 1150 patients with acute myocardial infarction however showed that elevated IgG aPL and low IgM aPL were independent risk factors for recurrent cardiac events (Bili et al., 2000).

1.3 Diagnostic criteria for the diagnosis of APS

In an attempt to consolidate consensus, clarify diagnosis and treatment of the syndrome, an international and multidisciplinary symposium has been held every two years since 1984. Formulation of the preliminary classification criteria for APS ("Sopporo criteria") was undertaken during a post-conference workshop that took place in Sopporo, Japan on October 10, 1998 following the Eighth International Symposium on Antiphospholipid Antibodies. Extensive review of clinical and experimental evidence has reinforced the widespread opinion that aPL are significantly associated with arterial and venous thrombosis and recurrent miscarriage. Results of prospective studies and the strong experimental evidence defined vascular thrombosis and/or recurrent pregnancy loss as the clinical criteria for APS. The diagnosis of APS requires that aPL must be present in a medium to high titre and persistently present on two or more occasions at least six weeks apart (Wilson W.A. et al., 1999; Carreras, Forasterio, & Martinuzzo 2000). Definite APS is considered to be present in a given patient when at least 1 of the laboratory criteria (detection of either aCL or LA) and at least one of the diagnostic criteria (vascular thrombosis or fetal loss) are met (Table 1.1). Patients with either PAPS or secondary APS experience no major differences in clinical symptoms as a consequence of aPL (Levine, Branch, & Rauch 2002). Definite APS is considered to be present if at least 1 of the clinical criteria and 1 of the laboratory criteria are met (see Table 1.1).

Table 1.1: Criteria for the classification of definite antiphospholipid syndrome

Clinical criteria

1. Vascular thrombosis

One or more clinical episodes of arterial, venous or small-vessel thrombosis in any tissue or organ.
Thrombosis must be confirmed by imaging or Doppler studies or histopathology, with the exception of superficial venous thrombosis. For histopathologic confirmation, thrombosis should be present without significant inflammation in the vessel wall.

2. Pregnancy morbidity

a) One or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation, with normal fetal morphology documented by ultrasonography or by direct examination of the fetus, **or**
b) One or more premature births of a morphologically normal neonate at or before the 34th week of gestation because of severe pre-eclampsia or eclampsia, or severe placental insufficiency, **or**
c) Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation, with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded. In studies of populations of patients who have more than 1 type of pregnancy morbidity, investigators are strongly encouraged to stratify groups of subjects according to a, b or c above.

Laboratory criteria

1**. Anticardiolipin antibody of IgG and/or IgM isotype** in blood, present in medium or high titre, on 2 or more occasions, at least 12 weeks apart, measured by a standardized enzyme-linked immunosorbent assay for β2-glycoprotein-I–dependent anticardiolipin antibodies.
2. Anti-β_2 GPI antibodies of IgG or IgM isotype in blood (in titre above 99th percentile), present on two or more occasions, at least 12 weeks apart, measured by a standardized ELISA, according to recommended procedures (Miyakis et al., 2006).
3. Lupus anticoagulant present in plasma, on 2 or more occasions at least 6 weeks apart, detected according to the guidelines of the International Society of Thrombosis and Hemostasis (ScientificSubcommittee on Lupus Anticoagulants/Phospholipid-Dependent Antibodies) in the following steps:
a) Prolonged phospholipid-dependent coagulation demonstrated on a screening test, e.g., activated partial lthromboplastin time, kaolin clotting time, dilute Russell's viper venom time, dilute prothrombin time, Textarin time.
b) Failure to correct the prolonged coagulation time on the screening test by mixing with normal platelet poor plasma.
c) Shortening or correction of the prolonged coagulation time on the screening test by addition of excess phospholipid.
d) Exclusion of other coagulopathies, e.g., factor VIII inhibitor or heparin, as appropriate.
Note: Ig = immunoglobulin.

(Arthritis Rheumatism 1999; 42(7):1309-11)

1.4 Incidence of aPL in healthy individuals

The incidence of aPL measured in the healthy population have indicated that 4.7 percent of healthy young people and up to 52 percent of elderly are positive for aPL. Antibody titres are low-positive and transient usually in response to infection (Durrani, Gordon, & Murray, 2002). It is highly unlikely for individuals to go on to develop clinical symptoms of APS when antibody titres are low. Additional risk factors should be assessed if such individuals go on to develop thrombosis. aPL are likely part of the natural repertoire of antibody production in these individuals and are not implicated in autoimmune disease.

aPL can be present in numerous other conditions such as cancer, they can be drug-induced or found in patients undergoing hemodialysis, or with infections such as HIV (Greaves et al., 2000). These antibodies are not usually implicated in association with thrombosis since they are usually IgM, transient, and present in low concentration.

1.5 The catastrophic antiphospholipid syndrome

In most patients with APS, thrombosis occurs as a singular event in one anatomical site. A small number of patients may however present with an acute syndrome characterized by multiple vascular occlusions throughout the body. The catastrophic antiphospholipid syndrome presents as any combination of thrombocytopenia, disseminated intravascular coagulation (DIC), adult respiratory distress syndrome, liver failure, multi-organ involvement and often results in death (Asherson 1992). Its onset has been associated with infection, surgical procedures and withdrawal of anticoagulant therapy. It often results in widespread microvascular coagulopathy and has been coined a “thrombotic storm”. Diagnosis requires arbitrary involvement of at least three different organ systems over a period of days to several weeks. The syndrome typically involves acute thrombotic microangiopathy that affects small vessels in multiple organs including the kidneys, lungs, central nervous system, heart and skin (Levine, Branch, & Rauch 2002). Catastrophic APS results in DIC in 25% of cases characterized by bleeding or thrombosis, increased activated partial

thromboplastin time (aPTT), decreased fibrinogen, antithrombin III, Protein C and increased D-dimer.

1.6 Anti-endothelial cell antibodies (AECA)

Anti-endothelial cell antibodies (AECA) are present in a number of autoimmune disorders where endothelial cell (EC) damage is common and AECA alter the procoagulant and anticoagulant phenotype of EC *in vitro* (reviewed in Praprotnik et al., 2001). AECA can bind directly to structural antigens on the EC surface itself or to extraneous antigens bound to the EC surface. AECA from one individual can show variation in binding affinities to EC of different phenotypes resulting in disease pathology within different parts of the vasculature (Hill et al., 1998). Their presence is well-documented in SLE-associated vasculitis and anti-neutrophil cytoplasmic antibody (ANCA)-positive vasculitis (Meroni et al., 2005). Numerous studies have attempted to identify AECA antigens by examining membrane lysate preparations but results are seldom conclusive due to the heterogeneity of these antibodies. AECA may have specificity for antigens on the EC membrane, the nucleus or cytoplasmic antigens. Methods for detection of AECA are also poorly standardized and give discrepant results (Praprotnik et al., 2001). AECA are found in sera of APS patients, of which aPL are a sub-group (Cines et al., 1998).

1.7 Detection of antiphospholipid antibodies

APL immunoglobulins (IgG, IgM and occasionally IgA) are generally divided into sub-groups based on the method by which they are detected. Pathogenic aPL are mainly IgG1 and IgG3 isotypes (Loizou et al., 1992). The antibodies are heterogeneous and adequate screening for their presence requires more than one type of assay. The two methods used for detection are: (1) solid phase immunoassays (ELISA) and (2) phospholipid dependent clotting tests. aPL are generally divided into lupus anticoagulant (LA) antibodies and anticardiolipin antibodies (aCL) where aCL represent aPL detected in the conventional ELISA assay that incorporates the negatively charged phospholipid cardiolipin as the antigen. Commercial aCL assays detect both infection and autoimmune derived antibodies. The original standard used to measure aCL was a cardiolipin affinity

purified standard (Harris et al., 1987). Isotype concentrations are expressed as (Ig) GPL, MPL or APL units where one unit is defined as the binding activity of 1μg/ml of affinity purified aCL. Reference sera for the measurement of aCL in ELISA assays have improved inter-laboratory reproducibility when quantitating the antibodies and helped in the standardization of assays. The formation of the European Forum for the standardization of aPL has improved standardization for antibody measurement between centres (Tincani, Allegri, & Sanmarco 2001). LA are detected in phospholipid-dependent coagulation tests where they interfere with phospholipid dependent coagulation reactions resulting in prolonged clotting times (Koike et al., 1984; Triplett 1994). A single patient may have aCL, LA or a combination of both and there may be some overlap in their antigenic specificities.

1.8 Beta$_2$ -Glycoprotein I: Co-factor for aPL

Autoimmune aPL were thought to bind to negatively charged phospholipids, but it has become apparent that they are antibodies which bind to plasma proteins which bind to phospholipid and other anionic surfaces. In 1990, independent groups determined that a co-factor, Beta$_2$–Glycoprotein I (β_2GPI or apolipoprotein H) was necessary for autoimmune aPL to bind to acidic phospholipids such as cardiolipin and phosphatidylserine in ELISA (Galli et al., 1990; Matsuura et al., 1990; McNeil et al., 1990; Triplett 1994). β_2GPI was also found to be necessary for some LA's to express their anticoagulant activity *in vitro* (Roubey et al., 1995). It should be noted that both ELISA and clot based assays are required for adequate screening for the presence of aPL. The aCL ELISA detects both autoimmune aPL that are β_2GPI-dependent and infection mediated PL antibodies. LA tests detect antibodies to β_2GPI or prothrombin in association with phospholipids.

β_2GPI is a plasma glycoprotein with a molecular mass of 50 kDa that circulates at a concentration of ~200μg/ml. It is composed of five short-consensus-repeat domains (sushi domains) that are also common to complement factors and selectins (Takeya et al., 1997) (see Figure 1.1). β_2GPI is highly conserved between species implying an important biological function *in vivo*. It binds to

negatively charged phospholipids (Roubey 1996). The positively charged phospholipid- binding site of the protein also known as the lysine binding region, is located in the fifth domain of the molecule. β_2GPI also binds to the surface of EC through its fifth domain (Del Papa et al., 1998). The protein is largely synthesized in the liver but β_2GPI mRNA has been isolated from different cell types involved in the pathology of APS (Caronti et al., 1999). β_2GPI has anticoagulant properties *in vitro* including the inhibition of ADP-induced aggregation of platelets (Nimpf, Wurm & Kostner 1985) and inhibition of the prothrombinase complex (Roubay et al., 1995). An anticoagulant role for this protein *in vivo* has been suggested due to its ability to bind to negatively charged phospholipids, potentially blocking the molecules required for the activation of coagulation (Schousboe 1985). However, deficiency of the protein does not cause a thrombotic tendency and β_2GPI–knockout mice were not distinguishable from normal mice indicating that the protein may not serve a physiologically significant role associated with thrombosis (Bansci, van der Linden, & Bertina 1992).

In 1994, Matsura was able to show that some aPL could recognise β_2GPI in the absence of cardiolipin, indeed aCL and anti-β_2GPI were identified as two distinct populations of autoantibodies in that infection mediated aCL do not require the cofactor in order to bind to phospholipid (Roubey, Eisenberg, Harper & Winfield 1995; Sorice, Circella & Griggi 1996). Anti-β_2GPI have been found to have greater specificity and predictive value for clinical complications in APS when compared to assays for aCL (McNally et al*.,* 1995; Amengual et al., 1996; Sanmarco et al., 1997). Indeed β_2GPI is now regarded as the most appropriate candidate antigen for clinically significant aPL (Hojnik et al., 1996; Roubey, Eisenberg, Harper & Winfield 1995; Triplett 1994). Like aCL, anti-β_2GPI antibodies can be measured using an ELISA but both assays have proven difficult to standardize between laboratories. (McNally et al., 1995; Amengual et al., 1996; Carreras, Forasterio & Martinuzzo 2000). A number of commercial ELISA kits are available for measurement of IgG anti-β_2GPI. The assay for anti-β_2GPI is valuable since it can distinguish between β_2GPI dependent and independent aPL (McNally et al., 1995). Testing for aCL and LA still remain the methods of choice in the diagnosis of APS (Greaves et al., 2000).

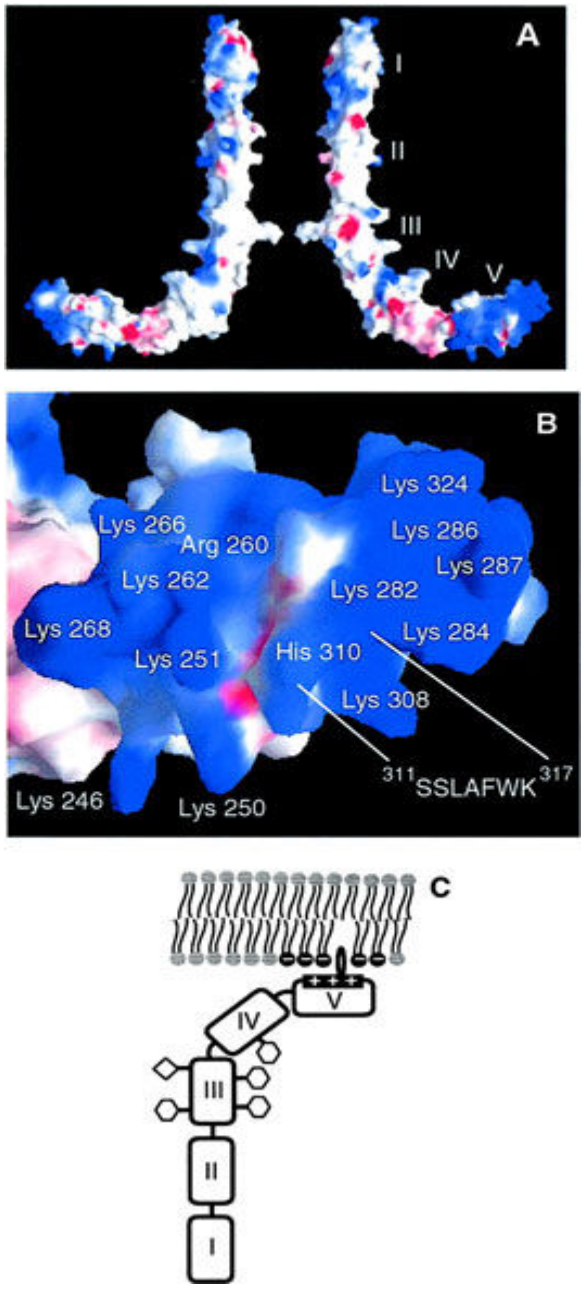

Figure 1.1 Beta 2 Glycoprotein I Structure Picture A shows two molecules of β_2GPI. The protein consists of a fish-hook like chain of five short consensus repeats (SCR), also called Complement Control Protein domains or Sushi domains. Each domain (I-IV) consists of around 60 amino acids (AA) and two di-sulphide bonds. Picture B- Domain V contains 82 AA including a six residue insertion and a 19 residue C-terminal extension which together form the PL binding site. Shown in B are the 14 positively charges AAs that allow interaction with negatively charged PLs. C- A hydrophobic loop (Ser311-Lys317) anchors the protein when it is inserted into the membrane. Taken from: The EMBO Journal (1999) vol. 18, 5166-5174 .

1.9 Antigenicity of anti-β_2GPI antibodies

The exact nature of the antigen/antibody interaction between aPL/anti-β_2GPI and β_2GPI has long been subject to debate. Opinions have differed as to whether the antibodies bind to the β2-GPI/phospholipids (PL) complex or to an epitope formed when the protein binds to negatively charged PLs or specially treated synthetic surfaces. γ-Irradiation of polystyrene plates increases the net negative charge enabling more β_2GPI to bind. It was proposed that the binding of β_2GPI to the PL surface caused a conformational change in the protein resulting in the formation of neo or cryptic epitope(s) for antibody binding. Preliminary studies in support of a cryptic epitope demonstrated that aPL were able to bind to β_2GPI coated on an oxidized polystyrene surface in the absence of cardiolipin, but did not bind to non-irradiated plates (Matsuura et al., 1994; Chamley et al., 1999). Binding of anti-β_2GPI to CL was inhibited by the simultaneous addition of CL-coated latex beads and β_2GPI but not addition of β_2GPI alone. This observation provided evidence that a cryptic epitope was formed as the result of conformational change in β_2GPI when the protein interacted with PL or irradiated plates.

In a ground-breaking study, Tincani and co-workers purified polyclonal anti-β_2GPI preparations from 5 patients with APS and characterized them in detail. They found that anti-β_2GPI were able to bind to β_2GPI in solution and in the absence of PLs. The study defined anti-β_2GPI as mono-reactive low-avidity auto-antibodies which react with epitopes located on β_2GPI (Tincani et al., 1996). Two other groups of investigators provided the first evidence that anti-β_2GPI bind to wild type protein and binding is dependent upon antigen density and bivalency of the antibody and not formation of a neo-epitope. Importantly, they showed that aPL and anti-β_2GPI bound to the protein in the absence of PL. Experiments demonstrated little detectable binding of anti-β_2GPI to fluid phase β_2GPI at low physiological concentrations or β_2GPI bound to ELISA plates at low density (Roubey et al., 1995; Sheng et al., 1998). Bivalent $F(ab')_2$ fragments bound more efficiently to immobilized β_2GPI than monovalent Fab'. Roubay concluded that low affinity aPL bind bivalently to β_2GPI on an irradiated plate or PL surface only when it was present at a sufficient concentration (Roubey et al., 1995). This observation is consistent with the more recent recognition of a threshold for β_2GPI

ELISA antigen coating concentration below which polyclonal anti-β_2GPI do not bind (Reddel, Wang, & Krilis 2003). Anti- β_2GPI binding to the native protein in solution provided important evidence against the theory of cryptic epitope formation.

In order to understand the pathology of these antibodies, it would be helpful to know the association between antibodies to certain epitopes and their contribution to thrombosis. A number of studies have been undertaken in order to determine the anti-β_2GPI antigenic targets on β_2GPI (Figure 1.1) (Giles et al., 2003). There is now evidence for anti-β_2GPI specificity on all five domains of the protein (reviewed in Giles et al., 2003). Domain V contains the positively charged lysine rich PL binding region and β_2GPI binds to human umbilical vein EC (HUVEC) through the same PL binding region in Domain V (Del Papa et al., 1998) (Figure 1.1). The crystal structure of β_2GPI shows that domains III and IV are heavily glycosylated and partially shielded from protein-protein interactions (Bouma et al., 1999) (Figure 1.1). They function as links for the N-terminal Domain I exposed to the blood and Domain V bound to the PL surface. Extensive work by Iverson and colleagues has suggested that Domain I of the molecule contains the major epitopes for anti-β_2GPI binding (Iverson, Victoria, & Marquis 1998; Iverson et al., 2002). Domain I is the one most accessible to circulating antibodies and it contains a pocket of negative charge which may help to attract clusters of positively charged arginine and lysine residues identified in anti-β_2GPI complimentarity determining regions (Bouma et al., 1999).

β_2GPI has a low affinity for PL, but interaction with some anti-β_2GPI can increase its affinity for PL up to 100 fold (Willems et al., 1996). Experiments with LA positive monoclonal anti-β_2GPI showed that only bivalent β_2GPI /antibody complexes were able to bind to PL surfaces and exert LA activity (LAC) (Arnout et al., 1998). The addition of a recombinant contruct of dimer-β_2GPI to clotting assays resulted in polonged clotting times and the same contruct had a much higher affinity for EC and activated platelets than the monomeric form (Bas de Laat, Derksen, & De Groot 2004a). Dimerization of the protein can occur when both Fab' antigen binding sites of the antibody were engaged with separate protein molecules. Anti-β_2GPI may cause formation of β_2GPI dimers *in vivo* resulting in

clustering of antigen, increased affinity for EC and resultant EC activation (See 1.17).

1.10 The Lupus Anticoagulant

The term "Lupus Anticoagulant" is universally recognized as a paradox since LA prolong blood clotting in the test tube but patients with LA's have a strong predisposition to thrombosis. A systematic review of the literature from 1988 to 2000 provided strong evidence that LA are a strong risk factor for venous and arterial thromboembolic events, odds ratio with 95% confidence interval, 5.7 to 9.4 (Ginsberg et al., 1995; Galli et al., 2003a). Lupus anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with SLE. Determination of LAC is more specific for APS than aCL (Galli et al., 2003a). Therefore each patient with clinical manifestations of APS should undergo testing for LA.

Internationally determined guidelines for the detection and diagnosis of LA have been defined. Recommendations for diagnosis of LA include (1): prolongation of a PL dependent clotting time (2): evidence of a PL- dependent inhibitor demonstrated in mixing studies and (3): phospholipid dependence of the inhibitor effect must be confirmed by the addition of excess phospholipid. Tests routinely used for detection of LA include a screening test such as the aPTT performed with a LA sensitive reagent (if prolonged checked with a 1 to 1 mix of normal plasma to rule out clotting factor deficiency), the dilute Russell's viper venom time (dRVVT), plus confirmatory testing with excess PL. Different possible mechanisms of interference with the PL-dependent reactions in the coagulation cascade in *in vitro* testing and low specificity mean that more than one assay for LA detection should be performed (Greaves et al., 2000).

LA are heterogeneous in terms of antigenic specificity although β_2GPI and prothrombin (Bevers et al., 1991) bound to the PL surface are the primary targets. LA's were thought, in part, to exert anticoagulant effect by inhibiting prothrombinase activity due to binding to the prothrombin activator complex (II, V, PL, Ca). But, just as β_2GPI was found to be required for the binding of autoimmune

aPL to cardiolipin**,** it was also determined that β_2GPI was necessary for some LA's to express their anticoagulant activity *in vitro* and its depletion resulted in the abrogation of LA activity from most LA positive plasmas (Roubay et al., 1992). Roubay postulated that autoimmune aPL, indeed anti-β_2GPI, amplified the interaction of β_2GPI with PL surfaces possibly by cross-linking molecules of β_2GPI on the PL surface resulting in impaired binding and activation of clotting factors. Subsequent work by Takeya *et al* also determined that anti-β_2GPI enhance the binding of β_2GPI to the endothelium and induce the formation of β_2GPI complexes on the PL surface due to their bivalent property enhancing LA activity via the $F(ab')_2$ fragment and not the Fab' fragment (Takeya et al., 1997). This suggests that the clustering of molecules on the PL surface and the bivalent nature of the anti-β_2GPI may hinder the interaction of clotting factors on the PL surface and exert LA activity. The paper stresses the heterogeneous LA activity of aCL. Some anti-β_2GPI will require β_2GPI in order to bind to cardiolipin but will not possess LAC. LA activity of anti-β_2GPI appears to be epitope dependent (Takeya et al., 1997). Those monoclonal antibodies directed against the carboxy terminal of the fourth domain and the fifth domain showed no LA like activity while those directed against the third and fourth domain inhibited thrombin generation in the dRVVT. The effect was blocked in the presence of excess PL confirming the LA activity of the antibodies. Increased levels of IgG anti-β_2GPI are associated with increased incidence of thrombosis in patients positive for LA (Zoghlami-Rintelen et al., 2005).

Recent studies have resulted in the advent of assay systems more specific for the detection of LA positive anti-β_2GPI. Bas de Laat *et al* outline the development of a modified aPTT incorporating cardiolipin vesicles that is capable of specifically detecting LA caused by anti-β_2GPI (Bas de Laat et al., 2004b). The addition of CL shortens the clotting time due to anti-β_2GPI and prolongs the times due to anti-prothrombin antibodies. Using the assay, they established that β_2GPI dependent LA was irrefutably associated with a history of thrombosis (odds ratio 42.3: 95% confidence interval 194.3-9.9) while an increase in thrombosis was not associated with LA in the absence of anti-β_2GPI (odds ratio 1.6). The authors suggested that

anti-β_2GPI with LA activity are those responsible for thrombosis in APS and their assay may be instrumental in identifying patients at risk of thrombosis.

A subsequent study by the same group divided IgG anti-β_2GPI from 52 patients with systemic autoimmune diseases into two groups using ELISA. Group A antibodies recognized domain I of β_2GPI coated onto hydrophobic plates and group B showed heterogeneous activity towards all domains. Group A (23 out of 25, 92%) antibodies recognized an epitope around Gly40-Arg43, had β_2GPI-dependent LAC activity and a strong association with thrombosis thereby identifying a subset of pathogenic anti-β_2GPI specific for domain I of the protein (Bas de Laat et al., 2004c).

1.11 Possible mechanisms of aPL induced thrombosis

The mechanism by which aPL cause thrombosis is uncertain although a number of mechanisms have been proposed. Since aPL bind to negatively charged surfaces, it is possible that they interfere with regulatory coagulation proteins that are associated with phospholipid on endothelial and platelet surfaces. This could result in an imbalance between the pro and anti-coagulant systems and result in thrombosis. Antibodies directed against β_2GPI and prothrombin are the most frequently occurring and best studied of these antibodies. Other candidate proteins addressed in the literature which include both activators and inhibitors of clot formation are: protein C, protein S, tissue plasminogen activator, thrombomodulin, high and low molecular weight kininogens, Annexin V, coagulation factor XII, complement component C4, complement factor H and coagulation factor VII/VIIa (Pierangeli, Gharavi & Harris, 2000). Protein C becomes activated when thrombin binds to thrombomodulin on the EC surface. Activated protein C acts as an anticoagulant in conjunction with its cofactor, protein S by inactivating factor Va and VIIIa in the cascade (Figure 1.2). ProteinC/S interactions occur on phospholipid surfaces. Antibodies to protein C, S and thrombomodulin found in some APS patients could interfere with this process (Pengo et al., 1996). Impaired fibrinolysis in association with thrombosis and aPL has been identified in some patients with SLE. This observation was attributed to

increased levels of circulating plasminogen activator inhibitor-1 and tissue plasminogen activator in these patients, and possibly the result of antibody induced EC perturbation (Jurado et al., 1992).

1.12 Tissue Factor

Tissue factor (TF) is the major physiological initiator of blood coagulation *in vivo*. (Mackman 2006)(Figure 1.2) It is a 47 kDa transmembrane glycoprotein and is structurally a member of the class II cytokine receptor family. Cells that are normally in contact with blood such as vascular endothelium and monocytes do not express TF in its functionally active form. These cells synthesize and express TF after being stimulated by lipopolysaccharide (LPS) or other inflammatory cytokines (Roubay RAS 2000). TF expression is imperative in order for normal endothelium to be converted to a procoagulant surface and increased procoagulant activity (PCA) due to TF is associated with thrombosis. Physiological activation of the coagulation pathway must be very rapid implying availability of preformed molecules. Functionally inactive TF is encrypted within vesicles in the cell membrane. Disruption of the membrane exposes PL necessary for the binding of coagulation factors and the TF-VIIa complex (Figure1.2) (Price et al., 2004). Several transcription factors that control the TF gene are activated by inflammatory cytokines such as interleukin1-β (IL-1 β), IL-6, and TNF-α.

In vivo, monocytes adhere to activated EC and become activated in turn, expressing TF and subsequent PCA. The possible association between TF and APS was first demonstrated in studies using sera from patients with SLE. Incubation of normal donor monocytes with patient derived monoclonal aPL, resulted in the induction of TF-like PCA (Kornberg et al., 1994). Monocytes from a healthy donor incubated with APS plasma expressed more TF antigen and PCA than monocytes incubated with control plasma (Amengual et al., 1998). A study by Cuadrado et al. (1997) showed that monocytes from PAPS patients with a history of thrombosis have increased expression of TF, TF associated PCA, cellular and soluble TF. Dobado-Berrios et al. (1999) collected freshly isolated monocytes from 14 individuals with PAPS, 9/14 had a history of thrombosis. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed on

monocyte total RNA in order to evaluate levels of TF gene expression. The 9 patients with thrombotic history showed increased levels of mRNA for TF gene compared to normal controls and patients with no history (Dobado-Berrios et al., 1999). A study by Williams suggested that monocytes from PAPS patients are primed to express more TF-associated PCA in response to a second stimulus such as LPS when compared to normal monocytes (Williams et al., 1998).

The complex formed between TF and VIIa initiates coagulation. TF functions as the co-factor in order for VIIa to cleave its substrates factor IX and factor X (Roubey 2000). Factor VIIa/TF is responsible for the initial induction of Xa generation. This initial response will produce enough thrombin to allow local aggregation of platelets in addition to factors V and VIII activation. However, activated clotting factors will be removed by circulating blood and anticoagulant mechanisms involving protease inhibitors and fibrinolysis will be inititated. Coagulation must be sustained and amplified on site by the actions of factors IXa, VIIIa and XIa stressing the importance of both intrinsic and extrinsic pathways. Factor IX and X activation by TF is controlled by tissue factor pathway inhibitor (TFPI). TFPI is a Kunitz-type protease inhibitor. The several Kunitz-type domains on the TFPI molecule are consistent with the two inhibitory targets, factor Xa and factor VIIa/TF. Increased plasma concentrations of TFPI have been identified in other diseases and may be considered a marker of EC dysfunction. TFPI regulates the beginning of coagulation by factor Xa-dependent feedback inhibition of the TF/VIIa complex. TFPI forms a quaternary complex (TFPI, TF, VIIa, Xa) and inhibits VIIa and Xa in the presence of calcium ions and negatively charged PL (Broze 1995). TFPI may also inhibit factor Xa directly in the absence of phospholipid. Three intravascular pools of TFPI exist. 10-50% of TFPI circulates in plasma complexed to lipoproteins (50-150 ng/ml), 50 to 90% of TFPI is bound to glycosaminoglycans on the vessel wall and can be released into the circulation by the introduction of heparin (Kato 2002). A soluble isoform of TF present in blood has recently been described called alternatively spliced human tissue factor (Szotowski et al., 2005). Endothelial cells incubated with TNF-α or IL-6 released asHTF into the supernatant and this soluble TF is thrombogenic in the presence of phospholipids. APS patients have increased levels of both TF and TFPI in their plasma (Cuadrado et al., 1997; Amengual et al., 1998). An association between

aPL and TF expression on EC and monocytes and upregulation of the TF/TFPI pathway in the pathology of thrombosis in APS is strongly implicated based upon these observations.

1.13 Expression of TF in EC

When the integrity of the endothelium is breached, EC are stimulated to express TF which functions as a high affinity receptor for coagulation factor VII/VIIa. TF expression can be induced on EC in response to thrombin, inflammatory cytokines, hypoxia, oxidized lipoproteins and LPS (Cines et al., 1998; Vega-Ostertag et al., 2005). Branch et al. (1993) reported that APS patient sera induced PCA on EC (Branch & Rodgers 1993). Vega-Ostertag et al. confirmed the upregulation of TF by demonstrating dose-dependent increased expression of TF on EC in response to aPL, the effects of which were negated by pre-treating the cells with fluvastatin. They were also able to show that aPL increased TF PCA (conversion of X to Xa) on EC and up-regulated TF mRNA transcription. aPL have been shown to induce increased TF transcription by activating the NF-κB pathway incorporating p38 MAPK phosphorylation. Vega-Ostertag et al (2005) were able to inhibit aPL induced upregulation of TF in a dose-dependent manner by pre-incubating HUVEC with p38 MAPK inhibitor SB203580. Incubation of HUVEC with aPL also induced IL-6, IL-8 and iNOS expression (Vega-Ostertag et al., 2005).

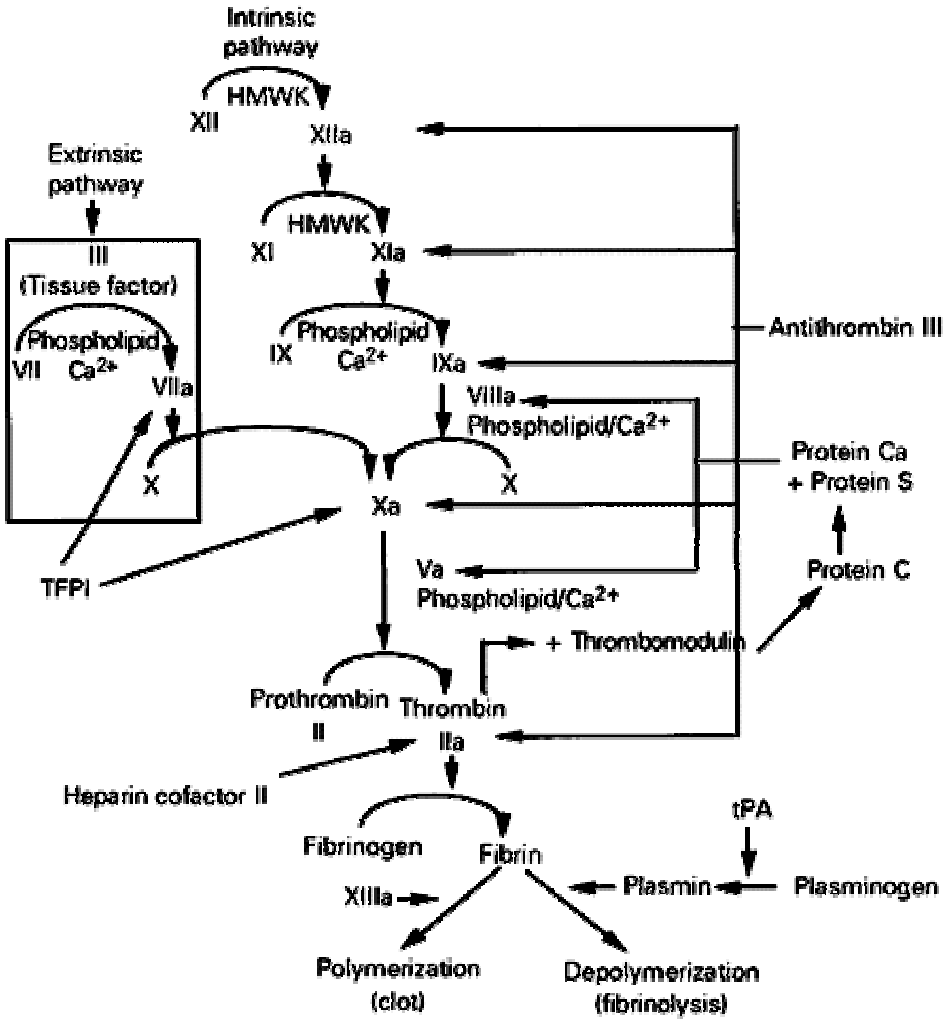

Figure 1.2 The coagulation cascade The extrinsic pathway of coagulation is initiated by TF while collagen and activation of Factor XII play a major role in the activation of the intrinsic pathway. The cascade organizes the sequence of protein interactions in the extrinsic, intrinsic and common pathway beginning with the activation of factor X to Xa. These events are summarized in Figure 1.1. In the common pathway the prothrombinase complex (Xa, II, Va, PL) catalizes the conversion of prothrombin to thrombin. Thrombin and Factor XIII act on fibrinogen and in combination with platelets, form a fibrin plug. PL surfaces are essential for the coagulation mechanism to be carried to completion. Protein C and Protein S function as anticoagulants by inactivating VIIIa and Va. Thrombomodulin on the EC surface is required for activation of thrombin. Plasminogen is cleaved to become plasmin, the enzyme required for fibrinolysis of the clot. All proteins contribute to the pro- and anticoagulant balance of normal hemostasis.

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1.14 Platelets and APS

Platelets have a primary role in hemostasis as they provide procoagulant PL surfaces for interaction of coagulation factors. Platelet adherence to injured endothelium is followed by platelet activation, and platelet granule release. This is followed by platelet shape change and exposure of PL in the platelet membrane. Platelets circulate in a more activated state in APS have a more prevalent role in arterial clots (Greaves 1999). Both polyclonal and monoclonal aPL have been shown to activate platelets *in vitro* (Joseph et al., 2001). Patients with PAPS often demonstrate mild thrombocytopenia that is not usually associated with bleeding. Platelets sensitized by autoantibodies may be removed from the circulation resulting in lower platelet number. *In vitro*, pre-treatment of platelets with low concentrations of another aggregation agonist such as thrombin is required before aPL can induce platelet activation. aPL can only bind to activated platelets and the binding is β_2GPI-dependent (Shi, Chong, & Chesterman 1993). IgG purified from patients with APS enhance thrombin induced platelet activation and thromboxane B_2 (TXB_2) formation and complexes of aPL and β_2GPI have been shown to induce increased platelet TXB_2 production (Robbins et al., 1998). TXB_2 is a potent eicosanoid responsible for vasoconstriction and platelet aggregation. It is possible that β_2GPI-anti-β_2GPI complexes may prime platelets to be more adherent and susceptible to involvement in clot formation.

Parallels have been drawn between heparin induced thrombocytopenia (HIT) and APS since both are characterized by autoantibody mediated thrombocytopenia and thrombosis in both venous and arterial sites. In both instances the target proteins (β_2GPI or Platelet factor 4) bind to negatively charged molecules resulting in autoantibody formation (Warkentin, Aird, & Rand 2003). Unlike aPL, the antibodies in HIT bind to the FcγRII where as antibodies in APS are $F(ab)_2$ dependent. In HIT PF4/heparin macromolecular molecules form and IgG binds only when critical concentrations of heparin and PF4 are reached (Warkentin, Aird, & Rand 2003).

1.15 Thrombosis in APS

aPL are associated with thrombosis in APS, indeed thrombosis is the most common clinical event manifested in APS (Cervera et al., 2002). Approximately 70% of events are venous and 30% are arterial (Hughes 1983; Cameron & Frampton 1990; Cuadrado et al., 1997; Asherson et al., 1998). Venous thrombosis, particularly deep vein thrombosis (DVT) of the legs, is the most common clinical manifestation in APS and occurs in 29 to 55% of APS patients. This is accompanied by pulmonary emboli in at least half of the patients (Levine, Branch, & Rauch 2002). Arterial thromboses are less common, with strokes and transient ischemic attacks in the brain being the most common manifestation of arterial events (50%). A further 23% of arterial events occur in the coronary vessels while the remaining 27% occur in renal, retinal, subclavian and pedal arteries. Cerebral events can be caused by emboli from mitral or aortic valve vegetations. Thrombosis can occur in vessels of any size. Capillary, arteriole or venule involvement can reflect a clinical picture similar to hemolytic-uremic syndrome, thrombotic thrombocytopenic purpura or other thrombotic microangiopathies. Notably, thrombosis in APS can occur in both the venous and/or arterial vasculature whereas thrombotic events occurring in other thrombophilias tend to occur in either venous or arterial parts of the circulation but not both (Rosenberg & Aird, 1999).

Not all patients with aPL go on to develop APS making it difficult to determine the risk of thrombosis associated with these antibodies. The development of animal models of APS has helped to confirm that aPL have a direct pathogenic role in APS. *In vivo* experiments by Pierangeli et al. (1995) showed that the infusion of APS patient immunoglobulins containing aPL into mice increased the size and duration of thrombi formed at the site of an artificially damaged vessel. The same group were able to repeat this observation in mice following immunization with β_2GPI (Pierangeli et al., 1996). Mice and rabbits immunized with purified human β_2GPI developed aPL and symptoms of APS including fetal wastage and readsorption (Blank et al., 1991; Blank et al., 1994). Mice and rabbits immunized with purified human β_2GPI developed aPL and symptoms of APS.

Laboratory measurement of LA and aCL provide clinicians with diagnostic markers for APS. Numerous studies have addressed the role of the measurement of different aPL and its usefulness when attempting to identify those at risk of developing venous and/or arterial thrombosis and subsequent recurrence. LA is the strongest risk factor for both venous and arterial thrombosis in patients with SLE, and LA positivity is also strongly associated with pregnancy loss in these patients (Ginsberg et al., 1995). Importantly, LA is a more specific test for APS than aCL. An extensive review of the relevant literature from 1988 to 2000 was undertaken by Galli et al., 2003. Comparison of 12 prospective studies in 1608 patients showed a significant association between LA and thrombosis (Odds ratio, OR of 7.3-10.7). Therefore LA are consistently associated with thrombosis, and screening for LA is necessary in order to identify patients at risk of developing thrombosis as well as aiding their therapeutic management. aCL were not as strong risk factors unless present in medium to high titres in the IgG isotype. ACL were more strongly associated with arterial events than LA. The review confirmed that LA should be included in the laboratory criteria in relation to venous and arterial thrombosis while the relevance of anticardiolipin antibodies was slightly less certain (Galli et al., 2003a). Retrospective studies are unable to determine whether aPL cause thrombosis or are actually a consequence of the thromboembolic event.

A prospective Canadian study measured LA and aCL in 256 patients suspected of having venous thromboembolism (VTE) or pulmonary embolism (PE). The study was undertaken in order to determine the clinical relevance of these antibodies in the absence of SLE. A strong association was seen between LA and VTE (OR 9.4) while no association was seen between aCL and VTE (OR 0.7) although this was perceived to be due to the high numbers of positive aCL assays in patients without VTE (Ginsberg et al., 1995).

An extensive prospective study of 360 patients with LA and/or elevated IgG aCL was undertaken in sixteen institutions included in the Italian Registry of Antiphospholipid Antibodies. These patients were investigated for the presence of aPL because they had a history of thrombosis, a disease related to the presence of aPL, or a coagulation abnormality that was suggestive of LA (Finazzi et al.,

1996). Patients were followed for a median of 3.9 years and 34 patients developed thrombosis. Patients with a previous vascular event showed a 5.4% patients/year incidence of recurrent events compared to 0.95% in patients with no symptoms thus identifying a previous thrombotic event as the most important clinical risk factor for the development of thrombosis. This data was confirmed by another European APS study in which 4.5% of patients per year suffered a recurrence (Vianna et al., 1994).

Pertinent to and in support of this study, a close association has been found between IgG anti-β_2GPI and venous thrombosis (VT) (Cabral, Cabiedes & Alarcon-Segovia 1995; Martinuzzo, Forastiero & Carreras 1995; Forastiero et al., 1997; Wahl et al., 1998). Persistently positive anti-β_2GPI identifies patients who have a higher risk of thromboembolic events (Carreras, Forasterio & Martinuzzo 2000; Triplett 2002). In a further systematic review and meta-analysis of the literature 27 (largely) retrospective studies analysed 60 associations between anti-β_2GPI and thrombosis (studies involved patients with SLE, LA or aCL, difficult to evaluate these antibodies as independent risk factors for thrombosis.) The odds ratio (OR) for anti-β_2GPI (95% confidence interval-CI) for thrombosis were available for 27 studies in 4,394 patients and 1973 controls. IgG anti-β_2GPI were significantly associated with thrombosis in 20 of 33 studies(61%), IgM in 7 of 15 studies (47%) and IgA in 3 out of 3. Overall, IgG anti-β_2GPI antibodies were associated with venous thrombosis and two multivariate analyses identified them as independent risk factors for vascular events. aCL were associated with arterial thrombosis (Galli et al., 2003b). It was noted by the author that the majority of aCL recognize β_2GPI bound to cardiolipin (as well as co-factor independent aPL) yet anti-β_2GPI and aCL show different associations implying that they are not identical. ELISAs for anti-β_2GPI are more specific for APS than aCL since only antibodies specific for β_2GPI are detected. (Martinuzzo et al.,1995). In another study, a cohort of 87 patients (55 with a history of thrombosis and 32 without) and persistently positive LA were tested for aCL and anti-β_2GPI. Patients with history of thrombosis had significantly higher levels of both antibodies compared to those without thrombosis. Increased risk of thrombosis was associated with elevated IgG anti-β_2GPI (OR 4.0, 95% CI 1.2-13.1, OR for VTE was 5.2). Therefore

patients with LA who have anti-β_2GPI have a significantly increased risk of risk of developing thrombosis. (Zoghlami-Rintelen et al., 2005).

Patients with aPL can be divided into two groups. Those who are asymptomatic require observation while those who have had previous thrombosis or miscarriage require treatment. Retrospective studies of patients after the initial thrombotic event have confirmed that these patients will not have recurrent events if they receive high dose oral anticoagulation (warfarin) with an INR (international normalized ratio) between 2.5 and 4.0 (Rosove & Brewer, 1992).

1.16 Fetal loss in APS

The classification criteria for APS include recurrent fetal loss as a clinical criterion for diagnosis (Wilson et al., 1999) (Table 1.1). Women with APS are most at risk of pregnancy loss from the 10^{th} week of gestation (fetal period). The dagnostic criteria for the obstetric manifestations of APS state that the patient must have one or more unexplained pregnancy losses in the fetal period (up to 34 weeks gestation) or three or more losses in the first 9 weeks of gestation in the presence of moderate to high titres of aPL. aPL are associated with recurrent miscarriage, fetal death and intrauterine growth restriction (IUGR). Pre-eclampsia, premature delivery and placental abruption are also associated with APS. (Pre-eclampsia is a common condition also associated with intrauterine growth retardation (IUGR) involving high blood pressure, kidney malfunction resulting in fluid retention and protein in the urine after the sixth month of gestation.)

Several *in vivo* murine studies have provided evidence for the pathogenic role of aPL in recurrent fetal death. An animal model of SLE, MRL/lpr mice with high levels of IgG aPL, and thrombocytopenia have intrauterine fetal death with smaller litter size compared to NZB/NZW F1 mice (another SLE model) and normal Balb/c mice. Branch *et al* infused pregnant Balb/c mice with IgG aPL fractions from women with pregnancy loss and this resulted in intrauterine death and miscarriage within 48 hours of injection (Branch, Dudley, & Mitchell 2006). Histopathology showed decidual necrosis, intravascular IgG aPL and fibrin deposition while the

infusion of IgG from normal pregnant subjects did not result in intrauterine death or histological evidence of infarction. High levels of aPL were induced in normal mice when they were infused with purified human β_2GPI. This resulted in pregnancy loss in some strains of mice (Gharavi et al., 1992).

The precise role that aPL play in pregnancy loss remains uncertain. The binding of affinity purified aPL to placental antigens suggests that they may cause placental damage. β_2GPI has been demonstrated on the surface of trophoblasts by *in vivo* histochemical staining and is present in higher concentration on trophoblastic villi of women with APS and fetal loss when compared to controls (LaRosa et al., 1994). It has been demonstrated that phosphatidylserine is exposed on the external cell surface during intertrophoblastic fusion (syncytium formation) and this would provide a negatively charged surface for the binding of β_2GPI. Human monoclonal and polyclonal anti-β_2GPI are able to bind to trophoblasts *in vitro* directly through exposed anionic PL (co-factor independent) or adherent β_2GPI. Binding altered *in vitro* trophoblast function by reducing the release of human chorionic gonadotrophin (hCG) and reducing matrigel invasiveness suggesting that aPL may alter trophoblast development and placentation (Di Simone et al., 2005). The same group demonstrated binding of β_2GPI to the trophoblast surface by the PL binding site in the fifth domain of the protein as in EC (Di Simone et al., 1999). This suggests that aPL may bind to adherent β_2GPI on trophoblasts *in vivo.* Incubation of polyclonal IgG containing anti-β_2GPI impaired fusion of cytoptrophoblasts. The authors postulated that binding of antibody to adherent β_2GPI may reduce hCG secretion and prevent normal syncytiotrophoblast formation resulting in the defective placentation seen in APS (Di Simone et al., 2005).

In APS, fetal death is preceded by intrauterine growth retardation, oligohydramnios, and abnormalities of heart rate all triggered by impaired fetal blood supply and hypoxia. These effects are thought in part to be a consequence of inadequate utero-placental blood flow (Branch 1994). Infarction is described as an area of ischaemic villous necrosis, aggregation of the villi and destruction of the intervillous space. Isolated case reports have attributed uteroplacental insufficiency to abnormality in the terminal branches of the spiral arteries that provide maternal blood to the

intervillous space in the placenta. In 1982, De Wolf *et al.* (1982) identified the absence of these normally occurring changes in the spiral arteries in the placenta from a woman with APS, LA and fetal death in the third trimester. The vessels were smaller and showed intimal thickening, fibroblastic proliferation, intraluminal thrombosis and infarction of greater than fifty percent of the placenta. The vasculopathy revealed accumulation of lipid laden macrophages similar to that seen in atherosclerotic lesions. In normal pregnancy fetal trophoblasts (cytoptrophoblasts) invade the wall of the spiral arteries replacing some of the endothelium. Muscular tissue is replaced by fibrous material and as a consequence, the arterioles lose their elasticity. This allows maternal blood to freely enter the intervillous space where nutrients and oxygen are absorbed by fetal blood (Branch 1994). Defective trophoblast invasion has been implicated in some SLE, pre-eclampsia and APS pregnancies with IUGR.

A previous study of 47 women experiencing fetal death (16 had aPL) concluded that women with APS showed no distinct placental pathology but most showed signs of infarction, thrombosis, fibrosis and hypovascular villi (Out et al., 1991). This is in agreement with a recent prospective study of placental biopsies from 12 women with PAPS. The results did not show absence of remodelling in spiral arteries compared to controls, however third trimester APS placental bed biopsies with adverse pregnancy outcome showed evidence of failed endovascular trophoblast invasion, more prominent synctial knot formation than controls, and more parenchymal infarction or thrombosis (Stone et al., 2006). Of note, a significant increase in the concentration of inflammatory cells, particularily macrophages, clustered around the blood vessels in APS biopsies was observed. The authors postulated that these inflammatory cells could be contributing to pregnancy complications (Stone et al*.,* 2006). In conclusion, there does not appear to be a distinct histological lesion for APS in the placenta. Histologic findings in pre-eclampsia and SLE with secondary APS are difficult to distinguish from those of APS in pregnancies with poor outcome.

Thrombotic manifestations are a major contributing factor in aPL mediated pregnancy loss (Holers et al., 2002). Annexin V is a PL binding protein with anticoagulant properties (displaces other coagulation proteins from PL surface)

Rand *et al*. (1997) reported that annexin V expression (placental anticoagulant protein I) was markedly reduced on placental villi of patients with APS. A subsequent study by the same group showed that incubation of trophoblasts and endothelial cells with IgG aPL resulted in reduced expression of annexin V on the surface of both cell types resulting in a procoagulant phenotype *in vitro* (Rand et al., 1997).

aPL have been shown to induce fetal loss *in vivo*. Recent studies have shown a direct association between complement activation in the placenta, tissue damage, and fetal loss in aPL treated mice. *In vivo* blocking of the complement cascade with C3 convertase inhibitor complement receptor-1 elated gene/protein y (Crry) blocked fetal growth retardation and fetal loss (Holers et al., 2002). The major complement components involved are C3 and C5, mice deficient in these were resistant to enhanced white cell adhesion and thrombus formation induced by aPL (Pierangeli et al., 2005). Products of complement activation such as C5a and C3a can trigger the release of TF, recruitment and activation of neutrophils as well as other inflammatory cells such as monocytes (Girardi et al., 2003). Girardi proposed that treatment with heparin prevents obstetric complications by blocking activation of complement rather than preventing thrombosis (Girardi, Redecha & Salmon, 2004). This observation further lends support to the finding that placenta of APS patients demonstrate infiltration with large numbers of macrophages indicating that the aPL-mediated mechanism of fetal loss may in fact be mediated primarily by inflammation (Stone et al., 2006).

1.17 aPL mediated activation of EC

EC activation was defined as “altered synthesis of proteins that mediate functional characteristics of cells in response to stimulation with cytokines.”(Cines et al., 1998). Upon EC activation, production of mRNA generally results in protein synthesis and change in the phenotype of the cell, this is called endothelial type II activation (Kaplanski et al., 1997). However, EC activation can include alterations in cell function that do not require transcription and translation. Therefore, EC activation is better defined more generally as a change in cell phenotype or function in response to external stimuli (Zimmerman et al., 1999).

It is well documented that aPL cause EC activation. Initial experiments assessed the effect of APS patient IgG on endothelial cell activation. The ability of affinity purified IgG aPL to bind to EC and upregulate adhesion molecule expression was first demonstrated by Del Papa *et al.* (Del Papa et al., 1995). This effect was shown to be due to the binding of the antibodies and autocrine expression of IL-1α. Activation resulted in expression of IL-6 and IL-1β which upregulate adhesion molecule expression in turn. Upregulation of adhesion molecules (ICAM-1, VCAM-1, E-selectin) in the presence of anti-β_2GPI was confirmed by Simantov *et al.* who were able to further support their findings by showing increased adhesion of monocytes to the EC (Simantov et al., 1995). Incubation of EC with β_2GPI and rabbit anti-β_2GPI resulted in significant increases in a monocyte cell line (MM6) cell adhesion and EC surface E-selectin expression. Cells were only activated in the presence of both β_2GPI and anti-β_2GPI confirming co-factor requirement for EC activation. Subsequent experiments demonstrated a dose-dependent increase in the expression of ICAM-1, VCAM-1 E-selectin, IL-6 and 6-keto prostaglandin F1α in response to affinity purified IgG anti-β_2GPI and monoclonal anti-β_2GPI -derived from an APS patient (Del Papa et al., 1997).

Experiments by Pierangeli et al. (1995) provided the first *in vivo* evidence that immunoglobulins from patients with APS increased adhesion of white cells to endothelium. They developed a mouse model of the microcirculation that enabled measurement of changes in adhesiveness of leukocytes to the postcapillary venules of the cremaster muscle in mice. After 72 hours, mice infused twice with aPL showed an increase in the number of adherent white cells when compared to mice infused with normal control IgG (Pierangeli et al., 1995). Subsequent experiments in mice immunized with β2GPI or human IgG aPL antibodies showed dose-dependent formation of significantly larger thrombi *in vivo* at the site of vascular injury when compared to normal control IgG infusion (Pierangeli et al., 1996). Thrombi also took longer to lyse. Similar results were noted in response to IgM and IgA APS immunoglobulin (Pierangeli et al., 1995). Importantly, these experiments demonstrated the association between aPL induced EC activation and thrombus formation *in vivo.*

It has been proposed that several pathways may be involved in anti-β_2GPI induced EC activation. One pathway through which aPL activate endothelial cells is the transcription factor NF-κB. NF-κB is linked to its inhibitor IκB in the cytoplasm. When EC are activated by inflammatory cytokines, IκB becomes phosphorylated and degraded by IκB kinases. NF-κB is then free to move to the nucleus where it induces transcription in a large number of genes that possess the corresponding promoter (Tak & Firestein 2001). Inflammatory mediators such as TNF or IL-1 activate EC via NF-κB resulting in increased transcription of TF and leukocyte adhesion molecules. Incubation of EC with anti-β_2GPI results in the movement of NF-κB from the cytoplasm into the nucleus (Dunoyer-Geindre et al., 2002). Preincubation of the cells with the nuclear translocation inhibitor BAY11-7085 prevented the increase in adhesion molecule expression normally seen in response to these antibodies. Dunoyer-Geindre *et al.* concluded from these observations that NF-κB is key to at least part of the inflammatory response triggered by anti-β_2GPI. (Dunoyer-Geindre et al., 2002). Incubation of EC with anti-β_2GPI resulted in a partial translocation of NF-κB to the nucleus after several hours although this delay could be due to suboptimal concentrations of antibody used in the experiments. TNF triggers complete translocation that occurs within 30 minutes. This suggests that there may be differences as well as similarities between signal transduction pathways activated by anti-β_2GPI and other inflammatory cytokines. Fluvistatin was shown to inhibit upregulation of adhesion molecules in HUVEC in the presence of aPL by impairing the migration of NF-κB transcription factor to the nucleus and its subsequent binding to pro-inflammatory genes (Meroni et al., 2001). Activation of EC by anti-β_2GPI results in activation similar to that induced by cytokines (IL-1β or TNF-α or LPS) (Meroni et al., 2001). Anti-β_2GPI and LPS activate EC via IL-1 receptor-activated kinase (IRAK) phosphorylation and the MYD88 pathway, suggesting the involvement of the toll-like receptor family (Raschi et al., 2003a).

Endothelial activation by aPL is not mediated by Fc receptor binding. Thrombus formation and EC activation were studied in mice deficient in the FcR γ (-/-) in the presence of aPL IgG and IgG-NHS. FcR γ (-/-) mice injected with aPL IgG had significantly larger thrombi than those injected with normal IgG. These mice also

showed increased white cell adhesion compared to controls (Pierangeli, Gharavi, & Harris 2000).

1.18 Origin of anti-β_2GPI antibodies- An autoimmune response mediated by molecular mimicry?

In general, autoimmune diseases are thought to be caused by a combination of genetic and environmental factors. Autoimmunity can be defined as the breakdown of mechanisms responsible for self tolerance. As a consequence, immune cells launch an immune response against self-antigens. All individuals have small numbers of autoreactive T cells that recognize self antigens with low avidity. Some autoreactive T cells may have a beneficial role such as elimination of malignant cells. When normal regulatory mechanisms fail, loss of tolerance to self antigens can lead to the production of organ specific and/or systemic autoreactive antibodies.

The healthy immune system is exposed to a variety of bacterial, viral and parasitic antigens, and many are representative of highly conserved protein families. Molecular mimicry occurs when similar tertiary protein conformation or similarities in amino acid sequences occur between self and foreign proteins. This can result in cross-reactivity between B and T cells and subsequent autoimmune disease. B or T cells initially reactive against an infectious agent can cross-react with self tissues or proteins leading to tissue damage and chronic autoimmune disease (Baum et al, 1996). Molecular mimicry will only lead to autoimmunity when the host and infectious agent are similar enough to cross-react but different enough to break immunological tolerance (Oldstone et al., 1987).

Molecular mimicry has been proposed as the primary cause of a number of autoimmune disorders such as rheumatoid arthritis, diabetes, inflammatory bowel disease as well as multiple sclerosis (Kohm, Fuller, & Miller 2003). Infection is now considered to be the major trigger for the development of anti-β_2GPI antibodies. It has been suggested that molecular mimicry between pathogens and the β_2GPI molecule may result in APS. Protein data bases have revealed strong

sequence homology between target epitopes for anti-β_2GPI antibodies and a number of common pathogens (Shoenfeld et al., 2006). A number of bacterial and viral infections result in elevated titres of aCL and anti-β_2GPI resulting in the clinical manifestations of APS (Blank et al., 2004a; Blank et al., 2004b). Examples include Hepatitis C, EBV, CMV, HIV, *S.aureus*, *Streptococcus*, *Salmonell*a and *E. coli.* Molecular mimicry following infection has been implicated as a major mechanism responsible for development of Catastrophic APS (Blank et al., 2002).

Molecular mimicry at the PL binding site on β_2GPI may result in loss of self tolerance and the development of anti-β_2GPI. Initial experiments involving infusion of three synthetic peptide sequences complementary to antigens recognized by patient derived monoclonal anti-β_2GP, showed inhibition of EC activation *in vitro* and inhibition of induction of experimental APS in naïve mice in the presence of anti-β_2GPI. (Peptide A ^{58}LKTPRV^{63} corresponds to the first and second domain of the ß$_2$-GPI molecule, peptide B ^{208}KDKATF^{213} the fourth domain, and peptide C ^{133}TLRVYK^{138} the third domain) (Blank et al., 1999). Subsequent experiments by Gharavi et al. (1999, 2003) showed induction of anti-β_2GPI production in naïve mice immunized with synthetic peptides with sequence homology to 72 kDa human adenovirus type 2 DNA-binding protein, CMV, HCMVA and *Bacillus subtillis* (Gharavi et al., 1999; Gharavi, Pierangeli & Harris 2003). Immunization of mice with GDKV, a 15 AA synthetic peptide with sequence homology to Gly^{274} –Cys^{288} representing a major PL binding region of β_2GPI induced aPL production *in vivo.* These antibodies induced thrombus formation. The same group of investigators were able to induce aPL in mice using synthetic peptides of bacterial and viral origin with AA sequence similar to GDKV (Gharavi et al., 1999).

Several studies have demonstrated that bacteria and viruses with sequence homology similar to β_2GPI induce production of pathogenic anti-β_2GPI and manifestations of APS *in vivo* (Gharavi et al., 1999; Blank et al., 2002; Gharavi, Pierangeli & Harris 2003). Anti-β_2GPI production was induced in naïve mice immunized with microbial pathogens sharing sequence homology with peptide C ^{133}TLRVYK^{138} from the third domain of β_2GPI identified by phage display. The

highest levels of anti-β_2GPI resulted in animals immunized with *Haemophilus influenzae, Neisseria gonorrhoeae* or tetanus toxoid. Resultant anti-TLRVYK antibodies were affinity purified and infused into naïve pregnant mice. Recipient mice developed significant thrombocytopenia, prolonged aPTT and increased percentage of fetal loss. This study demonstrated that bacteria with structural homology to β_2GPI could induce production of pathogenic anti-β_2GPI (Blank et al., 2002).

The development of pathogenic aPL involves the production of autoantibodies by autoreactive B cells and T cell help. CD4+ HLA class II restricted T cells are responsible for antibody production from B cells (Hattori et al., 2000). T cell clones from APS patients preferentially recognise the antigenic peptide containing the major PL binding site and stimulate B cells to produce anti-β_2GPI through IL-6 dependent helper activity and CD40-CD40 ligand engagement (Arai et al., 2001; Kuwana 2002). Subsequent studies have confirmed that TCRß chain expression is highly restricted in $ß_2$-GPI reactive T cells from APS patients and suggest that targeting the Vß7+TCR region may be a possible therapeutic strategy to prevent the production of anti-β_2GPI (Yoshida et al., 2002). It was postulated that since toll-like receptors (TLR) are the ligands for microbial structures, the protein may interact with TLR and anti-β_2GPI may cross-link the receptors resulting in activation of EC.

1.19 Heredity of PAPS

Molecular mimicry may be one of the mechanisms responsible for development of autoimmune disease in response to a virus or bacteria. However, it is clear that not all individuals exposed to a pathogen go on to develop APS. It has been suggested that the disease occurs through a two hit mechanism (Pierangeli, Chen, &. Gonzalez 2006). Although PAPS is considered to be an acquired disease, this implies that genetic predisposition may also be a contributing factor. Certain HLA haplotypes are associated with SLE (DR4, DR5, or DR7) and other autoimmune diseases (Arnett, Olsen, Anderson & Reveille 1991). Cantalapiedra et al. described a father and daughter with PAPS who both suffered episodes of venous thrombosis

and thrombocytopenia (Cantalapiedra et al., 1999). Another study described 12 families with inherited PAPS (Durrani, Gordon, & Murray 2002). Older family members of PAPS patients show a higher incidence of autoimmune diseases including SLE and RA and patients initially presenting with PAPS can go on to develop features of SLE. This suggests expression of familial autoimmunity (Andrews, Frampton, & Cameron 1993).

1.20 Microarray Background

The completion of the Human Genome project has provided scientists with limitless potential in their quest for insight into the interactions of genes and their functions within cells and biological systems (Venter et al., 2001). In the context of human health and disease, this invaluable knowledge can be utilized in order to determine the mechanisms of disease, how drugs interact with cells and also aid in the identification of genes that may themselves be therapeutic targets. New methods of statistical analysis are enabling the comparison of genomic and proteomic data leading to a better understanding of biological processes. As a consequence of this, the drug discovery industry will benefit from the technology. Microarray can be used to identify mutations/polymorphisms within genomic DNA. It is also one of a number of methods that measure gene expression. The methodology is exceptional in that it enables the analysis of the expression of thousands of genes in a cell type or tissue type simultaneously rather than one gene at a time.

It is now possible to determine and compare differential gene expression between diseased and healthy cells or tissue, perhaps at different time points, in response to drug treatment or under variable conditions. A number of large studies have used microarray methods to gain insight into tissue specific regulatory genes potentially involved in the development of cancer. This endeavour has been facilitated by the availability of homogeneous cell samples in biopsy material. One such study has lead to the identification of markers of gene expression (mRNA) capable of distinguishing between different types of B cell lymphoma. In addition, these diagnostic markers were predictive of likely prognosis in the corresponding patients and therefore helpful for therapeutic strategy (Alizadeh et al., 2000). The

results of this study were particularly powerful in that large numbers of samples were tested on homogeneous cell populations. In another example, microarray technology was used for compiling a list of genes associated with and specific to the 6 major subclasses of acute lymphoblastic leukaemia (ALL) and their prognostic indices (Mitchell et al., 2004).

Several studies have used microarray to investigate gene expression in SLE and identified prominent up-regulated expression in a number of interferon-regulated genes. These studies looked at gene expression in heterogeneous cell populations, peripheral blood mononuclear cells (PBMC). Even though proportions of different cell populations varied between individuals in these studies, a strong interferon regulated gene signature was observed in each (Baechler et al., 2003; Bennett et al., 2003; Crow, Kirou, & Wohlgemuth 2003; Han et al., 2003). Relevant to this study, a recent paper by Potti et al., (2006) presented the results of oligonucleotide array used to assess global genes expression in subsets of patients with APS and/or VTE in the presence or absence of aPL. Such information could possibly identify those patients at greater risk of developing thrombosis, enabling better clinical management and reducing the need for long-term anticoagulation with its inherent risk of bleeding. Using RNA from PBMC, the authors were able to detect subtle differences in gene expression potentially valuable in discerning those APS patients with thrombosis and patients with VTE and no APS (Potti et al., 2006). Although promising, these results were not totally definitive since evaluation of differential gene expression in monocytes and lymphocyte subsets may have been more informative. In addition, differences in gene expression related to previous thrombosis only could not be ruled out.

A number of studies have examined the effect of inflammatory stimuli on global gene expression in HUVEC. Septic shock is caused by the liberation of large amounts of bacterial endotoxin (LPS) into the circulation and is a leading cause of death. cDNA microarrays were used to determine the effect of LPS exposure on gene expression in HUVEC at a number of time points (1-24 hours) (Zhao et al., 2001). Results showed induction of a number of pro-inflammatory genes regulated primarily through the NF-κB transcription factor. Another study assessed the effect of interleukin 1 (IL-1) on gene expression in HUVEC at time points from ½ to 6

hours using Affymetrix 133A arrays (Mayer et al., 2004). This study provided evidence for both positive and negative IL-1 mediated feedback mechanisms that may have implications for treatment of chronic inflammatory conditions. Collectively, these studies revealed a number of genes and activation pathways potentially common to mediators of inflammation.

Selective nucleotide base pairing (hybridization) is the underlying principle behind microarray technology (Lennon & Lehrach 1991). Micorarray chips are prepared by attaching DNA of known sequence (**probe**) to a solid surface glass slide (microarray chip) at precise co-ordinates. The array is exposed to labelled (radioactive or fluorescent tag) sample (**target**) DNA or RNA in solution. The target DNA binds to complementary sequences on the array and the identity and abundance of the target sequences are determined. The two basic types of microarray technology are: (1) the two dye cDNA microarray using double-stranded cDNA fragments as probes and (2) the single dye oligonucleotide probe method (Lockhart & Winzeler 2000). This project used commercially prepared oligonucleotide labelled GeneChips (Affymetrix, Santa Clara. CA). The starting material for analysis was HUVEC derived messenger ribonucleic acid (mRNA). mRNA provides the expression profile of a cell in that it is a quantitative representation of those genes that have undergone transcription. The Affymetrix protocol amplifies and converts cellular mRNA into cRNA that is then hybridized to the GeneChips. Stringent adherence to protocol and quality control ensure that the amount of target cRNA is representative of the relative amounts of each mRNA molecule in the original sample.

In both methods, probe sequences are spotted onto glass slides at precise coordinates. Probes on Affymetrix oligonucleotide arrays are synthesized *in situ* one nucleotide at a time by a process called photolithography. This technology was pioneered by Affymetrix Company. Microarray technology has been refined to the extent that an Affymetrix GeneChip can scan for approximately 15,000 known genes within the human genome. One GeneChip contains 400,000 features or oligonucleotide binding sites in an area of 1 square centimetre. Evolving

technology will ultimately enable the entire human genome to be downloaded onto a single chip.

Numerous experiments in a large variety of disciplines have lead to the accumulation of vast amounts of gene expression data. As the use of the technology becomes more widespread, increasing attention is being paid to details of experimental design and methods of data analysis in the interests of optimal use of information and the benefit of the scientific community at large. The minimum information about a microarray experiment (MIAME) has been compiled as the result of an international initiative to enforce standards for microarray data (Brazma et al., 2001). The greatest challenge posed by microarray technology lies in the ability to consolidate and share new knowledge in a mutually beneficial way within the scientific and medical communities.

1.21 Aims of the study

Patients with APS are prone to thrombosis and recurrent miscarriage and these manifestations are thought at least in part, to be due to interaction of aPL and the endothelium throughout the vasculature. APL, and more specifically anti-β_2GPI activate EC *in vitro*. To date, investigation into the mechanisms by which such activation occurs, have been limited to measurement of levels of individual genes/proteins in EC and phenotypic changes in the cells themselves. An increase in leukocyte adhesion molecules on vascular EC and stimulation of pro-inflammatory cytokines in response to anti-β_2GPI has been well-documented in a number of *in vitro* studies. Preliminary experiments to investigate the possible activation pathways triggered by anti-β_2GPI have provided some interesting insights.

To date, there have been no studies to formally investigate the extent and diversity of gene regulation in response to anti-β_2GPI. This is the first study to look at global gene expression in HUVEC in response to anti-β_2GPI using microarray technology. Briefly, the aims of the study were:

1. To collect sera from PAPS patients with history of thrombosis and high titre β_2GPI-dependent aPL. To affinity purifiy anti-β_2GPI and characterize the antibody preparations.
2. To design and carry out microarray experiments to assess global gene expression in HUVEC after incubation with patient polyclonal anti-β_2GPI preparations. Particular reference will be made to inter and intra-experiment technical reproducibility and meticulous care in following Affymetrix protocol.
3. To perform appropriate analysis and statistical analysis on the microarray data using available software in order to identify those genes differentially expressed in response to polyclonal anti-β_2GPI when compared to HUVEC incubated with normal control IgG.
4. To confirm results of microarray analysis with another method appropriate for assessment of changes in gene expression.
5. To look for possible associations between those genes differentially expressed and consider their potential contribution to the pathology of APS.

Chapter 2

Materials and Methods

2.1 Patients

Sera were collected from patients in the Louise Coote Lupus Clinic at St. Thomas' Hospital in London. Samples were taken and saved with written patient consent and formal hospital ethical approval. Patients had APS with SLE or PAPS and were diagnosed using the Sopporo classification criteria (Wilson et al., 1999). 5 ml of sera were collected from 13 APS patients for initial HUVEC activation studies. Diagnostic criteria are given in Table 2.1. This serum was used for the development of antibody purification techniques and antibody classification. IgG purification followed by affinity purification of anti-β_2GPI were performed according to the method of Tincani et al., (1996).

Table 2.1 APS diagnostic criteria for patients whose sera were used for IgG purification and HUVEC activation studies.

Sample	Clinical History
1	PAPS, 3 recurrent miscarriages, PE
2	PAPS, DVT, 2 stillbirths,PE, cerebral vascular disease, catastrophic APS
3	PAPS, 3 fetal losses, MI, microinfarct CNS, aortic stenosis
4	PAPS, 3 recurrent miscarriages, PE
5	APS, cerebral SLE
6	SLE, recurrent miscarriages
7	PAPS, DVT
8	PAPS, retinal arterial thrombosis, cerebral thrombosis
9	APS, SLE, arterial thrombosis, lupus nephritis
10	PAPS, DVT
11	PAPS, PE, microangiopathy
12	PAPS, 2 recurrent miscarriages
13	APS/ SLE

APS= antiphospholipid syndrome, DVT= deep vein thrombosis, PAPS= primary antiphospholipid syndrome, PE=pulmonary embolism, SLE=systemic lupus erythematosus

Table 2.2 Clinical profiles of patients from whom polyclonal anti-β_2GPI antibody preparations were made for microarray. These are designated P1-P5 in methods.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Sex / Age	F/33	F/53	F/54	F/38	F/59
Diagnosis	PAPS	PAPS	PAPS	PAPS	PAPS
Clinical Features of APS	1 DVT, 1 PE, PET, TIAs and stroke	1 DVT, 3 PE	1 DVT, 2 stillbirths, 1 PE, CVD, catastrophicAPS	PVD, TIAs, brachial artery thrombosis	3 fetal losses, microinfarct CNS, MI, abnormal MRI, aortic stenosis
IgG aCL (GPL U/ml)	350	223	142	257	308
Lupus Anticoagulant	+	+	+	+	+
Experimental Procedures	Microarray, Real Time RT-PCR, ELISA	Microarray, Real Time RT-PCR, ELISA	Microarray, ELISA	Microarray , Real Time RT-PCR, ELISA	Microarray, Real Time RT-PCR

aCL= anticardiolipin , CVD = cerebral vascular disease, DVT= deep vein thrombosis, MI=myocardial infarction, PE= pulmonary embolism, PET= pre-eclampsia, PVD = peripheral vascular disease, TIA= transient ischemic attack

Sera from 5 additional PAPS patients were collected (diagnostic criteria are listed in Table 2.2). Approximately 25ml of serum was collected from each of these patients and IgG and anti-β_2GPI were purified from the total volume of sera for each. The larger volume of serum was required in order to obtain adequate amounts of anti-β_2GPI for characterization and subsequent microarray experiments. Samples were collected into red top vacutainer tubes, allowed to clot at room temperature (RT) and spun for 15min at 2000g. Serum was removed and frozen at -70°C until use.

2.2 ELISA for the detection of anticardiolipin antibodies (aCL)

Microtitre plates (Immulon B, Fisher Scientific, UK) were coated with bovine cardiolipin (Sigma Chemical Company, Poole UK) (30µl of 52µg/ml cardiolipin in absolute ethanol per well) and incubated overnight at 4°C uncovered in order to allow the ethanol to evaporate. Plates were washed 3X with PBS and blocked with PBS containing 10% adult bovine serum (Sigma) for one hour at RT. Plates were washed 3X with PBS. Patient serum samples were diluted 1/50 in PBS containing 10% adult bovine serum and 50µl of each sample was placed on a 96 well microtitre plate in duplicate. Dilutions of IgG aCL standard (1/50 – 1/6400 corresponding to serial dilutions, 261- 0.1 GPL U/ml, the aCL standard is a patient serum with high titre IgG aCL which has been calibrated using the Harris standards (Harris et al., 1987) were applied to the plate in duplicate and incubated for 3 hours at RT. Plates were washed 3X with PBS. Wells were washed and incubated with 50µl alkaline phosphatase conjugated goat anti-human IgG (Sigma) diluted 1/8000 in PBS/10% ABS for 1.5 hours at RT. Plates were washed 3X with PBS and incubated with 50µl P-nitrophenyl phosphate substrate per well (1 mg /ml, Sigma) until the OD of the 1/50 dilution of standard was greater than 1.0. Plate ODs were read at 405 nm using a microtitre plate reader (Titertek Multiscan PLUS MKII, ICN Flow). Patient values were determined from the standard curve. The cut-off for normal was <2 GPLu/ml, moderate titre 20-80 GPL and high titre >80.0 GPL.

A modified aCL assay (George et al., 1998) was used to test β2GPI (co-factor) dependence. Microtitre plates were coated with bovine cardiolipin as in the standard aCL assay. Samples were diluted and microtitre wells were blocked in PBS/10% adult bovine serum. In parallel, on the same plate, the same samples were also diluted in and microtitre wells were blocked in PBS/0.6% gelatin (Sigma) for comparison. The IgG aCL standards were not run in the absence of cofactor (10% adult bovine serum). The assay was taken through to completion as for the standard aCL assay (See Appendix 1.1 for buffers). Sample OD's with and without co-factor were compared.

2.3 HUVEC tissue Culture

Human umbilical cords were obtained from the labour ward at St. Thomas' Hospital London, following Hospital ethical approval and written patient consent. HUVEC were isolated from normal full term umbilical cord vein using collagenase enzyme (Sigma, Poole,UK.) and cultured as previously described at 37°C in 5% CO_2 in a humidified incubator (Jaffe et al., 1973). (See Appendix 2 for materials and reagents required.)

2.3.1 Method

Cords were collected by the mid-wives and stored in storage solution at 4°C. (See Appendix 1.3) Cords were used within 24 hours of collection. The cord was rinsed in Hank's Balanced Salt Solution (HBSS)(Sigma) and a sterile bone marrow needle was inserted into the vein at one end and secured with string. Using a sterile 20ml syringe, the vein was thoroughly rinsed with DMEM, (Dulbecco's Eagle's Medium, Gibco Life Technology Ltd., Paisley, UK) to remove as much blood as possible. A second bone marrow needle was secured in the vein at the other end of the cord and a 20 ml syringe was placed into the needle. 50µl of collagenase enzyme 100 mg/ml (Sigma) was added to 50ml of DMEM. This solution was filtered with a syringe driven 0.22µm filter (Millipore) and the vein was filled with approximately 25ml of the collagenase solution. The cord was left for 10min at RT with occasional gentle massaging of the vein to loosen the cells. The second syringe was used to remove the cells and the vein was rinsed with DMEM containing 10% FCS in order to inactivate the collagenase. The media containing

cells was divided between two sterile universal tubes. The cord was flushed with DMEM + 10% FCS to remove remaining cells. The tubes were spun at RT for 5min at 800g. The supernatant was carefully decanted and the cell pellets were gently resuspended. The bottom of a small tissue culture flask (25 cm^2) was thoroughly coated with 3ml of 0.1% gelatin (w/v)(Sigma) and excess was removed. The cells were resuspended in 5 ml of M199 (Gibco) supplemented with 10% FCS (Biowest, East Sussex, UK), Penicillin/Streptomycin (100µg/ml, Sigma), Fungizone (l2.5µg/ml, Gibco) and 2mM/l glutamine (Sigma) – further referred to as complete media. The flasks were labeled with the date, cord number and passage zero. The flask was placed in a 37°C incubator with 5% CO_2. The flask lid was loosened if not vented.

2.3.2 Splitting the cells: Passage 1

Passage zero HUVEC were checked for confluence after 48 hours incubation. using a phase contrast microscope. Confluent cells were split and placed in a larger tissue culture flask (75 cm^2) as follows. Media was removed and cells were gently washed with HBSS. 3 ml of 0.5% Trypsin EDTA (Sigma) was warmed to 37°C and placed on the cells. The cells were exposed to the trypsin for no longer than 20-30 sec, the cells should round up and lift off of the flask surface and this was checked under the microscope. The flask was tapped on the bench to loosen those cells still adherent. 5 ml DMEM + 10% FCS was added to the trypsinized cells in order to inactivate the trypsin. Cells were resuspended and divided between two sterile universals. The tubes were spun for 5min at 800g at RT. The supernatant was decanted and cells resuspended in complete media. The bottom of a large tissue culture flask was coated with 0.1% gelatin and the cells were transferred to the labeled flask. The cells were incubated at 37°C with 5% CO_2 until reaching confluence. The large confluent flask was spit into two 75cm^2 flasks following the same protocol and labeled passage 2. HUVEC in passage 3 were used in both HUVEC activation assays and microarray experiments.

2.4 Method for determination of HUVEC (EC) activation as assessed by E-selectin and ICAM-1 ELISA

HUVEC from passage 2 were seeded onto 96 well plates (1 X 10^4 cells per well) and grown to confluence (therefore used in passage 3) in complete media. Cell numbers were determined using a haemocytometer. EC were washed gently 2X with sterile PBS and incubated with either purified IgG (500µg/ml) or affinity purified (AP) anti-β_2GPI (various concentrations) for 4 hours at 37°C in 5% CO_2. Supernatants were removed and cells were washed carefully 2X with PBS. EC were checked under the microscope for structural integrity and fixed in 1% w/v glutaraldehyde (Sigma) for 5min at RT. EC were washed 3X with PBS. Monoclonal antibodies to E-Selectin and/or ICAM-1 (R&D Systems, Abingdon, Oxford, UK) were diluted to a working concentration of 1µg/ml in PBS and 200µl was incubated individually with the EC for 1½hours (37°C in 5% CO_2). EC were washed 3X with PBS. HRP conjugated goat anti-mouse IgG (Sigma) was diluted 1/500 in PBS. 200µl of conjugate was added to each well and incubated at 37°C for 1hour. EC were washed 3X with PBS. 100µl of ABTS chromogenic substrate (Sigma) was added to each well and optical density (OD) was read at 410 nm. Unstimulated EC served as a negative control and positive control was rTNF (R&D Systems) at a working concentration of 10ng/ml (Carvalho et al., 1996).

Calculation:

The average of the ODs of negative control non-stimulated cells was taken and used as a blank value. The OD for the blank was subtracted from the sample OD and the normal control OD. The ratio of sample OD to Normal control OD was calculated. A ratio of 1.0 represented 0% EC activation.

2.5 Detection of anti-β_2GPI antibodies

Anti-β_2GPI titres on the first 13 APS patients collected were determined using the Genesis Beta2 Glycoprotein I IgG ELISA kit for the determination of anti-β_2GPI (Genesis Diagnostics, Ely, Cambridgeshire, UK). Results in the assay greater than 10 u/ml were considered positive. Because of the cost of the ELISA kit, further patient serum samples were screened for the presence of anti-β_2GPI antibodies using an in house ELISA. Immunlon B microtitre plates were incubated overnight

at 4°C with 100µl per well β_2GPI (10µg/ml) (SCIPAC Ltd., Sittingborne, Kent, UK) diluted in coating buffer (0.05M carbonate buffer, pH 9.6). Plates were washed 3X with wash buffer PBS/ 0.05% Tween (Sigma) and blocked at RT with 100µl of blocking buffer PBS/ 1%BSA(Sigma) for 1hour. Samples were diluted 1/50 in dilution/wash buffer and filtered through a 0.22micron filter (Millipore). Samples were applied to the microtitre plate (100µl) in duplicate and incubated at RT for 2hours. Plates were washed three times with 150µl of wash buffer. 100µl of phosphatase-conjugated anti- IgG (1/2000 in dilution buffer) was incubated 1½ hours at RT and washed 3X with wash buffer. Chromogenic substrate (PNPP, Sigma) was diluted (1 mg/ml) in substrate buffer (0.05M Mg Carbonate buffer pH 9.8) and 100µl per well was added. Plates were incubated at 37°C until the positive control had an OD of 1.0 at 405nm. A reagent blank and normal control serum were included in each assay. Results were reported as OD. (See Appendix 1.2)

2.6 Purification of normal control IgG and anti-β_2GPI antibodies from sera

IgG from patients or normal control age and sex-matched subjects were purified using 1ml or 5ml HiTrap Protein G HP affinity columns (GE Healthcare, Buckinghamshire, UK) as per the manufacturer's instructions. Purified human β_2GPI protein was purchased from SCIPAC Ltd. (Sittingborne, Kent, UK.) The protein was coupled to a HiTrap NHS-activated HP column as recommended by the manufacturer (GE Healthcare). (See Appendix 1.4)

2.6.1 Manual IgG purification using HiTrap Protein G sepharose columns

IgG from patients or normal subjects were purified using 1 ml or 5 ml HiTrap Protein G HP affinity columns (GE Healthcare) as per manufacturer's instructions. The column was equilibrated with 5-10 ml of binding buffer (20mM $NaP0_4$ pH 7.0). 1ml of serum was diluted with 7ml of binding buffer (1/8 diln). The diluted sample was filtered using a 0.22µm filter (Millipore) and applied to the column at a rate of approximately 1 ml per min. Applying the sample too quickly resulted in compression of the column. 3 to 5ml of elution buffer (0.1 M Glycine-HCl pH 2.7) was applied to the column and the eluate was collected 0.5 ml at a time into 1.5ml eppendorf tubes containing 60µl neutralizing buffer (1M Tris-HCl, pH 9.0).

Fractions containing the IgG were identified using the Bicinchoninic Protein assay (Sigma). IgG fractions were pooled and dialyzed overnight against PBS pH 7.0 at 4°C followed by a further protein assay to determine final protein concentration. The column was equilibrated with 5-10ml of binding buffer and filled with 20% ethanol in sterile endotoxin free water (Sigma) for storage at 4°C.

2.6.2 Preparation of β_2-GPI affinity columns

Purified human β_2GPI protein was purchased from and coupled to a N-hydroxysuccinimide (NHS) activated sepharose column following manufacturer's instructions (GE Healthcare) (Del Papa et al., 1997). (See Appendix 1.5) NHS activated agarose beads allow covalent coupling of the protein ligand containing primary amino groups. 1mg of β_2GPI was dissolved in 5ml of coupling buffer (1 mg/ml). 1 drop of ice-cold 1mM HCl was added to the top of the column. Using a 5ml sterile plastic syringe 6 column volumes of 1mM HCl were added to the column to remove isopropanol used for column storage (1 drop per 2 sec). β_2GPI was drawn into a syringe and passed through column at the same rate and was collected in another 5 ml syringe attached to the bottom of the column. The protein was passed through the column 3X over a 30 min period. The column was washed and free binding sites were inactivated with ethanolamine (buffer A) and acetic acid (buffer B) as follows: column was washed with 6 column volumes (6ml) of buffer A, column was washed with 6ml of Buffer B, column was washed with 6 ml of buffer A, and left at RT for 30min. Column was washed with 6ml buffer B, column was washed with 6 ml of buffer A, column was washed with 6ml buffer B. Column was washed with 5ml of storage buffer and stored at 4°C(See Appendix 1.5 for contents of buffers A and B).

2.6.3 Affinity Purification of anti-β_2GPI

The β_2GPI column was equilibrated with 3 volumes of start buffer and three volumes of elution buffer. Ten ml of binding buffer were applied to the column at a flow rate of 0.2-1.0ml per minute. Protein concentration of purified IgG was determined and the sample was diluted to 1mg/ml in binding buffer (pH 7.0). Diluted IgG was filtered with a 0.22µm filter (Millipore). The sample was applied to the column manually with a 3ml sterile plastic syringe, affinity-purified anti-β_2GPI was eluted with 3ml of elution buffer (0.1M glycine-HCL, pH 2.7). Sample

fractions were collected in 1.5ml eppendorf tubes containing 100µl of neutralizing buffer (1M Tris-HCL pH 9.0).

Subsequent to obtaining an AKTAprime 3 protein purification system (GE Healthcare) the protocols for IgG and antibody purification were programmed on the machine. For purification protocols on the AKTAprime 3, serum samples were diluted 1/8 in start buffer for purification of IgG and further diluted to a concentration of 1 mg/ml for affinity purification of anti-β_2GPI as in manual protocols. Protein fractions were collected into neutralizing buffer on a fraction collector. The AKTAprime incorporates a UV flow cell and a recorder so that fractions containing the protein could be visually identified. (See Appendix Figure 1A and 1B for examples of tracings)

The IgG and anti-β_2GPI were purified and buffer exchange was achieved by dialysis overnight at 4°C against 20mM phosphate buffer, pH 7.0. Occasionally the IgG or anti-β_2GPI would precipitate and we had difficulty re-dissolving the precipitate.

The IgG and antibody preparations were subsequently buffer exchanged with start buffer and concentrated using an Amicon Ultra-15 Centrifugal Filter device MW 30,000 (Millipore). Samples were spun 4 or 5 times at 800g at RT, adding 20mM phosphate buffer between spins. Samples were concentrated to a final volume of approximately 200µl. Concentration resulted in a total volume of approximately 250 to 300µl of antibody from 25ml of serum. When antibody purification was completed on a patient IgG preparation, fractions were pooled, the protein concentration was determined, and aliquots were frozen in sterile start buffer at -70°C until use.

2.7 Bicinchoninic protein assay

The principle of the Bicinchoninic acid protein assay (Sigma) relies on the formation of Cu^{2+}-protein complex under alkaline conditions. Cysteine, cystine, tryptophan, tyrosin and the peptide bonds are able to reduce Cu^{2+} to Cu^{1+} resulting

in a purple-blue colour with intensity proportional to the protein concentration. The Protein Standard (BSA 1mg/ml, Sigma) was diluted in HBSS buffered salt solution (Sigma) to give protein concentrations of 200, 400, 600, 800 and 1000µg/ml for a standard curve. Samples were run undiluted, and at 1/5 and 1/10 dilutions. 25µl of standards and samples were placed on a 96 well plate. 175µl of a 1/50 dilution of Copper (II) Sulfate Pentahydrate in Bicinchoninic acid was added to each sample. The plate was incubated at 37°C for 30min. The absorbencies were read at 490 nm using a microtitre plate reader (Dynex MRX II) and sample values were read from the standard curve. In order to determine the protein concentration in the affinity purified antibodies (µg/ml), the protein standard was diluted 1/10 in HBSS to give a concentration of 100µg/ml and dilutions of 20, 40, 60, 80 and 100µg/ml were made and run as a standard curve.

2.8 ELISA method for the measurement of IL-8

The concentration of IL-8 in cell supernatants was determined according to manufacturer's instructions (BD Biosciences). Microtitre plates (Immulon B, Fisher Scientific, UK) were coated with 100µl of capture antibody diluted 1/250 (dilution specified for each lot number) in coating buffer (0.1 M Sodium Carbonate, pH 9.5). Plates were incubated with capture antibody overnight at 4ºC. Plates were washed 3X with wash buffer (PBS pH 7.0 with 0.05% Tween). Plates were blocked for 1hour at RT with 200µl assay diluent (PBS pH 7.0 with 10% FCS). Plates were washed 3X with wash buffer. The concentration of the IL-8 standard in the kit was provided and the standard was reconstituted in 1.0ml deionized water. The standard was further diluted in assay diluent to a concentration of 200ng/ml. Serial dilutions of the standard (200ng/ml) were made to a final dilution with an IL-8 concentration of 3.1pg/ml. Several dilutions of each sample were made in assay diluent to ensure that a value could be read from the standard curve. 100µl of each dilution of standard, sample and control were added to appropriate wells and the plates were incubated at RT for 2hour at RT. Plates were washed 5X with wash buffer. 100µl (1/250 dilution of detection antibody biotinylated anti-human IL-8 antibody in assay diluent) was added to each well and incubated for 1hour at RT. Wells were washed 7X with wash buffer allowing buffer to soak wells for 1min after each wash. 100µl of substrate solution (1/250

dilution of streptavidin-horseradish peroxidase conjugate in assay diluent) was added to each well and incubated for 30min at RT in the dark. 50µl of stop solution (2N H_2S0_4) was added to each well. OD was read at 450 nm using a microtitre plate reader (Titertek Multiscan PLUS MKII, ICN flow) within 30min of stopping the reaction. The concentrations of IL-8 in cell supernatants were determined from the standard curve. Sample diluent was included in each assay as a negative control. TNF-α (R&D Systems) was included in each assay as a positive control (10ng/ml in assay diluent).

2.9 Precautions taken to prevent endotoxin contamination of IgG and anti-β_2GPI preparations

As a precaution, buffers were prepared with endotoxin free water (Sigma W3500) and filtered using vacuum system 0.22µm stericup filters (Millipore). Diluted serum samples were also filtered before putting them through columns. Amicon ultra centrifuge tubes used for concentrating and buffer exchange were sterile, collection tubes containing neutralization buffer were sterile. Polymixin B (Sigma) (5µg/ml) was added to the anti-β_2GPI/HUVEC incubation step before RNA extraction for microarray analysis to minimize the possibility of endotoxin effects as previously described (Raschi et al., 2003a).

2.10 Measurement of Endotoxin- *Limulus* Amebocyte Lysate Assay

Endotoxin in antibody preparations was measured using the Pyrochrome® Chromogenic Test Kit incorporating *limulus* amebocyte lysate (LAL) (Associates of Cape Cod Inc., Falmouth MA). In the presence of endotoxin, LAL activates a proteolytic cascade that results in the cleavage of a colourless peptide substrate present in Pyrochrome LAL. Cleavage of the substrate liberates P-nitroaniline which is yellow in colour. The intensity of colour is proportional to the endotoxin concentration in the sample and the absorbance is read at 405 nm. The kit contained pyrochrome LAL reagent, pyrochrome reconstitution buffer, LAL reagent water and a 2.0 Endotoxin Unit (EU) vial of control standard endotoxin. The standard was reconstituted with 0.5ml LAL reagent water (4.0 EU/ml). The pH of antibody preparations to be tested was checked using universal indication

paper. Values were acceptable for inclusion in the LAL assay (pH ranged from 6.0 to 8.0). 100µl of antibody and standards were applied to an endotoxin free 96 well microtitre plate.

Antibody samples were diluted 1/5 and 1/50 in LAL water on the plate. Serial dilutions of endotoxin standard (4 EU-0.0625 EU) were prepared in LAL reagent water in lidded FACS tubes and applied to the plate. All samples and standards were tested in duplicate. 100µl of pyrochrome LAL reagent was added to the sample in each well and the plate was incubated at 37ºC for 30min. The colour reaction was stopped with 50µl of 50% acetic acid. The absorbance was read at 405nm. Endotoxin concentrations in the antibody samples were calculated from the standard curve. LAL reagent water was included as a negative control and gave an OD lower than the lowest concentration of the standard curve.

2.11 Anti-β2GPI antibodies and normal control IgG incubation with HUVEC-preparation for microarray analysis

Experiment 1

HUVEC were grown to confluence at passage 3 in six well tissue culture plates incubated with complete media (see 2.3.1) containing 10% FCS. A single well of a six well tissue culture plate contained approximately 1 X 10^6 cells (counted with hemocytometer). A single well of confluent HUVEC at passage 3 was incubated with anti-β2GPI antibody preparation from P5 (Table 2.2) or normal control IgG (both at 50µg/ml) for four hours at 37°C in a humidified incubator with 5% CO_2. The experiment was repeated three times on different HUVEC donors on three separate occasions. Total HUVEC RNA was then extracted using the RNeasy Kit Section 2.12) (Qiagen, Crawley, West Sussex, UK). The quality of the RNA was checked using a 1% agarose gel after each experiment.

Experiment 2

Confluent HUVEC at passage 3 were incubated with four independent PAPS-derived anti-β_2GPI antibody preparations (P1, P2, P3, P4, 50µg/ml) or four normal control IgG (N1, N2, N3, N4, 50µg/ml) for four hours at 37^{o}C in a humidified incubator with 5% CO_2. Three independent experiments using HUVEC from three different donors were carried out on different occasions and samples were processed as above.

2.12 RNeasy Mini protocol for the isolation of total RNA from HUVEC

RNA was extracted from HUVEC using an RNeasy Mini kit (Qiagen, Crawley, UK). (See Appendix 3.0 for kit contents) After the 4hour incubation time (see 2.11), all supernatant was carefully removed from the cells and cells were gently washed once with HBSS. The cells were trypsinized with 0.5ml 0.25% Trypsin/EDTA in PBS for 30sec and 0.5ml M199 + 10% FCS was added to neutralize the trypsin. Cells were transferred to a sterile 1.5ml eppendorf tube and pelleted by centrifugation (2200g for 2min). β-mercaptoethanol (β–ME) was added to RLT Buffer in the fumehood before use (10µl per 1.0ml Buffer RLT). 350µl of buffer RLT/β–ME was added to the cells and mixed in order to lyse the cells. (The buffer contains denaturing guanidine isothiocyanate and inactivates RNases.) The cells were homogenized by passing the lysate through a 20-gauge needle fitted to a 1 ml RNase-free syringe five times. An equal volume of 70% ethanol was added to the sample and mixed by pipetting. The sample was applied to an RNeasy mini-column, placed in a collection tube and centrifuged for 15sec at 2200g. Flow-through was discarded. The column was washed with 700µl of RW1 wash buffer, centrifuged again at 2200g for 15sec, and the flow-through was discarded. The RNeasy column was transferred to a new collection tube, 500µl of RPE buffer was added to column and the column was centrifuged for 15sec at 2200g, flow-through was then discarded. The last step was repeated but the column was centrifuged for 2min at 2200g. The column was transferred to a new 1.5ml collection tube and 30µl of RNase-free water was carefully applied to the column membrane. RNA was eluted from the column by centrifuging for 1 min at 2200g. RNA was frozen at -70°C until use.

2.13 Preparation of RNA Sample for Affymetrix Microarray

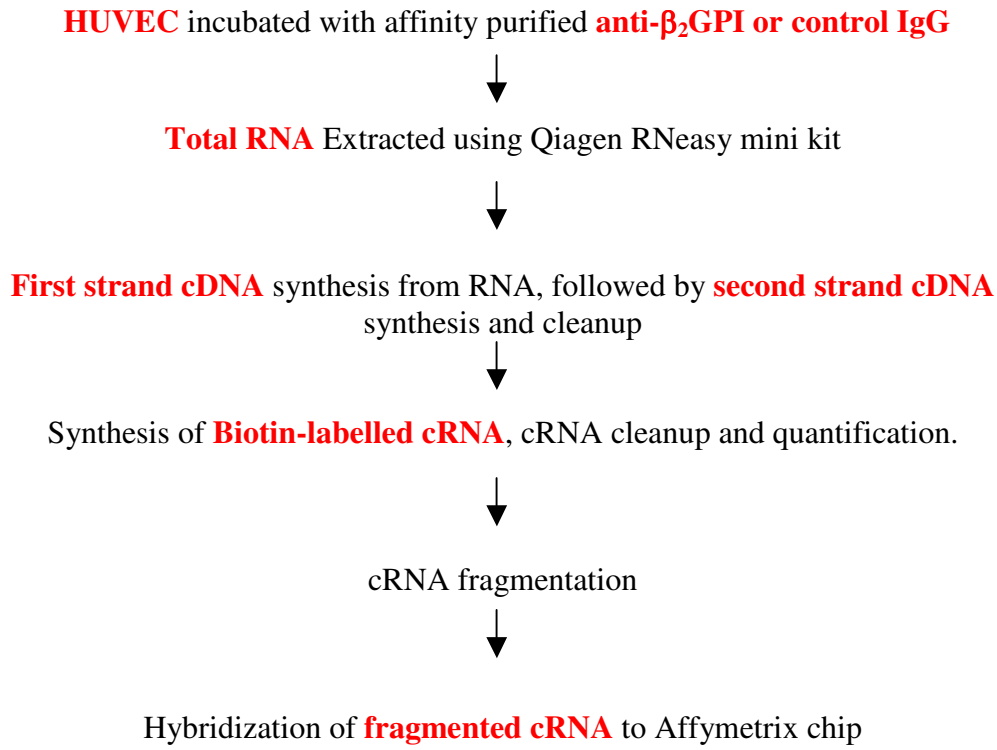


Figure 2.1 Method scheme for Affymetrix microarray sample preparation

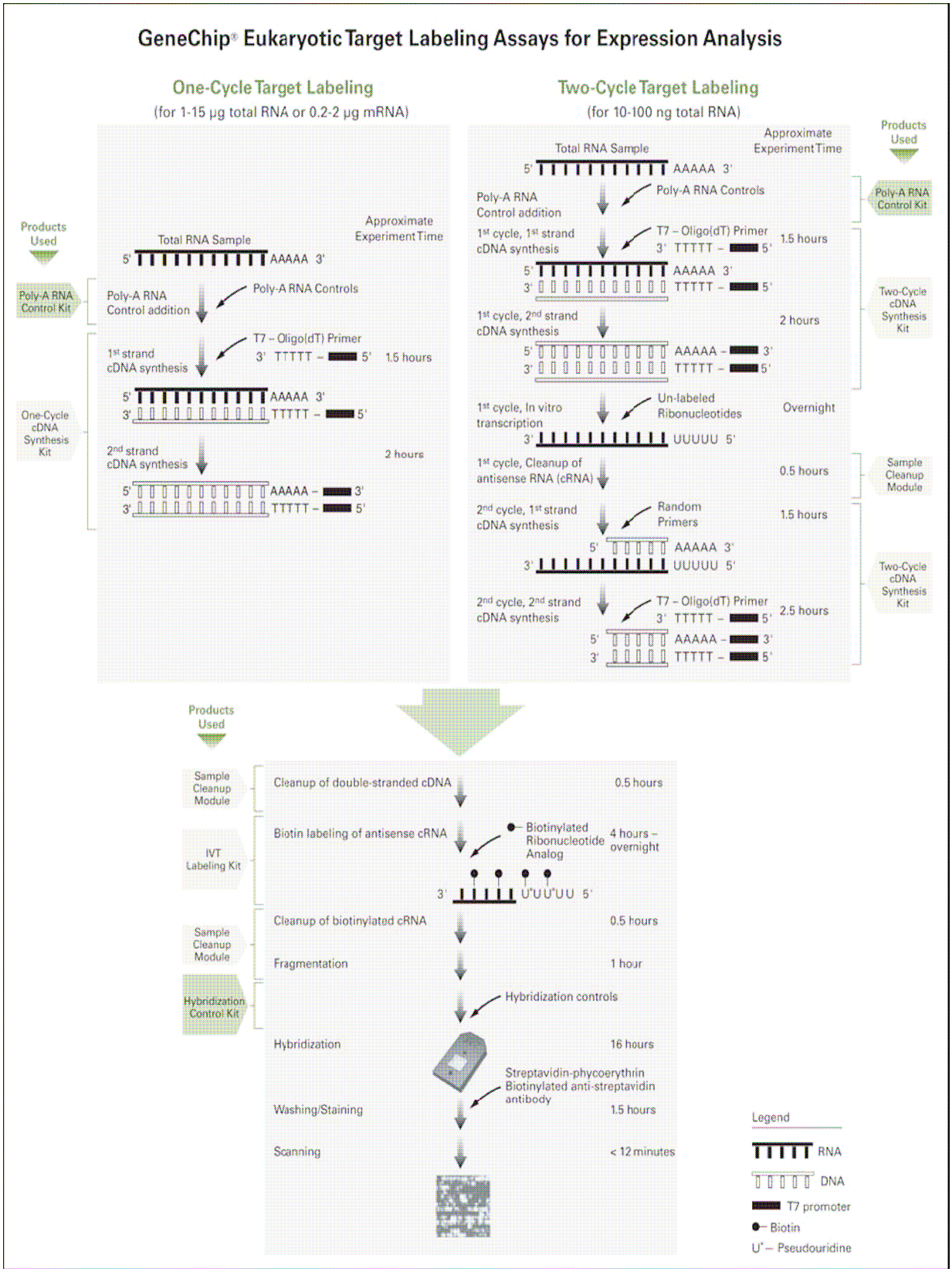


Figure 2.2: Scheme for preparation of labeled cRNA for Genechip microarray cRNA samples for microarray hybridization were prepared following manufacturer's instructions (Gene Chip® Expression Analysis: Data Analysis Fundamentals, 2004. Affymetrix, Santa Clara CA). The overall scheme is outlined in Figures 2.1 and 2.2.

In summary: Total RNA quality and concentration were determined using the Nanaodrop spectrophotometer and further assessed on RNA 6000 Nano Assay micro-capillary gel (Agilent Technologies, Santa Clara, CA) (See Appendix Figure 3). This was followed by cDNA synthesis using RT oligo-dT primer T7-$(dT)_{24}$ (Genset Corporation, LaJolla, CA) and an *in vitro* transcription reaction for cRNA synthesis incorporating biotinylated ribonucleotides. Full-length cRNA was then fragmented to 35 to 200 base fragments by metal-induced hydrolysis. The quality of cRNA was then assessed along with the fragmented RNA on a micro-capillary gel (See Appendix Figures 4, 5 and 6). Fragmented cRNA was hybridized overnight to gene chip arrays at 45°C for 18 hours. Control cRNAs were then added to the hybridization mix. Human Genome 133A (HGU133A) arrays were used in Experiment 1 and HGU133-2.0 arrays were used for Experiment 2. Both types of chips screen for 18,400 human transcripts, but HGU133-2.0 are a slightly smaller format and require slightly less RNA sample. Experiment 1 incorporated 6 chips in total. In one of the 3 independent experiments from Experiment 2, one anti-β_2GPI antibody (P2) treated sample and one control IgG treated (N2) sample were not processed beyond initial RNA quantitation due to low RNA yield. Therefore, a total of 22 chips were hybridized and scanned in Experiment 2. Gene chips were washed and stained on the GeneChip Fluidics Station 400 (Affymetrix). Fluorescent signals were detected using the HPG2500A Gene Array Scanner (see Figure 2.2).

The quality of the total RNA must pass the minimum requirements for the standard Affymetrix labeling protocol; minimum concentration = 0.5µg/µl, minimum total amount per sample = 5µg (slightly less for the HG133A_2 chip) The RNA must be clean, pure and undegraded. The approximate number of confluent HUVEC that would yield sufficient RNA for an Affymetrix chip was calculated (See Appendix Table 2 and Figure 2)

Before proceeding to cDNA synthesis, the concentration and purity of each total RNA sample was assessed using the Nanodrop Spectrophotometer (Agilent Technologies, Santa Clara, CA). It was imperative that precisely the same amount of RNA from each sample for comparison was incorporated into the cDNA

synthesis step. The Nanodrop measured the RNA OD at A260, provided the OD ratio of 260/230, 260/280 and calculated the concentration in ng/µl.

2.13.1 First strand cDNA synthesis

The total amount of RNA in each sample was calculated (30µl of RNA from extraction protocol X concentration in ng/µl) eg; experiment carried out on 20.9.05 , 29 X 90.5 = 2.6µg total RNA. Therefore 2.6µg of RNA would be used for analysis for all samples, the identical amount. Of this, approximately 5% was mRNA. (Samples with low concentration were concentrated using the speed vac) All reactions were carried out in 0.2ml PCR tubes.

The T7 oligo sequence for reverse transcription: 5'- GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG $(T)_{24}$- 3'

The appropriate volume of total RNA (and molecular biology grade RNase-free water if required to make up to the maximum allowable volume) and 1µl of T7 primer (200ng/µl) were mixed (Table 2.3)**.** Samples were incubated at 70°C for 5min in a PCR block (with heated lid), then put on ice for 5min and pulsed down (2200g for 10sec).

Table 2.3 Reagents for first strand synthesis (FSS)

Amount of RNA	**Maximum volume of RNA**	**Total volume of RT reaction**	**Total amount of T7 oligo**	**Volume of RT premix (µl)**
3-10 µg	**11µl**	**20µl**	**200ng (1µl)**	**8**

Samples were placed on ice until ready to add premix. RT premix was prepared (Table 2.4). A larger volume was prepared, ½-1 reactions extra to compensate for pipetting inaccuracies. 8µl of premix was added to each reaction tube to make a total of 20µl. Samples were pipette- mixed, pulsed down, and incubated at 42°C for 1hour in a PCR machine (with heated lid).

Table 2.4 RT premix constituents

Component	Volume for 8µl RT premix (µl)
5X First strand buffer	4
0.1 MDTT	2
10 mM dNTPs	1
Superscript II reverse transcriptase (200U/ml)	1

2.13.2 Second strand synthesis (SSS)

First strand synthesis reactions were incubated on ice for 5min immediately after 42°C incubation. Second strand premix was prepared 5-10 min before the end of the first strand incubation (Table 2.5). 130µl of SSS premix was added to each 20µl 1st strand synthesis reaction. Samples were pipette mixed well and pulsed down. Samples were incubated at 16°C for 2 hours in a PCR machine (with lid open). 12µl of 0.5M EDTA was added to each tube and mixed well to stop the reaction. The cDNA was now in a total volume of 162µl. The cDNA synthesis reactions were placed in the -20°C freezer or processing was continued.

Table 2.5 SSS premix constituents

Component	Volume for 130µl SSS preparation (µl)
RNase-free water	91
5X 2nd strand buffer	30
10 mM dNTP mix	3
DNA ligase (10U/µl)	1
DNA Polymerase (10U/µl)	4
RNase H (2U/µl)	1

2.13.3 cDNA extraction and clean-up

The cDNA products were transferred from 0.2ml tubes into 1.5ml tubes. The Affymetrix sample clean-up kit was used for the next step. The kit contained cDNA binding buffer, cDNA wash buffer (with ethanol added), cDNA elution

buffer, cDNA spin columns in 2ml collection tubes, extra 2ml collection tubes, extra 1.5ml elution tubes.

600µl of cDNA binding buffer was added to the 162µl of cDNA. The sample was vortexed for three sec. 500µl of sample was applied to a cDNA spin column. Sample was spun for 1min at 8000g in a microcentrigfuge, flow-through was disgarded. The remainder of the sample (262µl) was applied to the column and the spin was repeated. The flow-through and collection tube were discarded. The column was placed in a new 2 ml collection tube, 750µl of cDNA wash buffer was added and the column was spun for 1min at 8000g. Flow-through was discarded. The columns and tubes were placed back in the centrifuge with open caps. The columns were spun at full speed 16000g for 5min. Collection tubes were discarded. Columns were placed in fresh 1.5ml eppendorf tubes. 14µl of cDNA elution buffer was carefully applied to the column membrane and incubated for 1min. The column was spun for 1min at full speed to elute the cDNA. The samples were stored at -20°C or we proceeded with the IVT (*in-vitro* transcription) reaction.

2.13.4 In vitro transcription reaction (IVT)

The Affymetrix IVT kit was used for this step. All solutions except the IVT enzyme mix were brought to RT, including the cDNA. The tubes containing IVT buffer and dNTPmix were fully vortexed to eliminate precipitates. The volume of eluted cDNA was measured. We routinely began with less than 5µg of total RNA so all of the cDNA was transferred to a 1.5ml screw-cap microcentrifuge tube for the IVT reaction. The total volume of cDNA was brought up to 20µl using the water from the IVT kit. The IVT reagent mastermix was prepared (Table 2.6).

Table 2.6 IVT reagent mastermix

Volume per sample	Reagent
4µl	10X IVT Labelling Buffer
12µl	IVT Labelling NTP Mix
4µl	IVT Labelling Enzyme Mix
20µl	TOTAL VOLUME

2.13.5 Clean-up of biotin cRNA

The Affymetrix/Qiagen kit was used for this step. The kit incorporated cRNA binding buffer, molecular biology grade ethanol (100%), cRNA wash buffer, 80% ethanol (made up with molecular biology grade ethanol and water), cRNA spin columns in 2ml collection tubes and extra 2ml collection tubes and 1.5ml elution tubes.

The 20µl IVT reaction was made up to 100µl with RNase-free water. 350µl of cRNA binding buffer was added and contents of tube mixed on vortex for 3sec. 250µl of absolute ethanol was added and vortex mixed. Each whole sample (700µl) was applied to a cRNA spin column and spun for 15sec at 8000g. The flow through and collection tubes were discarded. Columns were placed in new collection tubes and 500µl of cRNA wash buffer was added to the columns. Columns were spun for 15sec at 8000g and flow-through was discarded. 500µl of 80% ethanol as added to the columns, spun for 15sec at 8000g and flow-through was discarded. The caps on the columns were opened and columns were spun dry by spinning at full speed (16000g) for 5min. Flow through and collection tubes were discarded. Columns were placed in fresh 1.5ml elution tubes and 11µl of RNase-free water was piptetted onto the column membrane and spun at 16000g for 1min to elute the cRNA. Another 10µl of RNase-free water was placed onto the membrane and spin was repeated. Purified biotin-cRNA (20µl) was stored at -80°C until use or used straight away in the hybridization protocol.

2.13.6 cRNA quantification and fragmentation

The concentration of the cRNA samples was determined at an absorbance of 260nm on the Nanadrop spectrophotometer. The IVT reaction should yield at least 15µg of biotin-cRNA in the 20µl at a concentration of ~2.5µg/µl. If the IVT reaction yielded sufficient cRNA for hybridization, cRNA fragmentation was the next step.

At least 15µg of fragmented cRNA from each sample was required for preparation of the hybridization cocktail. It was necessary to prepare enough fragmented cRNA in order that 100-250ng (0.1-.25µg) were available for quality control on a

NanoLab gel electrophoresis chip. The maximum final volume of the fragmentation reaction was 31µl. Based on the cRNA concentration, the volume required for 15.5µg cRNA was calculated for each sample, 6.2µl of 5X fragmentation buffer (31/5) was added and RNase-free water was added top make up a total volume of 31µl. Samples were incubated at 94°C for 35min in a PCR machine with heated lid.

The biotinylated cRNA and the fragmented biotinylated cRNA were analyzed on a gel chip using the Agilent bioanalyser. cRNA should show a symmetrical smear of average length 1000-2000 nuceotides (Appendix Figure 5) (http://www.affymetrix.com/support/technical/manual/expression_manual.affx., http://microarrays.berkeley.edu/affy_services.php). Fragmented cRNA should show a single RNA peak of around 100 nuceotides in length (Appendix Figures 4 and 6).

2.13.7 Preparation of the hybridization cocktail

When the acceptable quality of the cRNA and fragmented cRNA were confirmed on the gel chip, the hybridization cocktail was prepared for hybridization to the GeneChips. The 20X Genechip Eukaryotic control and B2 Oligos were heated at 65°C for 5min to ensure that the cRNA was completed resuspended after being frozen. The appropriate number of gene chips were removed from the storage bags and left to warm to RT. The fragmented cRNA was transferred to an RNase-free screw-cap 1.5ml tube. The hybridization cocktail mastermix was prepared including volume for an extra reaction (Table 2.7).

Table 2.7 Hybridization cocktail mastermix

Component	Volume (µl)
Control Oligo B2	5
20X Eukaryotic Hybridization Controls	15
Herring Sperm DNA (10mg/ml)	3
Acetylated BSA (50mg/ml)	3
2X Hybridization Buffer	150
DMSO	30
RNase-free water	64
Total volume	270

270µl of hybridization cocktail mastermix was added to the fragmented cRNA in a fresh 1.5ml screw-cap tube and mixed. The completed hybridization cocktail was heat-denatured at 99°C for 5min in a heat block, followed by 45°C for 5min in the pre-heated hybridization oven.

2.13.8 Preparation of Genechips and sample hybridization

While the hybridization cocktail was denaturing, the chips were prepared. 1X hybridization buffer was made up from stock. The chips (1 per sample) were filled with 1X hybridization buffer and pre-hybridized in the Affymetrix rotisserie oven for 10min at 45°C rotating at 60 rpm. The tubes containing the hybridization cocktail were spun at max speed in a microcentrifuge for 5min to remove any insoluble or precipitated material from the hybridization mixture, being careful not to disturb any pellet that may have formed. The pre-heated chips were removed from the oven and the pre-hybridization solution was removed and replaced with 200µl of the hybridization cocktail containing fragmented cRNA. Hybridization takes place in the rotisserie oven for ≥16 hours at 45°C rotating at 60 rpm.

2.13.9 Washing, staining and scanning chips

Buffers A and B (commercially prepared by Affymetrix) for the fluidics station must be filtered before use. Scanner, computer and Fluidics station were switched on in that order and allowed to warm up for 15min before use. Individual files for

the chips were set up in Microarray suite. The Fluidics station was primed with buffers A and B, using the prime protocol. The chips were removed from the rotisserie oven and the hybridization cocktail was removed from the chips and saved at -20°C. The chips were filled with 250µl wash buffer A and placed in the Fluidics station. 2400µl of stain 1 and 1200µl of stain 2 were prepared for each chip. After preparation, the stain was aliquoted into 1.5ml eppendorf tubes each containing 600µl and placed on the fluidics station. Staining protocol was 1 application of Stain 1, 1 application of stain 2, followed by a second application of Stain 1.

Table 2.8 Stain 1

600µl	2X stain buffer
540µl	Water
48µl	Acetylated BSA(5mg/ml)
12µl	Streptavidin-phycoerythrin (SAPE) (1mg/ml)

Make up 600µl of antibody solution for each chip.

Table 2.9 Stain 2

300µl	2X stain buffer
266.4µl	Water
24µl	Acetylated BSA (50mg/ml)
6µl	Normal Goat IgG (10mg/ml)
3.6µl	Biotinylated anti-streptavidin antibody (0.5mg/ml)

The appropriate stain protocol was run for each chip on the Gene Chip Fluidics Station 400 (Affymetrix), four chips at a time. The chips were removed from the machine and checked for air bubbles. When chips were bubble free, they were scanned.

2.14 Statistical analysis of microarray data

After scanning the gene chips, images were analyzed using the Affymetrix microarraysuite (MAS) 5.0 to generate raw data in the form of '.cel' files. Further analysis was carried out using a combination of the MAS 5.0 and GeneSpring (Agilent) software programmes. The detection of a particular gene as 'present, absent or marginal' was carried out using the MAS 5.0 software. The .cel files were imported into GeneSpring and normalized by GC-Robust Multichip Average (GCRMA), an algorithm that normalizes the data by quantile normalization, in order to minimize the biological variation between samples. Further analysis was carried out on genes identified as present or marginal. Genes with statistically different expression between the control IgG and the anti-β_2GPI antibody treated cells ($p<0.05$) were identified by the Kruskal Wallis test (non-parametric one way analysis of variance (ANOVA) with the Benjamin and Hochberg multiple testing correction. Filtering the gene list on the criteria of a two-fold or more increase or decrease in expression identified a panel of genes that were significantly changed in HUVEC by anti-β_2GPI antibody treatment compared to normal control IgG treatment. Average-linkage hierarchical clustering (using the Pearson Correlation) was carried out separately on the genes and the samples generating a genetree and condition tree, respectively, to highlight any distinct patterns in gene expression and the relationships between the samples (Eisen et al., 1998).

2.15 Agarose gel electrophoresis

Extracted RNA and PCR reactions were run on agarose gels to ensure that the RNA was of good quality and that the PCR reactions had been successful. % gels were prepared by dissolving 1g of agarose (Invitrogen, Paisley, UK) in 50ml .045M TBE by heating the solution in a microwave. The melted agarose was cooled to approximately 50°C and 20µl (10mg/ml) of ethidium bromide (EtBr) was added before pouring the gel. 3µl of sample was mixed with 6µl loading dye (Promega) and each sample was applied to a well on a gel and electrophoresed in parallel with 100bp ladder (Promega). Gels were electrophoresed for approximately 30min at 100V.

2.16 Quantitative real-time RT-PCR analysis of gene expression

Quantitative real time PCR was used to confirm the microarray results for the expression levels of selected genes. Primers were purchased from MWG-Biotech AG(98% pure). The primer pairs used for the following genes were: CSF3 (75nt), forward 5'- CGCTCCAGGAGAAGCTGT-3', and reverse 5'-CCAGAGAGTGTCCGAGCAG-3',
CX3CL1 (76nt), forward 5'-ATCTCTGTCGTGGCTGCTC-3', and reverse 5'-TCACACCGTGGTGCTGTC-3',
E-selectin (91nt), forward 5'-TGAAGCTCCCACTGAGTCCAA-3', and reverse 5'-GGTGCTAATGTCAGGAGGGAGA-3',
FGF18 (113nt), forward 5'-CTCTACAGCCGGACCAGTG-3' and reverse 5'-CCGAAGGTGTCTGTCTCCAC-3',
ID2 (100nt), forward 5'-CAGCATCCTGTCCTTGCAG-3', and reverse 5'-AAAGAAATCATGAACACCGCTTA-3',
SOD2 (85nt), forward 5'-CAAATTGCTGCTTGTCCAAA-3', and reverse 5'-CGTGCTCCCACACATCAAT-3',
Tenascin C (108nt), forward 5'- GCTCAAAGCAGCCACTCATT-3', and reverse, 5'-CCCATATCTGGAACCTCCTCT-3',
β-actin (100nt), forward 5'-CCAACCGCGAGAAGATGA-3', and reverse 5'-CCAGAGGCGTACAGGGATAG-3'.

β-actin was used as an internal control as no changes were found in levels of expression of this housekeeping gene when cells were treated with antibodies in microarray experiments. Primers for the genes were designed using the Roche universal probe library (MWG Biotech, UK). Primers were reconstituted with sterile endotoxin free H_2O (Sigma) to a concentration of 100μM, and frozen in aliquots at -20°C. Primers were further diluted to 10μM concentration in sterile endotoxin free H_2O for PCR.

Concentration and quality of the total RNA was determined on the Nanodrop spectrophotometer. 1 μg of total RNA from HUVEC incubated for four hours in complete media alone (blank), or with 2 normal control IgG preparations (N3, N4) or 4 anti-β_2-GPI antibody preparation (P1, P2, P3, P5), or with TNF-α were reverse

transcribed into cDNA with the Quantitect reverse transcription kit (Qiagen) using oligo-dT primers. DNA wipeout buffer (2µl) (for removal of any contaminating genomic DNA) was combined with 1µg RNA and made up to a total volume of 14 µl with RNase-free water. The reaction was incubated at 42°C for 2min, and placed on ice.

Next, each reverse transcription reaction included 1µl of reverse-transcriptase, 4µl of RT buffer, 1µl RT primer mix and 14µl template RNA from previous incubation (Table 2.10). These were combined and incubated at 42°C for 15min. The reactions were then incubated for 3min at 95°C to inactivate the reverse transcriptase. A PCR using primers for β-actin was performed on each cDNA sample and run on a 2% agarose gel to ensure that the cDNA synthesis was successful.

Table 2.10 Reverse-transcription reaction components

Component	**Volume per reaction**
Reverse-transcription master mix- Quantiscript Reverse Transcriptase	1µl
Quantiscript RT Buffer 5X- includes Mg^{2++}	4µl
RT Primer Mix	1µl
Template RNA **Entire genomic DNA elimination reaction**	14µl
Total Volume	20µl

2.16.1 Preparation and purification of cDNA standards

HUVEC were incubated with TNF-α (10ng/ml) (R&D Systems, Abingdon, Oxford, UK.) for four hours, RNA was extracted and cDNA was prepared as previously described. cDNA was used for PCR in order to produce enough of each gene specific target to make DNA standards for absolute real time PCR. Forward and reverse primer pairs for selected genes were initially tested with DNA in a

25µl PCR reaction and checked on a 2% agarose gel to ensure that amplification of PCR product was successful. A 100µl reaction mixture was used to obtain enough gene specific product for standard preparation. PCR master mix contained Taq DNA polymerase 50units/ml, 400µM dNTPs, 3mM $MgCl_2$ and reaction buffer (Promega). A 100µl reaction mixture contained 2X PCR master mix, upstream primer, downstream primer, (primer concentrations 0.3µM), template DNA and nuclease-free water. A blank control including water rather than cDNA was run in parallel to each PCR reaction to check for DNA contamination in the reagents. PCR was performed in a DNA thermocycler (Biometra UNOII- ThermoblockNR 9611222, Whatman Biometra GMbH, Niedersachen, Germany) with 1 cycle of 2min at 95°C; 30 cycles of 30sec at 94°C, 30sec at 58°C (57°C or 61°C depending on the melting temperature) and 30sec at 72°C; and a final extension of 5min at 72°C. 3µl of PCR products and blanks were run on a 2% agarose gel to verify success of reaction. cDNA was checked on gel and the remainder was purified by electrophoresis and ethanol preciptitation based on methods described respectively in Chapter 5 and Appendix 8.14 of Molecular Cloning- A Laboratory Manual, J. Sambrook and D. W. Russell (eds.), CSHL press, New York, 2001. 20 µl of loading dye was added to 95µl cDNA. A 2% agarose gel (Invitrogen) was prepared with 50mM TBE plus EtBr (5µg/ml), with a well in the agarose large enough to contain the whole PCR sample volume. The sample was added to the well and 100 base pair (bp) ladder was run in parallel for reference. Gel was run at 100V for 1hour in 50mM TBE with EtBr added (5µg/ml). The gel was examined on a transilluminator (See Appendix Figure 7). The cDNA incorporated EtBr and was visible in the UV light. The gel containing the cDNA was carefully removed using a scalpel and the slice was placed in a small piece of dialysis tubing previously prepared by boiling and stored in water at 4°C (Appendix 8.4, Molecular Cloning- A Laboratory Manual). 500µl of 25mM TBE was placed in the tubing with the gel, bubbles were carefully removed and the other end clamped securely. The dialysis bag was placed in an electrophoresis chamber with the gel positioned parallel to the current and the current was run for approximately 1hour at 100V. The cDNA concentrated along the side of the tubing was carefully removed and the inside of the tubing was rinsed with a small volume of 25mM TBE to collect the remainder. The sample was placed in a 1.5ml eppendorf tube and the volume was measured. An equal volume of phenol chloroform (Sigma) was added and the sample

vortexed. The sample was spun at 8000g for 3min, the top aqueous layer was removed, the volume measured and placed in a clean eppendorf tube. 1/10 the volume of sodium acetate (3M) was added and mixed. An equal volume of absolute ethanol was added and the sample was mixed and placed at -50°C for 20min. The sample was spun at 8000g for 10min taking note of where the pellet should be in the tube. The tube was carefully decanted and 250µl of cold 70% ethanol was added. The tube was spun for 2min at 8000g, decanted and any remaining ethanol was allowed to evaporate. 15-20 µl of RNase-free water was added to the sample and quickly vortexed to dissolve the cDNA. The product was quantitated on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

The concentration was used to calculate the copy number/µl taking into account the molecular weight and length of the PCR product in base pairs.

Example: CX3CL1

1. length of PCR product 76bp
2. 660 is the average molecular weight of a nucleotide,

   660 x 76= 50160 (relative molecular mass)
3. the concentration of the cDNA was 40 ng/µl or 0.04 µg/µl

   0.04 divided by 50160 gives n =7.97448 x 10^{-7} where n=moles

   n x Avagadro's number (6.022 x 10^{23}) = number of molecules per litre divided by 10^6 gives the copy number per microlitre.

   7.97448 x 10^{-7} x 6.022 x 10^{23} / 10^6 = 4.8 x 10^{11}

This copy number was diluted in sterile dH_2O to give 1 x 10^8 copies per µl

2.16.2 Quantitative RT-PCR

The cDNA standard curves began with 1 X 10^8 copies per µl. Serial dilutions of the standards were prepared in sterile dH_2O down to 1 X 10^1. (Antibody preparations N3, N4, P1, P2 and P4 were previously used in the microarray experiments.) Quantitative real-time PCR (qRT-PCR) was carried out with the QuantiTect SYBR Green PCR Kit (Qiagen) in the ABI 7000 sequence detector (Applied Biosystems). The SYBR Green PCR Master Mix contained HotStarTaq DNA Polymerase, PCR buffer (contains $MgCl_2$ to give final concentration of

2.5mM) and Syber Green I fluorescent dye. The real time PCR was performed in 96 well microtitre plates. We used a final reaction volume of 25µl per well. This included 12.5µl of Master Mix, Primer A and Primer B at a 0.3µM concentration, 2µl template DNA and RNA'se free water. Dilutions of standards were run in duplicate as were dilutions of unknown samples (See Appendix Figure 8). The PCR program on the ABI Prism 7000 was: 1 cycle of 10min at 95°C; 40 cycles of 15sec at 95°C, 15sec at 58°C, 30sec at 72°C (Sample volume 25µl).

Gene expression levels were calculated with the absolute quantitation method (Bustin 2000) and normalized to the β-actin level. All PCR reactions were carried out in duplicate, and repeated at least twice for each gene. The specificity of the PCR reactions was verified with dissociation curve analysis (See Appendix Figure 9).

All dilutions of standards were run in duplicate and tested on two different occasions. All samples were run in duplicate. β-actin copy number was determined for each sample three times and the mean was established. For each gene the copy number per µl produced in each sample was established twice (on two occasions). The mean and SD were determined. Each value was normalized to β-actin, (divided by the copy number for β-actin). This assumes that the levels of β-actin in the cells are constant (microarray data did not show change in expression in response to antibody). This is relating the value of the unknown back to a constant in order to compensate for the fact that slightly different amounts of RNA may have been added in different tests or runs. Sample 5 (normal control) was arbitrarily given a fold change of 1 as a reference (in order to compare). In order to calculate fold change for the samples, we took the copy number/µl for the unknown and divided by the copy number of the reference normal (given copy number 1). Standard DNA concentrations were measured at 260 nm and the copy number per µl was then calculated based on the molecular weight of the PCR product in question. This provides information on the relative amounts of mRNA used in the qRT-PCR reaction. Small differences in the amount of RNA used for different samples in the reaction were corrected for by normalization of all values to a housekeeping gene, in this case β-actin. (See Appendix 5.0 for qRT-PCR data)

2.17 Cell cycle analysis by propidium iodide staining of HUVEC

HUVEC were seeded and grown at P3 to 70-80% confluence in complete media. Cells were incubated with anti-β_2GPI (50µg/ml) or normal control IgG (50µg/ml), (37°C with 5% CO_2) and complete media alone as a reagent blank for 18 hours. Cells were trypsinised and carefully washed twice with HANKS (Sigma) and fixed with cold 70% ethanol on ice for 30min. Cells were washed 2X with HANKS and spun for 5min at 800g. The cell pellets were treated with 30µl RNase (100µg/ml) (Sigma) for 30min at RT. 200 µl of propidium iodide (50µg/ml) was added to each sample and samples were incubated at RT for 1hour. Propidium iodide fluorescence of stained cells was measured using a FACScan™ flow cytometer (Becton Dickinson). FACS analysis of cell DNA content allows identification of relative numbers of cells in different stages of the cell cycle. Fluorescence data is acquired with linear amplification and plotted on a histogram where the DNA content of cells is plotted on the x axis and relative number of cells on the y axis. Cells are devided into G0/G1, S and G2/M phases (Krishan, 1975).

2.18 ^{3}H-thymidine incorporation- proliferation assay

HUVEC at P3 were seeded in a 96 well microtitre plate and grown to 70-80% confluence. Cells were incubated with anti-β_2GPI (50µg/ml) or normal control IgG (50µg/ml), (37°C with 5% CO_2) and complete media alone as a reagent blank each in a volume of 500µl for 18hours. In addition, 1.25 µCi of ^{3}H-thymidine (specific activity 70-85 Ci/mMol; Amersham International Ltd., Amersham, UK) was added to each sample for the 18hour incubation. After incubation, the cells were harvested onto glass-fibre filter mats (Wallac, Turku, Finland) using a Mach III harvester 96 (Tomtec, orange, USA). Conventional liquid scintillation procedures (Scintillant-Beta plate scint, Wallac) were used to measure the amount of ^{3}H-thymidine incorporated by HUVEC with a rackbeta counter (1450 Microbeta plus, Wallac). Results were presented as the mean counts per minute (CPM) for quadruplicate samples.

(See Appendix for Buffers and Formulations)

Chapter 3

Purification and Characterization of Anti-β_2GPI

3.0 Introduction

It is well documented that aPL cause activation of EC but aPL encompass a large group of heterogeneous antibodies with different specificities and binding affinities. Upregulation of adhesion molecules was taken as evidence of EC activation and their detection served as a measurable marker (Carlos et al., 1994). ICAM-1 is expressed in low levels in quiescent EC while E-Selectin is not constitutively expressed (Cines et al., 1998). Early *in vitro* studies using whole IgG fractions from APS patients containing high titre aCL antibodies demonstrated activation of HUVEC reflected in an increase in monocyte adhesion after four hours incubation. The same HUVEC also showed upregulation of adhesion molecules E-selectin, VCAM-1, and ICAM-1 (Simantov et al., 1995). *In vitro* monocyte adhesion was not induced when antibodies were in serum free media but did occur when purified β_2GPI was added (Simantov et al., 1995). This was the first evidence that the procoagulant phenotype on the EC may at least in part be induced by antibody mediated activation and that the activation may be β_2GPI dependent. Studies by Pierangeli et al. (1999) showed *in vitro* increase in adhesion molecules expression and enhanced *in vivo* adhesion of leukocytes to endothelium in the presence of affinity purified aPL. The Simantov paper (1995) also showed that IgG isolated from a rabbit immunized with purified β_2GPI stimulated monocyte adhesion and the effect was diminished when the IgG was pre-adsorbed with β_2GPI. But, IgG fractions from APS patients contain a number of antibodies including aCL, LA, anti-β_2GPI and AECA. Extensive characterization of anti-β_2GPI concluded that anti-β_2GPI were low affinity, monoreactive autoantibodies directed against β_2GPI as the primary antigen (Tincani et al., 1996). Subsequent studies looked more specifically at polyclonal and monoclonal anti-β_2GPI from APS patients and showed that the antibodies recognized co-factor on the EC surface and induced adhesion molecule upregulation that was co-factor dependent (Del Papa et al., 1997; George et al., 1998; Del Papa et al., 1999; Pierangeli et al., 1999).

3.1 Chapter objectives

Initially, the main objective of this project was to assess global gene expression in HUVEC in response to aPL. However, it was decided to be more specific and investigate the effect of anti-β_2GPI on gene expression due to the strong association of these antibodies with occurrence and increased risk of thrombosis. In order to proceed to microarray, it was first necessary to purify patients IgG, affinity purify anti-β_2GPI and demonstrate that we had in fact purified the appropriate antibodies. In short, correct protocols were developed and appropriate experiments were set up in order to achieve the following:

- To establish satisfactory methodology for the isolation and culture of HUVEC for subsequent experiments.
- To collect patient blood samples.
- To isolate patient IgG.
- To prepare β_2GPI affinity column(s) with subsequent purification of PAPS patient polyclonal anti-β_2GPI.
- To show that the affinity purified polyclonal anti-β_2GPI antibody preparations required co-factor in order to bind to cardiolipin.
- To show that the affinity purified polyclonal anti-β_2GPI antibody preparations were able to bind to β_2GPI and activate EC.
- To prepare polyclonal anti-β_2GPI antibody preparations from PAPS patients for use in microarray Experiments 1 and 2.

3.2 Results

3.2.1 Screening of APS patient sera for aCL and anti-β_2GPI antibodies

ACL titres in sera from 13 APS patients were measured using an aCL ELISA (developed in the Lupus Unit at St. Thomas' Hospital in London) (cut-off for normal value, < 2 GPL u/ml, where 1 GPL unit is the binding activity of 1μg/ml of IgG aCL affinity purified aPL). All patients fulfilled diagnostic criteria for definitive APS (see Table 1.1). Anti-β_2GPI titres were determined using an anti-β_2GPI ELISA kit for IgG antibodies (Genesis Diagnostics Ltd.). Anti-β_2GPI were measured in arbitrary units where results great than 10u/ml were considered

positive. All patients tested showed significantly elevated levels of aCL (high titres) and anti-β_2GPI (Table 3.1). Sample 14 was a normal control serum. A patient serum sample previously determined to have high titre IgG aCL was included in all aCL assays as a positive control.

NB. For clarity, patient samples other than those used in microarray experiments were designated as samples, not patients.

Table 3.1 aCL and anti-β_2GPI antibody concentrations of 13 patients used for IgG purification

Sample	IgG aCL	Anti- β_2GPI
	GPL u/ml	Arbitrary units (u/ml)
1	552	352
2	736	656
3	308	184
4	350	800
5	557	184
6	286	368
7	179	89
8	352	224
9	252	600
10	136	256
11	50	124
12	552	352
13	140	80
14	<2.0	<2.0

3.2.2 Effect of antibodies on EC activation

Initial investigations by other groups used IgG purified from APS patients to ascertain the affects of aPL on EC. Preliminary experiments in this chapter use the same approach to begin the investigation of the effects of aPL on EC activation. IgG from 13 APS patient samples and normal control IgG were manually purified on Protein G-Sepharose columns (1ml column). Protein concentrations were determined using the Bicinchoninic Acid Protein assay. Protein concentrations for IgG preparations ranged from 3.3 to 5.8mg/ml.

E-selectin is not constitutively expressed by vascular EC and increased expression is an indicator of EC activation (Zimmerman et al., 1999). ICAM-1 is expressed constitutively at low levels. Purified IgG from 13 patients containing anti-β_2GP1 and normal control IgG at a protein concentration of 500μg/ml were incubated with HUVEC for 6 hours. Activation was measured using a HUVEC activation ELISA. Expression of ICAM-1 and E-selectin were calculated as the ratio of patient optical density (OD) to that of the normal control where the normal control was given a ratio of 1.0 (100% activation baseline). OD for negative control (unstimulated cells incubated with media only) was subtracted from all values before calculations were done (Figure 3.1). Results of ratios are found in Table 1 in the Appendix.

The HUVEC activation ELISA showed upregulation of E-Selectin expression in response to all patient IgG preparations with the exception of patient sample 13, when compared to normal control IgG (500μg/ml) (Figure 3.1). Patient IgG contained IgG aCL and anti-β_2GPI (Table 3.1) Percent upregulation of E-selectin ranged from 0 to 215 percent compared to normal control IgG. The HUVEC activation ELISA showed ICAM-1 upregulation in response to patient IgG in all samples (n=13). Upregulation ranged from 27 to 308 percent compared to normal control IgG (Figure 3.2).

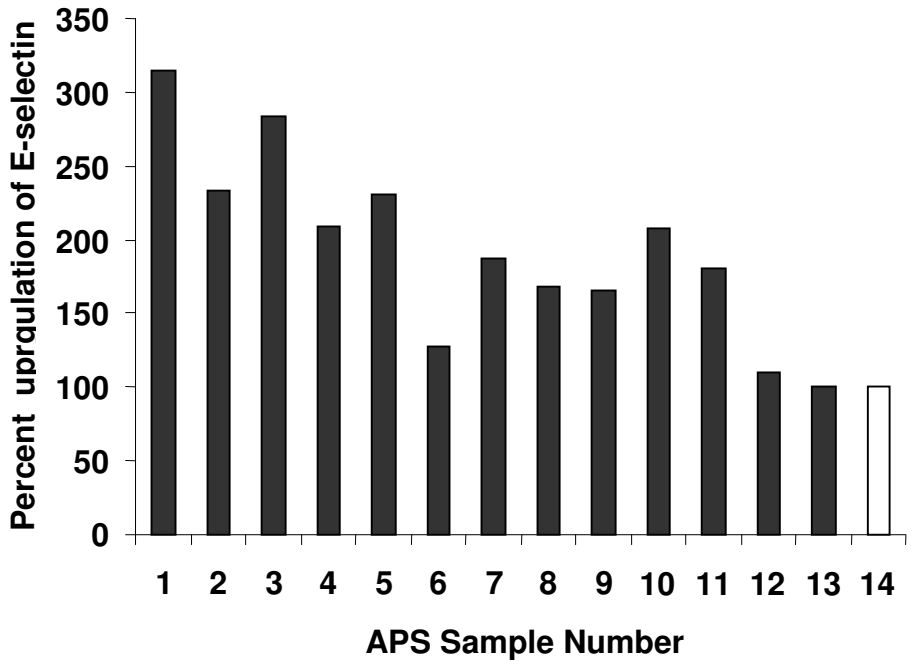


Figure 3.1 Percent upregulation of HUVEC E-selectin expression in the presence of patient IgG. The percent activation of HUVEC E-selectin expression in the presence of patient IgG containing anti-β_2GPI compared to normal control IgG was determined using a HUVEC activation ELISA assay. IgG from patient samples (n=13) and normal control IgG (n=1, sample 14) were incubated with cells at a concentration of 500μg/ml for 6 hours at 37°C with 5% CO_2. Unstimulated cells incubated with complete media only were included as a negative control. Basal OD values for unstimulated cells were subtracted from all OD values. Upregulation was calculated as the ratio of patient IgG OD to normal control IgG OD. Percent upregulation was calculated by comparing patient ratios to control IgG where normal represented a ratio of 1.0 or 100 percent baseline.

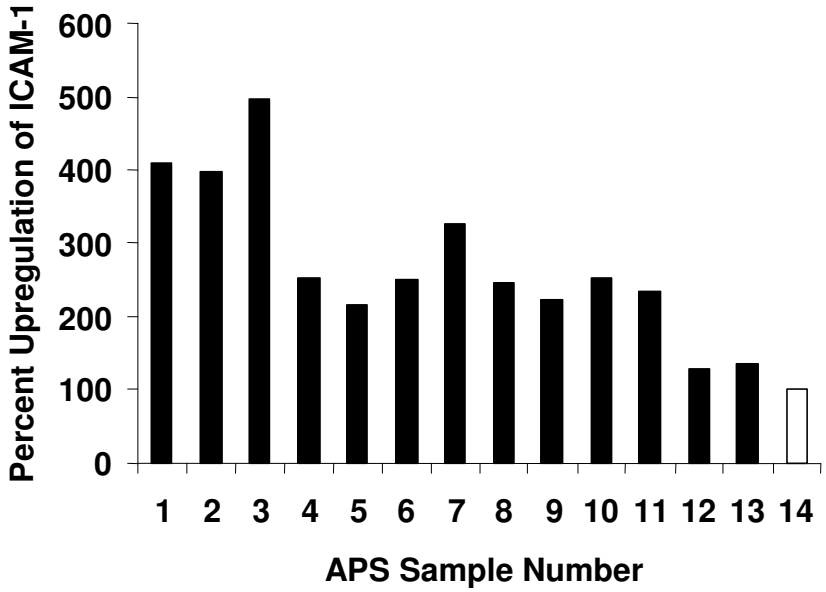


Figure 3.2 Percent upregulation of HUVEC ICAM-1 in the presence of patient IgG. The percent activation of HUVEC ICAM-1 expression in the presence of patient IgG containing anti-β_2GPI (n=13) compared to normal control IgG (n=1) was determined using a HUVEC activation ELISA assay. Explanation as for Figure 3.1.

3.2.3 Affinity purified anti-β_2GPI and co-factor dependence

The first polyclonal anti-β_2GPI preparation was prepared from purified IgG from sample 1 in Table 3.1 (clinical history is given in Table 2.1). Affinity purification was carried out using the NHS-activated sepharose column (1ml) coupled to purified human β_2GPI. The antibody preparation was initially characterized by determining the ability of anti-β_2GPI to bind to cardiolipin in the presence or absence of β_2GPI co-factor. Dilutions of antibody were prepared in PBS containing 10% FCS (containing the β_2GPI co-factor) and the ELISA plate was blocked with PBS supplemented with 10% FCS for these samples. Antibody dilutions were also prepared in PBS containing 0.6%(w/v) gelatin in place of FCS (no co-factor) and the blocking step for these samples was done with the 0.6%(w/v) gelatin in PBS. The anti-β_2GPI antibody preparation was found to be co-factor dependent (no

binding in the absence of β_2GPI) at two concentrations of antibody (1µg/ml and 3µg/ml) and binding was dose-dependent (Figure 3.3). Anti-β_2GPI binding to β_2GPI in the absence of co-factor was not above background levels.

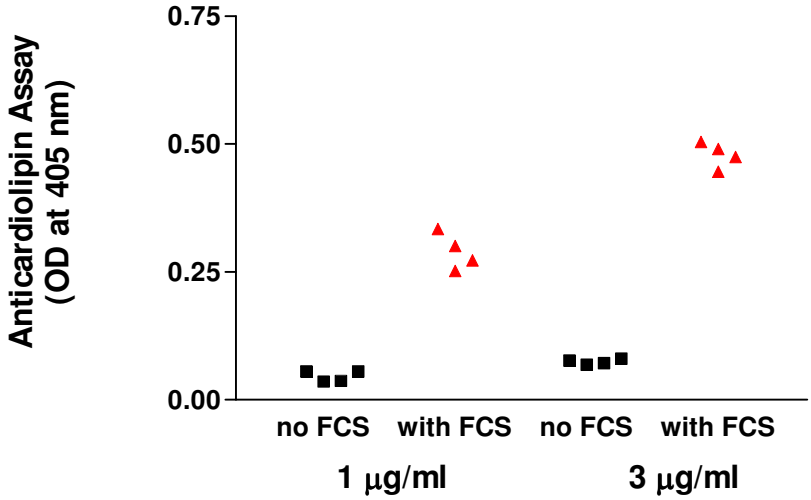


Figure 3.3 Anticardiolipin binding activity of anti-β_2GPI with and without co-factor. aCL binding of affinity purified antibody was tested in the presence and absence of co-factor β_2GPI known to be present in serum. The antibody preparation was tested at two concentrations (1 and 3µg/ml) with and without β_2GPI. Each test variable was tested in quadruplicate and the assay was done once. Anti-β_2GPI bound to cardiolipin only in the presence of fetal calf serum (FCS) containing β_2GPI.

3.2.4 Anti-β_2GPI binding to β_2GPI

The initial manipulations of affinity purified anti-β_2GPI were limited in that the amount of antibody yield was very small, so assays were performed only once. The antibody affinity purified from sample 1 (Table 3.1) was shown to bind to β_2GPI in a β_2GPI ELISA, and the binding was dose dependent (Figure 3.4). A 1/50 dilution of the serum from sample 1 (Tables 2.1 and 3.1) gave an OD of 0.827 and 1/50 of normal negative control serum gave an OD of 0.223 shown in Figure 3.4. This assay is highly sensitive and specific for anti-β_2GPI binding.

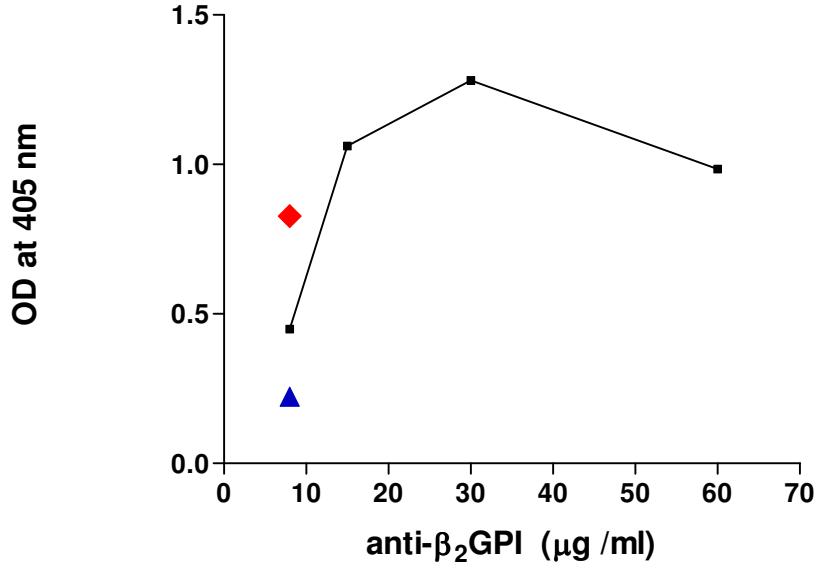


Figure 3.4 The binding of affinity purified anti-β_2GPI to β_2GPI. Binding affinity of purified anti-β_2GPI to β_2GPI in solid phase was tested using an anti-β_2GPI ELISA. The anti-β_2GPI preparation was tested singly at four different protein concentrations (8, 15, 30 and 60μg/ml). The blue triangle represents the OD for normal control serum used as a negative control (0.223). The red diamond represents the OD for a 1/50 dilution of serum from sample1 (O.827) in Table 3.1 used for the preparation of the anti-β_2GPI tested in this figure. The ODs for the controls are the mean of three values, the experiment was performed once.

The antibody used in Figure 3.4 was purified from 5ml of serum from sample 1 using a 1ml NHS activated sepharose column coupled to human β_2GPI. The antibody yield after dialysis was approximately 200μl with a protein concentration of 300μg/ml. After subsequent purification of two more anti-β_2GPI preparations from patient serum samples (samples 2 and 4, Table 3.1) it became apparent that larger volumes of patient sera would be required in order to have sufficient antibody yield for microarray and follow-up experiments. Over the next several months, 25ml of sera was collected from suitable patients with patient consent (Table 2.2). Subsequent IgG and antibody purification was achieved using the AKTAprime protein purification system.

3.2.5 Activation of HUVEC in response to anti-β_2GPI

Upregulation of E-selectin and ICAM-1 expression was shown in HUVEC in response to purified IgG from patients with APS containing anti-β_2GPI (Figures 3.1 and 3.2). In order to determine whether the affinity purified polyclonal antibody preparations would also activate HUVEC, we incubated HUVEC with two antibody preparations (Samples 1 and 4, Table 3.1) and then measured E-selectin expression in a cell activation ELISA. Four hours incubation with antibody resulted in a dose-dependent increase in E-selectin expression in the presence of both antibody preparations when compared to normal control IgG (Figure 3.5). Preparations were tested at three concentrations, 8, 15 and 30µg/ml.

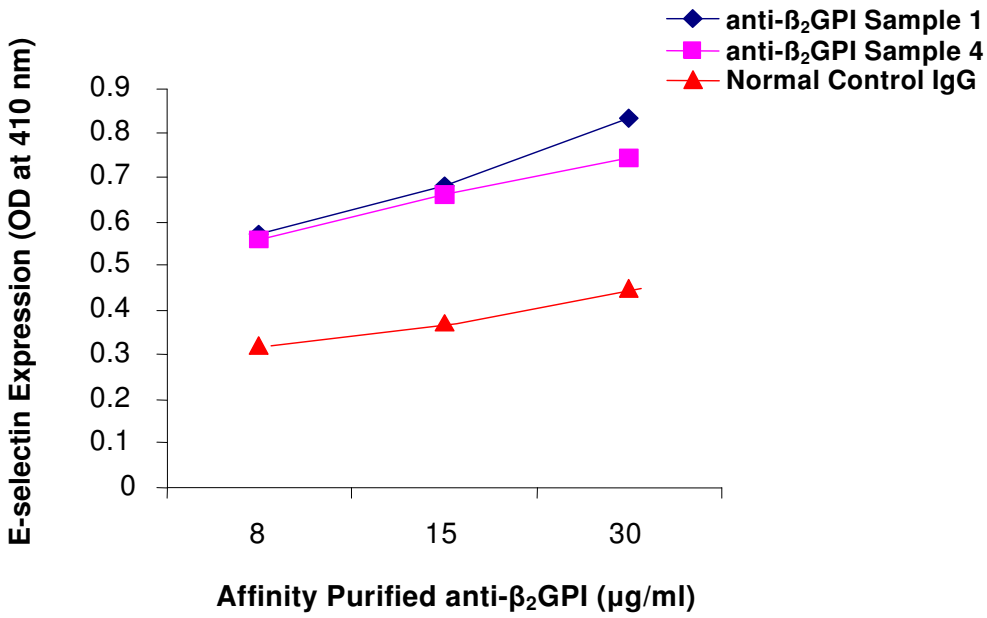


Figure 3.5 Upregulation of E-selectin in HUVEC in response to affinity purified anti-β_2GPI antibodies. E-Selectin expression in HUVEC in response to two affinity purified anti-β_2GPI preparations from two different patient serum samples (sample 1 and sample 4 from Table 3.1) was assessed using the HUVEC activation ELISA and compared to normal control IgG. The two anti-β_2GPI preparations and one normal control IgG were tested singly at three different protein concentrations (8, 15 and 30µg/ml). The experiment was performed once. HUVEC expression of E-Selectin was upregulated in the presence of anti-β_2GPI in a dose dependent manner.

Similarly, upregulation of ICAM-1 expression was assessed by incubating HUVEC for 4 hours with one antibody preparation (Sample 4, Table 3.1). HUVEC demonstrated a dose dependent increase in ICAM-1 expression in response to anti-β_2GPI compared to normal control IgG (Figure 3.6).

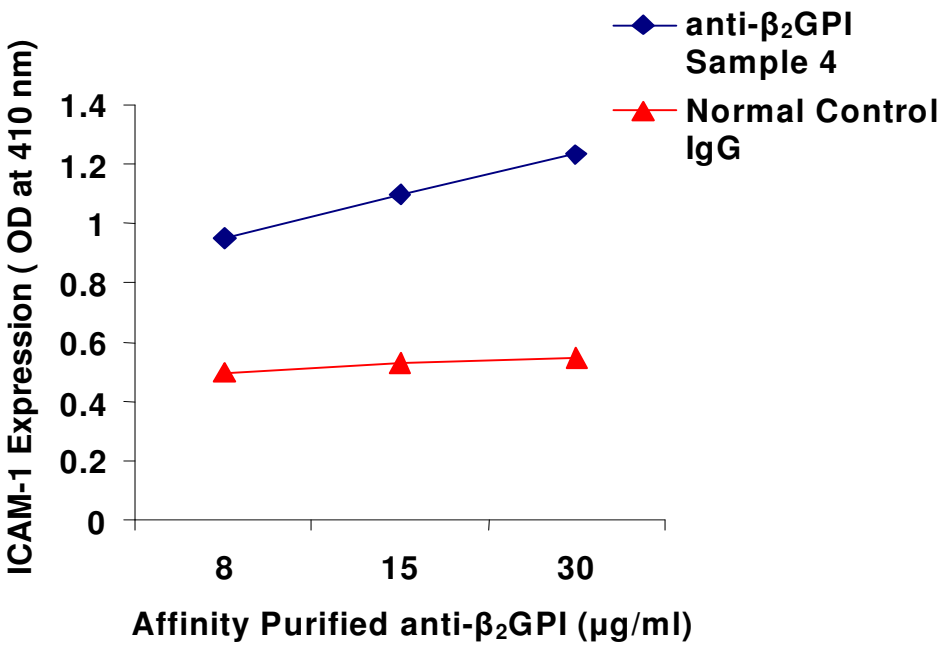


Figure 3.6 Upregulation of ICAM-1 in response to affinity purified anti-β_2GPI. The upregulation of ICAM-1 expression was assessed using the HUVEC activation ELISA. The anti-β_2GPI preparation (Sample 4) and one normal control IgG were tested singly at three different protein concentrations (8, 15 and 30µg/ml). The experiment was performed once. HUVEC expression of ICAM-1 was upregulated in response to affinity purified anti-β_2GPI from sample 4 in a dose dependent manner compared to normal control IgG.

3.2.6 IL-8 production in response to anti-β_2GPI

IL-8 is a well known pro-inflammatory chemokine produced in activated EC in response to a number of agents such as IL-1 and TNF-α (Kaplanski et al., 1997). In addition to the measurement of E-selectin and ICAM-1 expression in the cell ELISA, measuring IL-8 in cell supernatants of cells incubated with anti-β_2GPI was

used as an alternative measure of EC activation. In the first experiment, expression of IL-8 protein in cell supernatants was measured in response to three independent affinity purified anti-β_2GPI (25μg/ml) and compared to IL-8 concentrations in media alone and in response to normal control IgG (Figure 3.7).

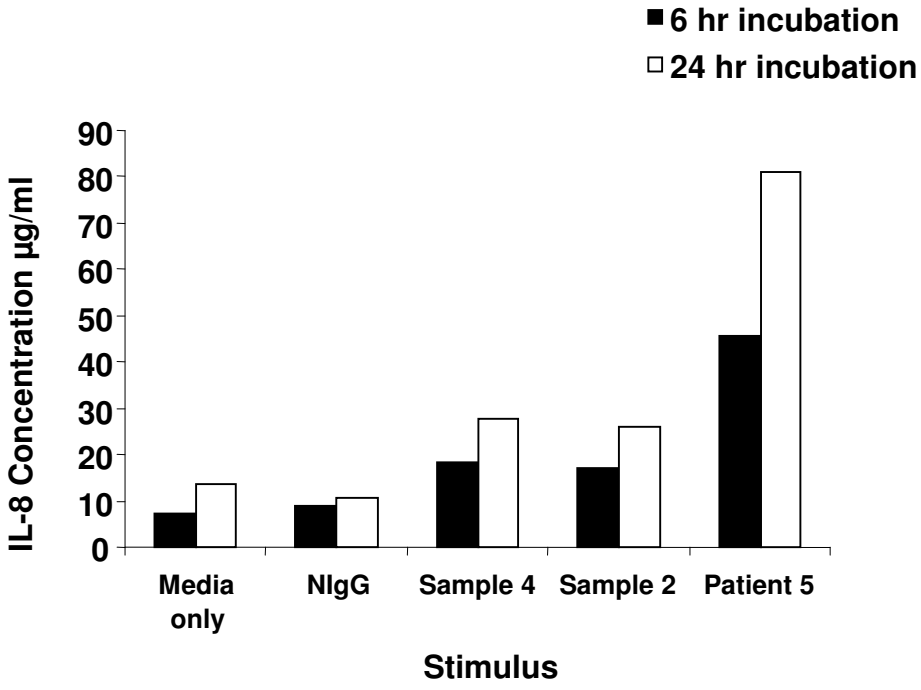


Figure 3.7 IL-8 concentrations in cell supernatants in response to affinity purified anti-β_2GPI. Three independent affinity purified anti-β_2GPI antibody preparations (25μg/ml) (Sample 4, Sample 2, and Patient 5) were incubated with confluent P3 HUVEC in 96 well plate for 6 hours (black) and 24 hours (white) at 37°C with 5% CO_2. Cell supernatants were removed after six and twenty-four hours incubation and frozen at -70°C. Each value is the mean of duplicate determinations. IL-8 concentrations were determined using IL-8 ELISA. Complete media and normal control IgG (25μg/ml) were included as negative controls. This experiment was done once.

IL-8 concentrations in the cell supernatants were checked after 6 and 24 hours incubation with the antibody preparations. Anti-β_2GPI from Patient 5 (Table 2.2) stimulated substantially more IL-8 than the other two preparations (Figure 3.7). (Patient 5 corresponds to sample 3 in Table 3.1. Patient 5 was used in microarray Experiment 1). EC IL-8 production was time-dependent in response to antibody exposure. TNF-α (10ng/μl) was included as a positive control and results were too high to read from the standard curve.

3.2.7 IL-8 induction with and without addition of Polymixin B

Increased IL-8 production in response to anti-β_2GPI from Patient 5 (Figure 3.6) was measured in a second experiment. HUVEC were incubated with increasing concentrations of anti-β_2GPI for four hours. Since LPS is a potent activator of EC, Polymixin B is routinely added to experiments to neutralize possible endotoxin contamination of reagents. IL-8 concentrations in HUVEC supernatants were measured in response to 4 dilutions of anti-β_2GPI and normal control IgG (25, 50, 100, and 150μg/ml) with and without the addition of Polymixin B (5μg/ml) during the incubation step in the protocol (Figure 3.8). The addition of Polymixin B did not appear to reduce the amount of IL-8 induction indicating that LPS contamination was negligible and not contributing to EC activation. This antibody preparation appeared to activate the cells in a dose-dependent manner with 50μg/ml giving optimum activation. This antibody preparation (Patient 5) was used in the first Affymetrix Experiment (1) at this concentration (see Chapter 4). This preparation was also found to bind to β_2GPI and was co-factor dependent. IL-8 was measured in complete media as a negative control and TNF-α (10ng/μl) as a positive control. TNF gave values too high to read from the standard curve.

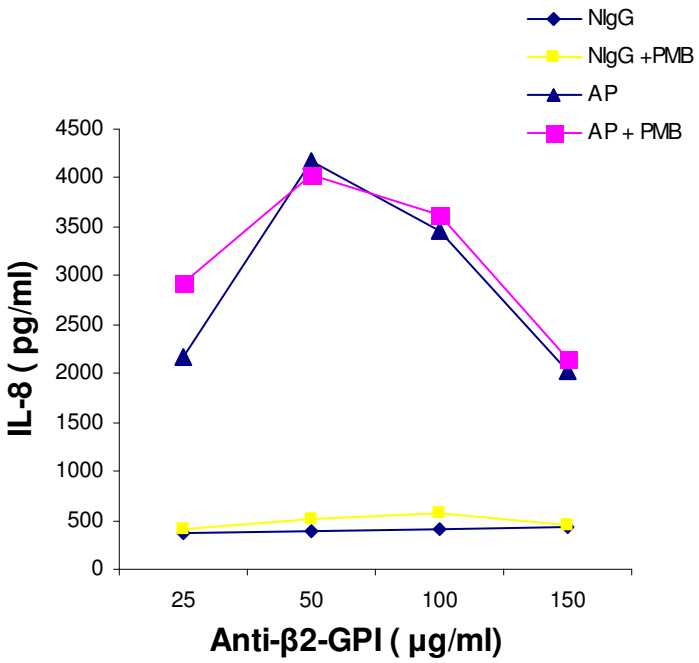


Figure 3.8 IL-8 up-regulation in the presence of anti-β_2GPI with and without the addition of Polymixin B. HUVEC were incubated with different concentrations of affinity purified (AP) anti-β_2GPI from Patient 5 (Table 2.1) and normal control IgG for 4 hours at 37°C with 5% CO_2 with and without the addition of Polymixin B (PMB) (5µg/ml). Cell supernatants were removed and frozen at -70°C. Samples were thawed and IL-8 concentrations of the supernatants were determined using an IL-8 ELISA.

3.2.8 Anti-β_2GPI preparations for Microarray Experiment 2

Approximately 25ml of sera were collected from four patients with PAPS (Patients 1-4, Table 2.2). IgG and anti-β_2GPI were purified using the AKTAprime protein purification system. After concentration and buffer exchange the final protein concentrations of antibody preparations were: Patient 1, 406µg/ml: Patient 2, 219µg/ml: Patient 3, 280µg/ml: and patient 4, 227µg/ml. In a modified aCL assay, all four antibody preparations (3µg/ml) were shown to bind to cardiolipin in the presence of co-factor (Figure 3.9). As in Figure 3.3, binding in the absence of co-factor was not above background levels.

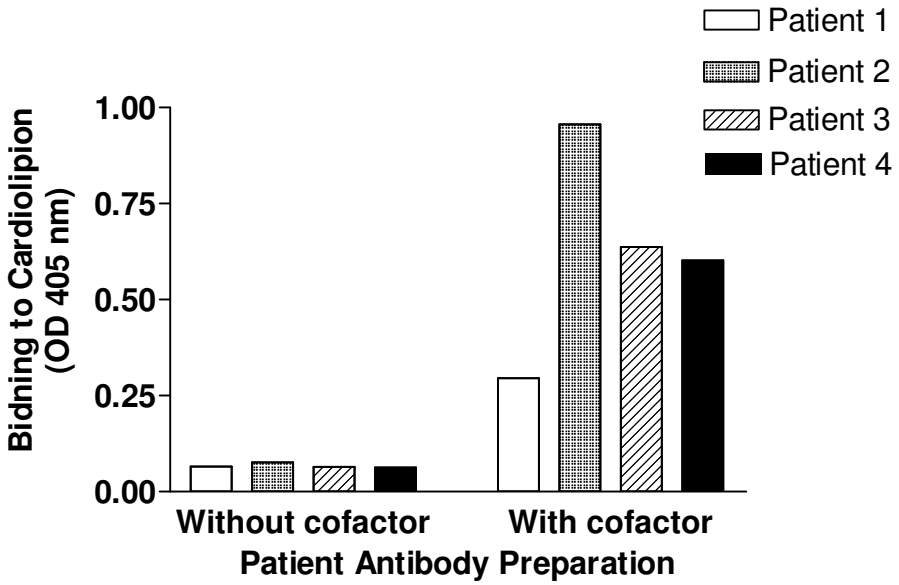


Figure 3.9 Co-factor dependent aCL activity of four anti-β_2GPI preparations. 4 anti-β_2GPI patient preparations (3μg/ml) were tested for binding to cardiolipin with and without the presence of co-factor (β_2GPI) in an ELISA previously described (see 3.2.3). Antibody preparations were tested in duplicate and values shown represent the mean of the two ODs. The experiment was done once.

It is interesting to note that the four antibody preparations at the same concentration show different abilities to bind to cardiolipin at the same concentration (Figure 3.9). This same observation was made when the same preparations were tested for their binding to β_2GPI (Figure 3.10).

All four antibody preparations were able to bind to β_2GPI. Normal control IgG at the same concentration showed binding at background levels. However, the amount of antibody bound to β_2GPI in the ELISA was highly variable between preparations at the same concentrations. This is in agreement with Del Papa et al. (1996) who affinity purified anti-β_2GPI from 6 APS patients and all 6 showed different EC binding activity at the same concentrations and negligible binding with normal control IgG in a β_2GPI ELISA (concentrations tested were from 1.5 to 100μg/ml).

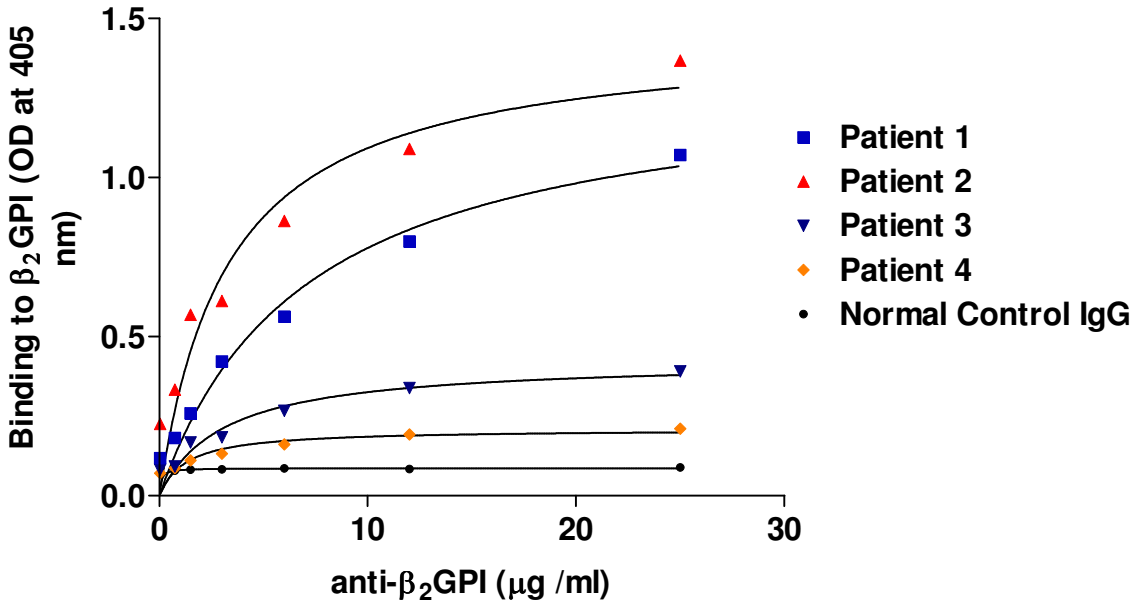


Figure 3.10 Binding of four anti-β_2GPI to β_2GPI. Binding of four affinity purified anti-β_2GPI to β_2GPI was assessed in a β_2GPI ELISA assay and compared to normal control IgG. Binding of four antibody preparations and normal control IgG were tested at concentrations of 0.75, 1.5, 3.0, 6.0, 12.5, and 25μg/ml. was coated on the ELISA plate at a concentration of 10μg/ml. Results were recorded as OD measured at 405 nm. These antibody preparations were used in Affymetrix Experiment 2.

3.2.9 Up-regulation of E-selectin and IL-8 expression in response to anti-β2GPI (P1-P4)

Another way to demonstrate activation of a gene (mRNA production) is to measure the protein or final expression product that results from transcription and translation in response to a stimulus (Lockhart & Winzeler 2000). The four antibody preparations and the four normal control IgG's to be used in Microarray experiment 2 (Patients 1-4) were tested for their ability to activate EC by measuring upregulation of E-selectin on the surface of the cells in the HUVEC ELISA (Figure 3.11). IL-8 was present in cell supernatants in higher concentration in response to antibody when compared to normal control IgG (Figure 3.11) Normal control IgG stimulated baseline amounts of E-selectin similar to incubation with media alone.

In order to check the antibody preparations for LPS contamination, E-selectin expression in response to antibody and normal control IgG was also tested with and without the addition of Polymixin B (5μg/ml). A paired t test showed no statistical difference between levels of E-selectin expression in the presence or absence of Polymixin B in three of the four anti-β_2GPI peparations (P1-P3) indicating LPS induced activation was negligible (Figure 3.11). P4 gave a p value of 0.04 ($p<0.05$). As a precaution, Polymixin B (5μg/ml) was added to the HUVEC/antibody and HUVEC/normal control IgG incubation step for microarray Experiment 2.

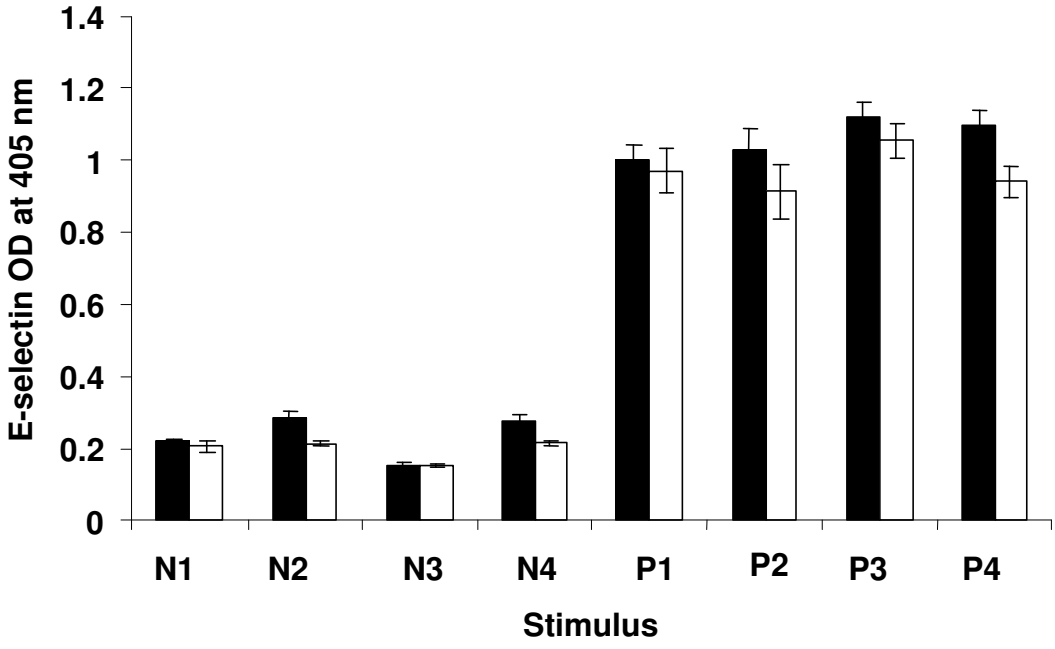


Figure 3.11 Effect of purified anti-β_2GPI on HUVEC E-selectin expression. The effect of the four normal control IgG and four AP anti-β_2GPI (used in microarray experiment 2) was determined using a HUVEC activation ELISA. Results are expressed in OD units at 405 nm. Mean ± SEM are shown, n=3. Antibodies were incubated with the cells for 4 hours (50μg/ml) at 37°C/5% CO_2 with (white) and without (black) the addition of Polymixin B (5μg/ml).

IL-8 levels were determined in HUVEC supernatants after four hours incubation with anti-β_2GPI preparations (P1, P2, P3 and P4), and normal control IgG all with the addition of Polymixin B (5μg/ml) (Figure 3.12). IL-8 concentration was increased in response to all four anti-β_2GPI preparations compared to normal control IgG, the difference being statistically significant ($p<0.03$ as determined by two-tailed Mann Whitney U Test).

Therefore, incubation of HUVEC with four anti-β_2GPI preparations from four different PAPS patients resulted in significant upregulation of E-selectin and IL-8 expression when compared to four normal control IgGs.

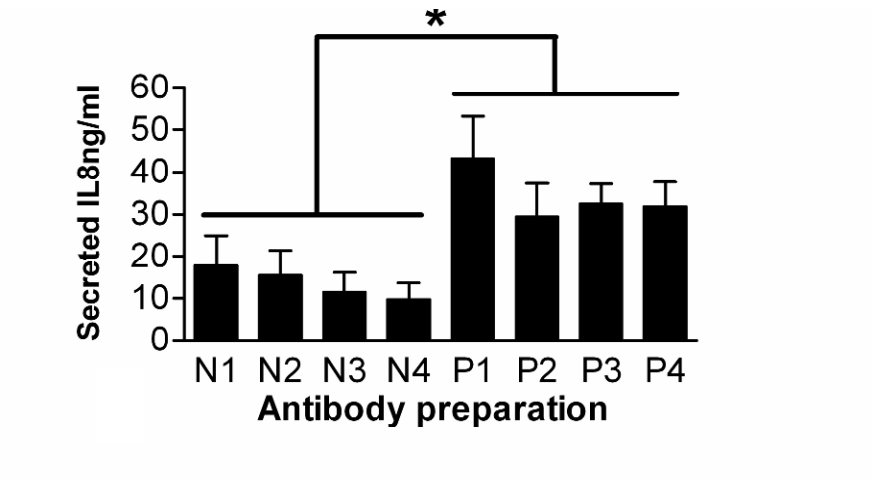


Figure 3.12 Anti-β_2GPI antibodies induce HUVEC activation. The effect of four normal control IgG and four APS-derived anti-β_2GPI preparations (used in Microarray experiment 2) on IL-8 secretion was determined by ELISA. Antibodies were incubated with cells for 4 hours at 50μg/ml. IL-8 was measured in cell supernatants. Results show mean ± sem of triplicate samples from a representative experiment (one of three). * =$p<0.03$ as determined by two-tailed Mann Whitney U Test. Polymixin B (5μg/ml) was added to the anti-β_2GPI incubation step.

3.2.10 Effect of endotoxin on EC activation

It was necessary to address the possibility that there was endotoxin contamination in the IgG and polyclonal anti-β_2GPI preparations since LPS could be at least partially responsible for activation of pro-inflammatory genes rather than the antibody preparations themselves.

The FDA defines the endotoxin unit (EU) as the endotoxin activity of 0.2ng of reference endotoxin standard (Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test for Human and Animal Parenteral Drugs, 1987). Levels of LPS in control and anti-β_2GPI antibody preparations were measured by a Limulus Amebocyte Lysate Test (Cape Cod Associates, Mass, USA) and they were very low (≤4ng/ml). In preliminary experiments to test the effectiveness of antibody preparation to induce E-selectin expression, all control IgG and anti-β_2GPI antibodies, which were used in the microarray experiments, were tested in the presence or absence of polymixin (See Figures 3.7 and 3.10). The addition of polymixin had no/minimal effects on control IgG or anti-β_2GPI antibody induced E-selectin levels, but nonetheless, as a precaution, it was decided to include this reagent in all experiments.

In order to test the effectiveness of Polymixin B (5μg/ml) on inhibiting endotoxin-induced EC activation, confluent HUVEC were incubated with increasing concentrations of LPS for six hours with and without addition of the standard recommended concentration of Polymixin B (5μg/ml) (Figure 3.13). In the presence of Polymixin B, IL-8 production was not increased beyond baseline levels (negative controls media and media /10% FCS) up to an LPS concentration of 25ng/ml. Finally, other investigators in the field have routinely used this concentration of Polymixin B in their experiments (Raschi et al., 2003a). These results support the conclusion that EC activation seen in the presence of anti-β_2GPI antibodies in the previous experiments was due to effect of antibody rather than endotoxin in the preparations.

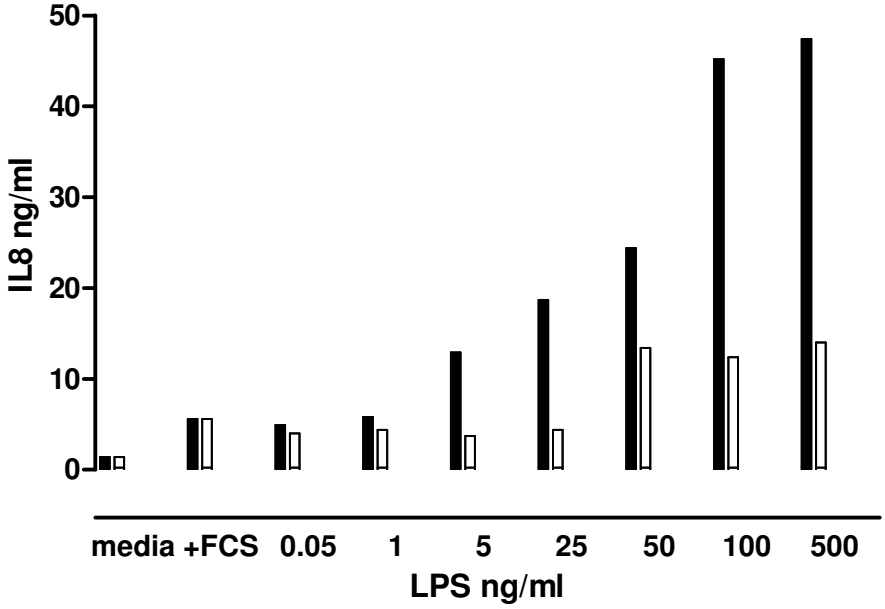


Figure 3.13 Affect of Polymixin B on IL-8 production in the presence of LPS. Confluent HUVEC were incubated with increasing concentrations of lipopolysaccharide (LPS) (0.05-500ng/ml) for six hours at 37°C/5% $C0_2$ with (white) and without (black) the addition of Polymixin B (5 μg/ml). IL-8 concentrations were determined using an IL-8 ELISA. Each value is the mean of three replicate samples. LPS stock was 0.5mg/ml.

3.3 Discussion

In order to achieve the objectives for this chapter a number of protocols had to be established and refined. It was necessary to isolate primary HUVEC, then grow and retain healthy HUVEC in culture until P3 when they would be used in experiments. Appropriate patient serum samples had to be collected from ongoing clinics in the Lupus Unit at St. Thomas' Hospital, London. IgG was purified from a number of patient sera, this was followed by preparation of β_2GPI affinity columns and subsequent purification of anti-β_2GPI.

EC activation in response to APS patient IgG containing anti-β_2GPI was confirmed by measuring upregulation of E-selectin and ICAM-1 on the HUVEC cell surface. Anti-β_2GPI was affinity purified from several PAPS patient IgG samples and these polyclonal antibody preparations were characterized by measurement in the standard aCL assay with and without the addition of β_2GPI co-factor and

measurement in a β_2GPI ELISA. Iverson et al., (1998) purified anti-β_2GPI from serum of APS patients and the antibody yield ranged from 10 to 190 µg per ml of serum. The first two affinity purified anti-β_2GPI preparations in this study were each purified from 5ml of sera and the yields were in the same range. Preparations gave 15 µg/ml of serum (Sample 1, Table 3.1) and 90µg/ml of serum (Sample 4, Table 3.1) (300µg/ml in a total volume of 200µl and 450µg/ml in a total volume of 250µl) respectively.

In preliminary EC activation experiments with anti-β_2GPI, polyclonal anti-β_2GPI and monoclonal anti-β_2GPI antibodies were tested at concentrations of 25-100µg/ml (Del Papa et al., 1997, George et al., 1998). Upregulation of adhesion molecules in response to antibody appeared to plateau at concentrations between 25 and 50µg/ml (George et al., 1998). In the same paper, monoclonal anti-β_2GPI binding to β_2GPI ELISA coated with 10µg/ml β_2GPI plateaued at antibody concentrations between 25 and 50µg/ml. Binding of affinity purified anti-β_2GPI from patients 1-4 in this study appeared to plateau at around a concentration of 25-30µg/ml at the same concentration of β_2GPI (10µg/ml)(Figure 3.10).

Preliminary experiments in this study indicated anti-β_2GPI induced EC activation occurred in a dose-dependent manner. EC activation with anti-β_2GPI appeared to be optimal at a concentration of 50µg/ml, so it was decided to use this concentration in the microarray experiments. EC activation was confirmed by measurement of increased expression of E-selectin and ICAM-1 on the EC surface and increased IL-8 concentrations measured in cell supernatants after incubation with anti-β_2GPI.

As a consequence of these encouraging results, anti-β_2GPI was affinity purified from sera of four additional PAPS patients, each of whom fulfilled the diagnostic criteria and had a previous history of thrombosis. These antibody preparations were characterized as above and shown to induce EC activation. Affinity purification of larger volumes of sera using the AKTAprime system meant that sufficient quantities of these four anti-β_2GPI as well as Patient 5 (Table 2.2) were obtained in order to proceed to microarray (Patient 5 corresponds to patient sample 3 in Table 3.1).

In conclusion, anti-β_2GPI antibodies were successfully isolated and characterized from PAPS patient sera. These antibodies demonstrated the ability to activate EC, making it feasible to proceed to microarray with confidence.

Chapter 4

Microarray Analysis of Global Gene Expression in HUVEC in Response to anti-β_2GPI from PAPS Patients

4.1 Introduction

In the previous chapter, IgG anti-β_2GPI from PAPS patient sera antibodies were successfully affinity purified and characterized. These antibodies are strongly associated with VT and have been identified as an independent risk factor for thrombosis (Cabral et al., 1995; Martinuzzo et al., 1995; Galli et al., 2003b). Purified polyclonal anti-β_2GPI preparations from APS patients have been used in numerous *in vitro* applications in order establish possible mechanisms of action of these antibodies. These studies were performed in order to identify anti-β_2GPI-dependent changes in EC phenotype that may be responsible for the prevalence of thrombosis in APS. It is well documented that anti-β_2GPI cause EC activation. Anti-β_2GPI antibodies have been shown to induce leukocyte adhesion molecules (Simantov et al., 1995; Del Papa et al., 1997; Pierangeli et al., 1999). aPL also upregulate TF on EC and monocytes, this increase in TF expression would clearly contribute to the prothrombotic EC phenotyope associated with APS (Kornberg et al., 1994; Cuadrado et al., 1997). EC activation has also been demonstrated in response to anti-β_2GPI *in vivo* (Pierangeli et al., 2000). Recent studies have reported that NF-κB transcription factor pathway, MyD88 pathway, and p38MAPK phosphorylation are involved in EC activation by anti-β_2GPI antibodies (Dunoyer-Geindre et al., 2002; Raschi et al., 2003; Bohgaki et al., 2004). Although these studies have identified a number of interesting associations between anti-β_2GPI induced EC activation and changes in EC phenotype, the extent and diversity of anti-β_2GPI-mediated gene regulation in EC is not well characterized.

The present study was undertaken in order to examine early global gene expression in EC in response to polyclonal patient-derived anti-β_2GPI using Affymetrix microarray gene profiling. Such an experimental approach would be able to screen for changes in regulation of large numbers of genes in EC simultaneously in response to affinity purified anti-β_2GPI. Microarray analysis can provide insights into those genes that may be co-regulated. Such information could lead to further understanding of gene interactions and provide insight into the complex mechanisms of EC activation in response to anti-β_2GPI. As well as confirming changes in those anti-β_2GPI regulated genes identified in previous studies, it was

hoped that the transcription profile obtained would identify a number of novel genes potentially involved in the pathology of PAPS.

4.1.1 Background- Affymetrix microarray technology and data analysis

The main goal of this project was to determine differential gene expression between EC exposed to anti-β_2GPI and those cells exposed to normal control IgG. Our microarray investigations incorporated the high-density oligonucleotide microarray platform, made by Affymetrix Inc. Experiment 1 used the HG U133A chip and Experiment 2 the HG U133A_2 chip. The Affymetrix methodology uses expressed sequence tags (ESTs). These represent segments of DNA of known sequence within a gene of interest and are selected for their specificity for the gene. These small segments of DNA also known as oligonucleotides are used as probes. In the case of Affymetrix, a probe is a 25-mer oligonucleotide of known sequence synthesized *in situ* on the surface of the chip using a technology called photolithography. Each oligonucleotide is synthesized on a feature or specific spot on the chip (Affymetrix Users Manual, 2006). The Affymetrix platform incorporates probe sets. These are collections of probe pairs that bind to the same sequence or set of sequences within a gene of interest. Affymetrix incorporates 11 probe pairs in a set (22 probes in total) per gene, using a number of different probes per gene makes results more reliable. A probe pair includes the complementary sequence or perfect match (PM) to the selected segment of DNA and a mismatch oligo (MM) that contains a single base mismatch in the centre of the oligo to the same target region. This base substitution compensates for non-specific hybridization of the target DNA. If the PM intensities are consistently higher than the MM intensities, there is a good indication that transcript for the corresponding gene was in fact present in the sample. The gene expression level or relative abundance of a transcript is obtained by taking the average hybridization intensity of the 11 probe pairs and this value is referred to as the signal. The unknown, labelled (biotinylated) fragmented cRNA, is applied to the probe array for hybridization and is called the target.

In order to achieve reliable results in gene expression analysis studies, stringent quality control (QC) measures were used throughout the process in order to monitor sample quality and successful hybridization. To begin, the overall quality

of the RNA sample was assessed by measuring the concentration and purity and assessing RNA quality on a gel using the Agilent Bioanalyser. The samples were checked visually twice more in the protocol by running them on a gel, the quantified cRNA and the fragmented cRNA were checked visually for overall quality and successful completion of cRNA synthesis and fragmentation.

Internal control probes on the array include genes Beta-actin and GAPDH and these are used to assess the RNA sample quality and assay efficiency. Probes at opposite ends of these genes (the 3' and 5'ends) are included on the array, the ratio of 5' to 3' intensities should be approximately 1 if the IVT reaction has been efficient. Other probes have a 3' bias to ensure complete transcription. Control probes within the array include eukaryotic hybridization controls (bio B, C and D biotin-labeled cRNA). These are added to the hybridization cocktail and are present in gradually increasing concentrations in order to evaluate sample hybridization efficiency. The number of probe sets called present (%P) in relation to the number of probes on the array is given as a percent. (HUVEC expressed approximately 55% of genes. Replicate samples should have a similar %P value as should samples from one cell type).

4.1.2 Processing the Scanned Image

After hybridization, the array is washed to remove unbound target and scanned. The scanned image must be translated into numeric data that can be used for statistical analysis. Immediately after scanning, the probe array image (.dat file) is examined visually for artefacts such as high/ low intensity spots, scratches, or high background staining. The oligo B2 hybridization control (Biotinylated control oligonucleotide) is incorporated into the hybridization cocktail and the binding of this control is assessed visually on the image. An alternating pattern of intensities around the border should be visible along with the array name located in the upper left corner. Using the oligo B2 as a reference, a grid is automatically placed over the image so that each probe corresponds to a specific location on the grid. Each spot on the array corresponding to a probe will be assigned an intensity value (single channel array). The *.cel file is created from the *.dat file where the cel file contains a single intensity value for each probe or feature on the grid. This data is filtered to remove background (electrical) noise in the system.

4.1.3 Detection Algorithm

Probe pair intensities are used to generate a Detection p-value followed by a Present, Marginal or Absent call. Each probe pair in a set (n=11) helps to determine whether the measured transcript is detected (present) or not detected (absent). The discrimination score (R) is calculated for each probe set and takes into consideration intensity values for the PM probe (perfect match probe) and the MM (mismatch) probe. R= (PM-MM)/ (PM+MM) where a PM much higher than the MM in a number of the probe pairs will result in a higher discrimination score and a lower or more significant detection p value. A lower p-value is a reliable indicator that the transcript is expressed and the result is valid. The discrimination scores are then tested against a user-defined threshold called Tau. Scores higher than Tau are in favor of the transcript being present, the higher the scores are above Tau, the smaller the p-value and the more likely that the transcript is present. The one-sided Wilcoxon's Rank test is used to determine the Detection p-value. (http://www.affymetrix.com/support/technical/technotes/statistical_reference_guide.pdf).

4.1.4 Detection Calls

The boundaries used to define Present, Marginal or Absent calls are detection p-value cut-offs called Alpha 1 ($p<0.04$) and Alpha 2 ($p>0.06$). A p-value <0.04 is given a Present call, p between 0.04 and 0.06 a Marginal call and $p>0.06$ an Absent call. The process of selecting Present and Marginal genes is called flagging. The flagged genes are used in further calculations of expression change. Genes should be present in half or more than half of the samples to be considered significant. In Experiment 2 we stipulated that genes had to be present in 11/22 samples or more. Signal is a quantitative measure of the relative amount of a transcript. It is calculated using the One-step Tukey's Bi-weight Estimate that gives a weighted mean. Each probe pair contributes to the determination of signal. The mismatch is used to determine background or stray signal. The signal is estimated by subtracting the MM intensity from the PM intensity, followed by taking the log of the value. The mean of the intensity values for the probe set is calculated, converted back to linear scale and given as the signal for the corresponding gene.

Fluorescent intensities from each spot are compared across arrays within an experiment. In order to be able to compare data between samples (different chips within an experiment) the data is normalized to remove inter-array heterogeneity. The purpose of normalization is to minimize the effects of experimental and or technical differences between the samples in an experiment. This assumes that the majority of the transcripts are not changing in a group of samples and overall intensities of the arrays should be similar. This process is known as global normalization (incorporates all probes). This method assumes that spot intensities on the arrays are linearly related and that the same scaling factor can be applied to each spot on an array. The normalization method used in Genechip software is called scaling. Scaling is the adjustment of the average intensity (signal) value across all arrays in an experiment to a common target intensity value (usually 100). Each sample will have a different scaling factor. The scaling factor is usually less than 1.0, a scaling factor of greater than 3 on an array will result in too much variation between the samples or sample degradation (Lu 2004). Scaling factors in the experiments reported here were consistently between 0.2 and 0.4. Affymetrix uses a trimmed mean including the intensities from 96% of the probe sets with the highest and lowest 2% of the values being eliminated to remove outliers. Flagged values (P and M) should be used in the calculation of the mean intensity. (The average intensity value (PM minus MM) for each probe pair in an array is calculated and compared to the means in the other samples.)

Hybridization and corresponding fluorescent intensities are influenced by the GC content of the probes. GC rich probes tend to have higher intensities than those with AT rich probes. A non-parametric between-array normalization technique for Affymetrix GeneChip data that corrects for GC content is the multivariate quantile normalization algorithim. This algorithm compensates for dispersion of the data and outliers and was applied to the raw cel. file data (Bolstad et al., 2003).

4.1.5 Log Transformation of Data

When comparing differences in an experiment between two or more conditions, change is the qualitative measure of increase or decrease in a transcript. Once the data has been normalized, the change in expression is taken as the ratio of the intensity for the probe in the treated sample compared to the intensity for the same probe in the baseline or control and this gives the fold change. Assuming that the

majority of transcripts are unchanged, the majority of values for this ratio will cluster around 1.0, the data tends to be heavily skewed. Taking the log ratio gives more structure to the values by enabling greater discrimination between small differences in expression. This means the data is dispersed more evenly around 1.0 and is more easily examined visually within the Affymetrix and Genespring programs. (Chapter 5, Processing microarray data- in Exploration and analysis of DNA Microarray and protein array data- Pg 61). Therefore, a quantitative estimate of the change in gene expression produced in the form of Signal Log Ratio is included in the analysis.

The ratio of expression intensities gives the fold change. In this study, fold change greater than 2.0 indicated upregulated expression and < 2.0 (ratios < 0.5) indicated down-regulated expression. The choice of the fold change cutoff is arbitrary, generally from 1.5 fold or greater up and down (see 4.1.8). The Affymetrix assay is able to ascertain fold changes of two fold or greater 98% of the time (Wodicka et al., 1997).

4.1.6 Significance Testing

The one way analysis of variance (ANOVA) allows you to determine if one given factor (eg; antibody treatment) has a significant effect on the expression of a gene across two groups of samples in a study. In this study the two sample groups include HUVEC incubated with anti-β_2GPI and HUVEC incubated with normal control IgG. Testing for statistical significance identified those genes that are differentially expressed and show similar patterns and/or levels of expression in treated samples when compared to untreated. Differential expression of such a gene would result in a significant p value in the one-way ANOVA. The one way analysis of variance (ANOVA) uses normalized log of ratio data and takes the average expression of each gene across all biological replicates. It then compares the mean difference for the gene between tests and controls (www.chem.agilent.com).

4.1.7 Clustering

Clustering of the data helps to identify patterns of regulation common to those genes that are altered in expression. Gene clustering approaches are applied to

microarray data in order to attempt to identify subsets of genes that display similar or coordinated expression patterns. The application is known as unsupervised pattern recognition (Boutros & Okey 2005). Clustering or unsupervised pattern recognition algorithms are able to identify patterns within a set of data. The three most common clustering algorithms are hierarchical clustering, k-means and self-organizing maps. Unsupervised algorithms that start with the raw data generally provide information about patterns within the data. Hierarchical clustering assumes that the genes can be organized in relation to their similarity to each other. If the expression of a gene is altered at the same time as another (co-expression), the assumption may be that both genes are required in order to achieve a biological effect. Co-expression of genes means that genes in a cluster display similar patterns of expression over several conditions. Regulation of genes that are co-expressed may be orchestrated through a common mechanism such as signaling through a common pathway, for example the transcription factor NF-κB. Clustering may help to identify sets of genes with such associations and give rise to new hypotheses about the mechanisms of regulation. Genes identified as having differential expression can also be grouped according to their function (see Figure 4.3).

4.1.8 Selection criteria for differentially expressed genes

This chapter presents the results of two separate microarray experiments undertaken in order to assess gene expression in response to anti-β_2GPI. In both Experiments 1 and 2, genes that show changes in expression in HUVEC when incubated with anti-β_2GPI compared to normal control IgG will be described as being differentially expressed. Only genes flagged as P or M by the Affymetrix MAS 5.0 software were included in the analysis. All expression values were normalized across the samples and fold change was calculated by taking the ratio of the mean expression for each gene in the treated samples compared to the controls. Non-changing genes were removed from the data before doing analysis of variance. Genes showing two fold change in expression (either up of down) were included in analysis of variance across the samples. So, those genes found to be statistically significant ($p<0.05$) were changed two fold or greater in expression.

Firstly, differential gene expression in Experiments 1 and 2 will be addressed individually. Two-fold changes in gene expression were chosen because generally two-fold changes in level of expression are regarded as biologically meaningful in more conventional biochemical assays such as Northern blot, Western Blot or Real-time PCR analyses. When it comes to microarray data, the two-fold cut-off is an arbitrary cut-off, however we restricted our analysis to this level of change in order to highlight those changes in gene expression that were likely to be biologically meaningful and also could be confirmed by other biochemical assays (such as those mentioned above).

Those genes statistically significant genes ($p<0.05$) common to both experiments are identified. A larger number of genes within the two experiments were changed two fold or greater in expression, but failed to meet statistical significance due to the variability in expression between samples. Not all genes altered in expression 2 fold or greater received individual comment. The focus was on those genes that were more highly expressed or those most likely to contribute to the thrombotic phenotype of PAPS based on their function.

4.1.9 Chapter Objectives

The principle objective of this chapter was to present, summarize and confirm data from Affymetrix microarray experiments designed in order to determine the effect of affinity purified anti-β_2GPI on global gene expression in HUVEC when compared to normal control IgG. The experimental approach in order to achieve this objective is summarized in the following points:

- Experiment 1 tested the effect of purified anti-β_2GPI from Patient 5 (Table 2.1) on differential gene expression in HUVEC. The antibody preparation was tested three times on three separate occasions using three different HUVEC donors. Each time, antibody treated cells were compared to normal control IgG treated cells and the samples were paired for analysis of the data.
- Experiment 2 incorporated four anti-β_2GPI antibody preparations from four additional PAPS patients and compared their regulated gene expression in

HUVEC to four additional normal control IgG samples. As in Experiment 1, the experiment was repeated on three different occasions using three different HUVEC donors.

- Data analysis was performed using Genespring software (http://www.silicongenetics.com).
- Those genes showing 2-fold or greater increase or decrease in expression, and statistically significant differences in expression ($p<0.05$) between antibody treated and normal control IgG treated cells were determined for both Experiment 1 and 2.
- Those genes statistically significant and changed in expression greater than two fold, common to both experiments were identified.
- Quantitative RT-PCR was used to confirm altered expression in several novel anti-β_2GPI regulated genes identified using microarray.
- Preliminary experiments to test the effect of anti-β_2GPI on DNA synthesis in HUVEC were performed.

4.2 Results

4.2.1 Experiment 1

Experiment 1 tested changes in global gene expression in response to anti-β_2GPI (treated) from Patient 5 (Table 2.1) and one normal control IgG (untreated) with confluent HUVEC from three different cell donors on three separate occasions. Cells were incubated with anti-β_2GPI and normal control IgG at a concentration of 50μg/ml for four hours. Polymixin B was not added to the cell anti-β_2GPI incubation step in Experiment 1.

After scanning the arrays, Affymetrix Microarray suite (MAS) X 5 software was used to generate expression values for each gene. Chip file data was imported into Genespring (Agilent Technologies) for further analysis. A paired t-test was performed on the 6 samples, pairing anti-β_2GPI treated sample to normal control IgG sample for each HUVEC donor. For paired comparison, all genes in the control samples were given the value 1.0. In Experiment 1, 43 genes up-regulated 2 fold or greater were statistically different ($p < 0.05$) between treated and untreated cells (Table 4.1). In this experiment, no genes down-regulated 2 fold or more were found to be statistically different between treatments.

Genes were divided into APS relevant categories; apoptosis/ anti-apoptosis, chemokines, metabolism, transcription factors/ signalling, adhesion molecules/ receptors and miscellaneous. For each gene, gene tables include the gene nomenclature, corresponding fold change, the number assigned to the gene by the National Centre for Biotechnology Information (NCBI) number, and a brief description.

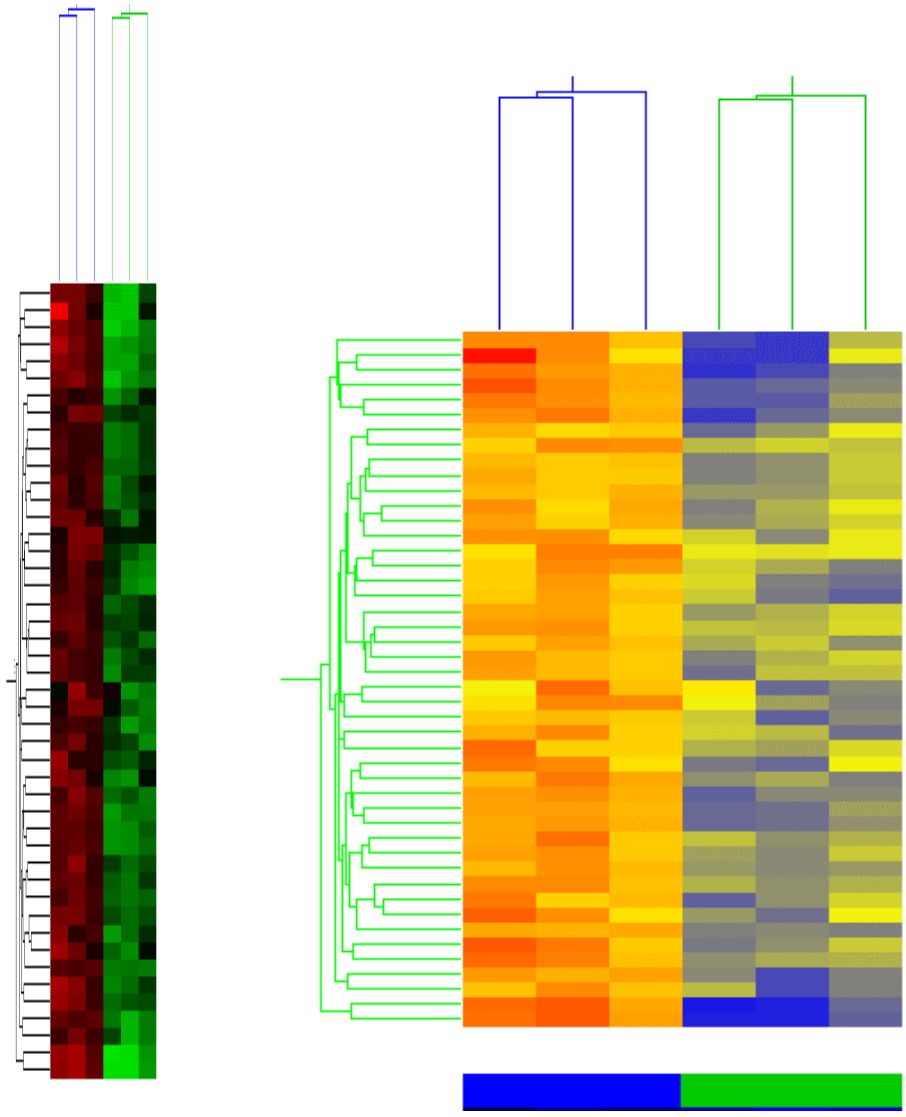
The results in Experiment 1 were very encouraging in that they confirmed EC activation in response to polyclonal patient derived anti-β_2GPI and the upregulation anticipated in several well documented genes, was in fact confirmed (ICAM-1 and VCAM-1, Table 4.1).

Table 4.1 Experiment 1- Anti- β_2GPI antibody-induced gene expression in HUVEC ($p<0.05$, n=43).

Gene	Fold Induction	Accession Number	Gene Description
Apoptosis/ anti-apoptosis			
RIPK2	3.6	AF027706	receptor-interacting serine-threonine kinase 2
TNFAIP8	2.6	NM_014350	tumor necrosis factor, alpha-induced protein 8
TRIB3	2.5	NM_021158	tribbles homolog 3 (Drosophila)
IFIH1	2.1	NM_022168	Interferon induced with helicase c domain 1
Adhesion Molecules/ Receptors			
ICAM	6.05	NM_000201	intercellular adhesion molecule 1 (CD54)
VCAM	5.0	NM_001078	vascular cell adhesion molecule 1
IL18R1	3.5	NM_003855	interleukin 18 receptor 1
TNFAIP3	3.4	NM_006290	tumor necrosis factor, alpha-induced protein 3
IFNGR1	2.1	AF056979	interferon gamma receptor 1
Cytokines/ Chemokines			
CXCL2	14.6	M57731	Chemokine (C-X-C motif) ligand 2
CX3CL1	6.3	NM_002996	Fractalkine
CCL7	2.4	NM_006273	Chemokine (C-C motif) ligand 7
CCL2	2.0	S69738	chemokine (C-C motif) ligand 2
Metabolism			
ASNS	2.4	NM_001673	Asparagine synthase
CTH	2.3	AL384572	Cystathionase (cystathionine gamma-lyase)
ABCG1	2.1	U34919	ATP-binding cassette, sub-family G (WHITE), member 1
CBR3	2.0	NM_001236	Carbonyl reductase 3
Transcription Factors/ signaling			
CEBPD	3.6	M83667	CCAAt/enhancer binding protein (C/EBP)
NFKBIA	3.4	NM_020529	Nuclear factor of kappa light polypeptide gene enhancer in B- cells inhibitor, alpha
VEGF	3.2	AF022375	Vascular endothelial cell growth factor
PMAIP1	3.2	NM_021127	Phorbol-12-myristate-13-acetate-induced protein 1
NKX31	3.1	AF247704	Transcription factor related, locus 1 Drospophila
SOD2	3.0	W46388	Superoxide dimutase 2, mitochondrial
HIVEP2	2.9	AL023584	Human immunodeficiency virus type 1 enhancer binding protein
IL-6	2.9	NM_000600	Interleukin 6
BCL3	2.8	NM_005178	B-cell CLL/lymphoma 3
RND1	2.7	U69563	Rho family GTPase 1
EFNA1	2.5	NM_004428	Ephrin-A1
RAPGEF5	2.4	NM_012294	Rap guanine nucleotide exchange factor (GEF)5
CEBPD	2.3	AL564683	CCAAT/enhancer binding protein (C/EBP), beta
ZBTB10	2.25	NM_023929	Zinc finger and BTB domain containing 10
MAP3K1	2.2	AA361361	Mitogen-activated protein kinase kinase kinase 1
DSCR1	2.15	NM_004414	Down syndrome critical region gene1
ATF3	2.15	NM_001674	Activating transcription factor 3
JUNB	2.1	NM_002229	Jun B proto-oncogene
NFKB1	2.1	M55643	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
RELB	2.1	NM_006509	v-rel reticuloendotheliosis viral oncogene homolog B
KLF9	2.0	NM_001206	Kruppel-like factor 9
Miscellaneous			
TNFAIP2	5.0	NM_006291	Tumor necrosis factor, alpha-induced protein 2
DD1T4	2.58	NM_019058	DNA inducible transcript 4
C8orf4	2.4	NM_020130	Chromosome 8 open reading frame 4
N4BP3	2.1	AW139448	Nedd4 binding protein 3
SDC4	2	NM_002999	Syndecan 4 (amphiglycan ryudican)

The majority of those genes induced in Experiment 1 ($p<0.05$) (Table 4.1) were transcription factors and genes coding for signalling molecules. Of note, both NFκB1 (nuclear factor of kappa light polypeptide gene enhancer in B cells) and one of its inhibitors NFκBIA were induced. The NFκB1 pathway is induced in response to pro-inflammatory stimuli and this will be referred to later in the discussion. Chemokines not previously associated with the syndrome included CXCL2, CXCL1, CCL2 and CCL7. Differential expression of TNF associated proteins TNFAIP8, TNFAIP3 and TNFAIP2 provided further evidence that anti-β_2GPI activate EC through inflammatory activation pathway(s). CTH is required for amino acid biosynthesis and metabolism and its' expression was upregulated.

The data in Table 4.1 has been presented in a different format in Figure 4.1. Gene trees are tree-like structures made up of branches that visually display similarities in gene expression as a dendrogram. Smaller branches are displayed within larger ones until eventually all branches are joined within one stem. The larger the branch, the more distantly related are the genes. Condition trees are gene trees that group similar samples (conditions) together based on similar expression profiles (http://www.microarray.okstate.edu/files/Genespring-tutorial.pdf). Figure 4.1 shows the 46 genes ($p<0.05$) from Experiment 1. The software has clustered the genes according to similarities in expression (not function) on the vertical axis. Each column on the horizontal axis represents a condition or sample (antibody treated versus normal control IgG treated) and the results pertain to one Affymetrix chip. The data is shown using coded colours for differences in gene expression. There are different formats available within the Genespring software, 4.1 A is defined as heatmap where red indicates up-regulation in expression. Figure 4.1 B is defined as default interpretation where red indicates up-regulation in gene expression, blue and yellow little or no change. The dendrograms clearly differentiate between anti-β_2GPI treated and normal control IgG treated samples.



A B

Figure 4.1 Hierarchical clustering of HUVEC genes changed two-fold or more in expression ($p<0.05$, paired t-test) by treatment with anti-β_2GPI antibodies in Experiment 1. In three independent experiments with three different HUVEC donors, the anti-β_2GPI antibody preparation from Patient 5 (Table 2.2) induced significant change in expression in 43 genes when treated samples were compared to one normal control IgG (N1). Genes have been clustered according to similarities in patterns of expression (vertical axis) as well as per condition (horizontal axis). Each row represents a gene and each column represents an experimental condition. Branches are color-coded for anti-β_2GPI antibody treated (blue) and normal control IgG (green) treated samples. **A** shows the clustering in heatmap format. **B** is colored by changes in expression where red indicates up-regulation and blue indicates little or no change. No down-regulated genes reached statistical significance in this experiment.

4.2.2 Experiment 2

In three independent experiments on different HUVEC isolate populations, cells were incubated for four hours with either anti-β_2GPI antibodies (P1, P2, P3, P4) or normal control IgG (N1, N2, N3, N4). Polymixin B (50µg/ml) was added to the incubation step in all replicate experiments. In the second of the three experiments, one patient sample (P2) and one normal control IgG sample (N4) were not included in the experiment because of insufficient RNA sample. Therefore, Experiment 2 consisted of 22 Affymetrix chips in total and all were included in the analysis. Genes included in the analysis had to be present in at least 11/22 of the samples (at least half). The approach to the statistical analysis of the data for Experiment 2 is included in Chapter 2.

The genechips incorporate 22,283 oligonucleotides that are representative of 18,400 human gene transcripts. Chips were scanned and cel. data was imported into Genespring. Genes defined as Present or Marginal by Affymetrix Mas 5.0 software and present in at least 11/22 samples were selected from all genes and numbered 13,727. From these 13,727 genes, those differentially expressed and statistically significant ($p<0.05$) when grouped by treatment type were determined by using non-parametric one way ANOVA. Those genes numbered 1792. Those genes changed in expression two fold or great up or down were selected as being of biological relevance (see 4.1.8). Of those 1792 genes, (anti-β_2GPI treated versus normal control IgG treated) a total of 101 genes were statistically significant ($p<0.05$) and upregulated by at least 2 fold or more by anti-β_2GPI antibodies (Table 4.2). 14 anti-β_2GPI antibody-regulated genes were downregulated in EC 2 fold or greater ($p<0.05$) (Table 4.3).

As in Experiment 1, genes in Experiment 2 were devided manually into APS relevant functional categories: apoptosis/ anti-apoptosis, chemokines, metabolism, transcription factors/ signalling, adhesion molecules/ receptors and miscellaneous.
In Experiment 2, of particular note is the high level of induction of the chemokines of both C-C and C-X-C sub-groups. Sub-groups of chemokines are based on structural, not functional similarities. The most highly anti-β_2GPI-induced gene was CCL20 (macrophage inflammatory protein-3 alpha or MIP-3) up 27 fold in

expression compared to normal control IgG treated cells. Other chemokines included CXCL3, Fractalkine (CX3CL1), CXCL5, CXCL2 and CXCL1 (human melanoma growth stimulating factor). A number of these inflammatory chemokines not previously associated with APS are angiogenic mitogenic factors. Genes classically associated with pro-inflammatory cytokine TNFα signaling such as TNFAIP6 (up 21 fold), TNFAIP2, TNFAIP8, TNFAIP3 were up-regulated. Functional groups containing the largest numbers of genes were transcription factors/signaling molecules reflecting complex mechanisms of EC activation, and adhesion molecules (Table 4.2).

Also in Experiment 2, expression of the anti-apoptotic genes BCL2A1 (up 14 fold) and BIRC3 was increased. Induced cell receptors included Tenascin C, OLR1 (oxLDL receptor) and IL-18 receptor 1 (IL18R1). Other induced genes of interest included growth factors CSF2 (GM-CSF, up 12 fold), CSF3 (G-CSF, up 7 fold), IL-6, IL-1β IL-8 and FGF18.

Table 4.2 Anti-β_2GPI antibody-induced gene expression in HUVEC

Gene	Fold induction	Accession no.	Gene description
Apoptosis/anti-apoptosis			
BCL2A1	14.4	NM_004049	BCL2-related protein A1
TNAIP8	3.8	NM_014350	tumor necrosis factor, alpha-induced protein 8
TNFAIP3	3.6	NM_006290	tumor necrosis factor, alpha-induced protein 3
TRAF1	3.5	NM_005658	TNF receptor-associated factor
BIRC3	3.4	U37546	baculoviral IAP repeat-containing 3
CARD15	3.0	NM_022162	caspase recruitment domain family, member 15
RIPK2	2.5	AF027706	receptor-interacting serine-threonine kinase 2
TRIB3	2.3	NM_021158	tribbles homolog 3 (Drosophila)
Adhesion molecules/receptors			
TNFAIP6	21.3	NM_007115	tumor necrosis factor, alpha-induced protein 6
TNFRSF1	5.0	NM_002546	tumor necrosis factor receptor superfamily, member 11b
LLT1	4.7	NM_013269	C-type lectin domain family 2, member D
BDKRB2	4.7	NM_000623	bradykinin receptor B2
TNC	4.5	NM_002160	tenascin C (hexabrachion)
TNFRSF9	4.4	NM_001561	tumor necrosis factor receptor superfamily, member 9
OLR1	4.4	AF035776	oxidised low density lipoprotein (lectin-like) receptor 1
IL18R1	4.3	NM_003855	interleukin 18 receptor 1
E-Selectin	3.8	NM_000450	selectin E (endothelial adhesion molecule 1)
ICAM	3.4	NM_000201	intercellular adhesion molecule 1 (CD54)
ICOSL	3.1	AL355690	inducible T-cell co-stimulator ligand
CCRL2	3.0	AF015524	chemokine (C-C motif) receptor-like 2
PDZK3	2.8	AF338650	PDZ domain containing 3
CD69	2.6	L07555	CD69 antigen (p60, early T-cell activation antigen)
IFRG28	2.5	NM_022147	28kD interferon responsive protein
JAG1	2.3	U61276	jagged 1 (Alagille syndrome)
PTHLH	2.2	BC005961	parathyroid hormone-like hormone
EB13	2.2	NM_005755	Epstein-Barr virus induced gene 3
VCAM	2.2	NM_001078	vascular cell adhesion molecule 1
CD83	2.1	NM_004233	CD83 antigen (immunoglobulin superfamily)
PDGFRA	2.1	NM_006206	platelet-derived growth factor receptor, alpha polypeptide
HRH1	2.1	D28481	histamine receptor H1
IL1R1	2.1	NM_000877	interleukin 1 receptor, type I
Coagulation			
F3	3.3	NM_001993	coagulation factor III (tissue factor)
Cytokines/chemokines			
>10 fold			
CCL20	27.0	NM_004591	Macrophage inflammatory protein- MIP-3
CXCL3	14.7	NM_002090	chemokine (C-X-C motif) ligand 3
CSF2	11.6	M11734	colony stimulating factor 2 (granulocyte-macrophage)
CX3CL1	10.5	NM_002996	Fractalkine
5-10 fold			
CSF3	6.9	NM_000759	colony stimulating factor 3 (granulocyte)
IL6	6.0	NM_000600	interleukin 6 (interferon, beta 2)
CXCL5	5.6	AK026546	chemokine (C-X-C motif) ligand 5
IL1Beta	5.1	NM_000576	interleukin 1, beta
2-5 fold			
CXCL2	4.1	M57731	Chemokine (C-X-C motif) ligand 2
FGF18	4.1	NM_003862	fibroblast growth factor 18
CXCL1	4.0	NM_001511	chemokine (C-X-C motif) ligand 1
LIF	3.6	NM_002309	leukemia inhibitory factor (cholinergic differentiation factor)
CXCL10	3.3	NM_001565	chemokine (C-X-C motif) ligand 10
CXCL11	2. 3	AF030514	chemokine (C-X-C motif) ligand 11
LTB	2.3	NM_002341	lymphotoxin beta (TNF superfamily, member 3)
IL8	2.2	AF043337	interleukin 8
CCL5	2.1	NM_002985	chemokine (C-C motif) ligand 5
CCL8	2.1	AI984980	chemokine (C-C motif) ligand 8
PBEF1	2.0	BF575514	pre-B-cell colony enhancing factor 1
CSF1	2.0	M37435	colony stimulating factor 1

Metabolism

SLC7A5	4.7	AB018009	solute carrier family 7 (cationic amino acid transporter)
PPAP2B	4.2	AB000889	phosphatidic acid phosphatase type 2B
ASNS	3.5	NM_001673	asparagine synthetase
INDO	3.3	M34455	indoleamine-pyrrole 2,3 dioxygenase
GCH1	2.9	NM_000161	GTP cyclohydrolase 1 (dopa-responsive dystonia)
S100A3	2.8	NM_002960	S100 calcium binding protein A3
SDC4	2.6	NM_002999	syndecan 4 (amphiglycan, ryudocan)
MSCP	2.5	BE677761	solute carrier family 25, member 37
GFPT2	2.5	NM_005110	glutamine-fructose-6-phosphate transaminase 2
MT1X	2.3	NM_002450	metallothionein 1X
MTIE	2.3	BF217861	metallothionein 1E (functional)
PDLM4	2.3	AF153882	PDZ and LIM domain 4
LIPG	2.3	NM_006033	lipase, endothelial
KCNMB1	2.2	U61536	potassium large conductance calcium-activated channel
OASL	2.2	NM_00373	2'-5'-oligoadenylate synthetase-like

Miscellaneous

TNFAIP2	9.7	NM_006291	tumor necrosis factor, alpha-induced protein 2
DD1T4	2.8	NM_019058	DNA-damage-inducible transcript 4
IFIT3	2.5	NM_001549	interferon-induced protein with tetratricopeptide repeats 3
IFIT2	2.3	BE888744	interferon-induced protein with tetratricopeptide repeats 2
MOX2	2.2	H23979	CD200 antigen
ZC3HV1	2.2	NM_020119	zinc finger CCCH-type, antiviral 1
CDC42	2.1	AI754416	CDC42 effector protein (Rho GTPase binding) 3
MSCP	2.1	NM_018579	solute carrier family 25, member 37
AIMI	2.1	U83115	absent in melanoma 1
CHST5	2.1	N32257	carbohydrate metabolism N-acetylglucosamine metabolism
NAV3	2.0	NM_014903	neuron navigator 3
ST5	2.0	NM_005418	suppression of tumorigenicity 5
FLJ23231	2.0	NM_025079	zinc finger CCCH-type containing 12A

Transcription factors/signaling

NKX31	6.3	AF247704	transcription factor related, locus 1 (Drosophila)
SOD2	5.7	AL050388	superoxide dismutase 2, mitochondrial
CEBPD	5.6	NM_005195	CCAAT/enhancer binding protein (C/EBP), delta
HIVER2	4.4	AL023584	human immunodeficiency virus type I enhancer binding protein 2
DSCR1	3.7	NM_004414	Down syndrome critical region gene 1
RAPGEF5	3.4	NM_012294	Rap guanine nucleotide exchange factor (GEF) 5
STC2	3.2	BC000658	stanniocalcin 2
NCF4	2.9	NM_013416	neutrophil cytosolic factor 4, 40kDa
APOL3	2.7	NM_014349	apolipoprotein L3
SNFT	2.6	NM_018664	Jun dimerization protein p21SNFT
RND1	2.6	U69563	Rho family GTPase 1
MAP3K8	2.6	NM_005204	Mitogen-activated protein kinase kinase kinase 8
FOXF1	2.5	NM_001451	forkhead box F1
IRF1	2.5	NM_002198	interferon regulatory factor 1
MSC	2.4	AF060154	musculin (activated B-cell factor-1)
RGS2	2.3	NM_002923	regulator of G-protein signalling 2, 24kDa
STAT5A	2.2	NM_003152	signal transducer and activator of transcription 5A
MEOX1	2.2	NM_004527	Mesenchyme homeo box1
NFKB1	2.2	M55643	nuclear factor of kappa light polypeptide gene enhancer
ISG20	2.2	NM_002201	interferon stimulated exonuclease gene 20kDa
ABTB2	2.2	AL050374	ankyrin repeat and BTB (POZ) domain containing 2

A smaller panel of anti-β_2GPI antibody-regulated genes were down-regulated in EC 2 fold or greater (Table 4.3). None of these genes has previously been reported to be anti-β_2GPI antibody-regulated genes in EC. The majority of the 14 down-regulated genes encode signaling and transcription factors/signaling molecules. Two receptor/adhesion molecules were also down-regulated. GJA4 (connexin 37) is a gap junctional protein and OCLN (Occludin) is a structural protein of tight junctions. ID2 (inhibitor of DNA binding and differentiation) was down-regulated.

Table 4.3 Anti-β_2GPI antibody mediated down-regulation of gene expression in HUVEC

Gene	Fold reduction	Accession no.	Gene description
Adhesion molecules/receptors			
GJA4	2.9	NM_002060	gap junction protein, alpha 4, 37kDa (connexin 37)
OCLN	2.1	U53823	occludin
Cytokine/Chemokine			
BDNF	2.1	NM_001709	brain-derived neurotrophic factor
Metabolism			
GFOD1	2.0	NM_018988	glucose-fructose oxidoreductase domain containing 1
Miscellaneous			
13CDNA73	2.2	NM_023037	hypothetical protein CG003
Transcription Factors/ Signaling			
MEOX2	3.1	NM_005924	mesenchyme homeo box 2
MAF	3.0	NM_005360	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)
BMP4	2.8	D30751	bone morphogenetic protein 4
TXB1	2.8	AF012130	T-box 1
ID2	2.3	NM_002166	inhibitor of DNA binding 2
DACH1	2.2	NM_004392	dachshund homolog 1 (Drosophila)
RUNX1T1	2.2	NM_004349	runt-related transcription factor 1
MAFB	2.0	NM_005461	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B
ZNF365	2.0	NM_014951	zinc finger protein 365

As in Experiment 1, genes altered in expression 2 fold or greater ($p<0.05$) in Experiment 2 were clustered according to similarities in patterns of expression and presented in a dendrogram (Figure 4.2). The expression patterns for all 22 Affymetrix chips are represented and Genespring software has divided the samples into antibody and normal control IgG treated samples. As branches indicate, samples were further divided into the three replicate experiments for treated and untreated samples.

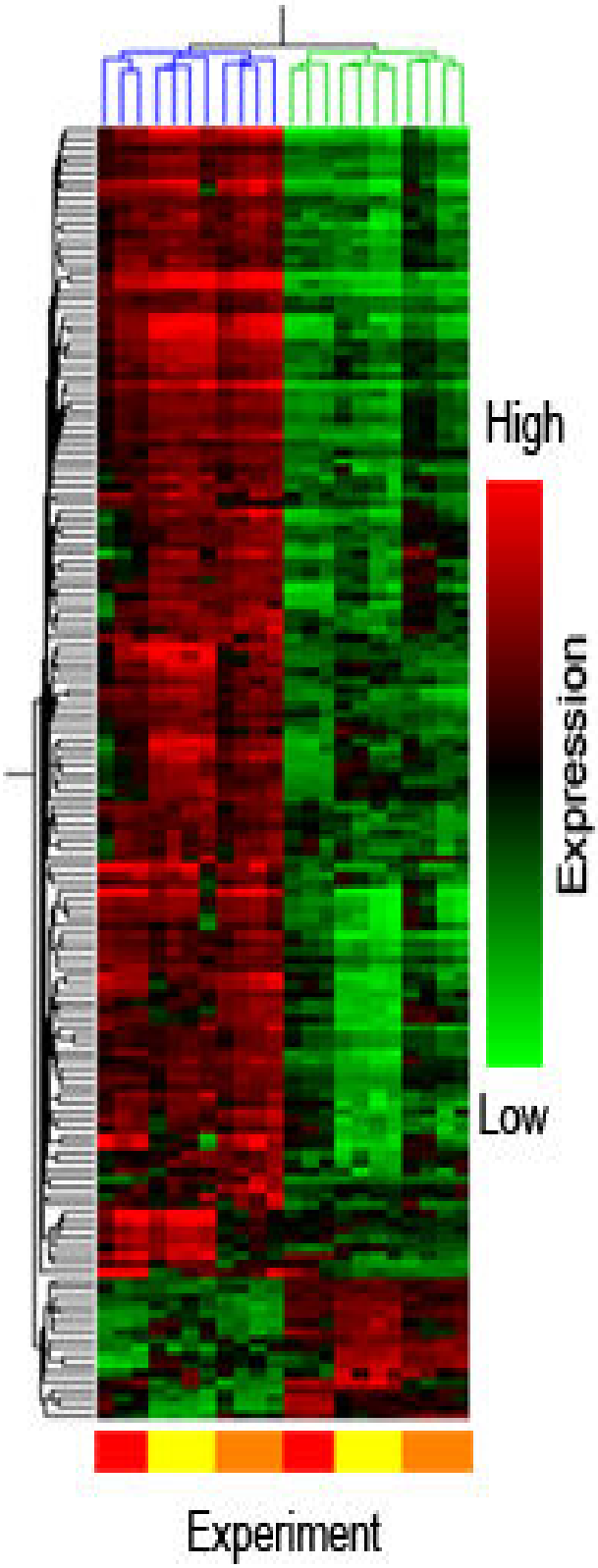


Figure 4.2 Hierarchical clustering of HUVEC genes changed two-fold or more in expression ($p<0.05$) by treatment with anti-β_2GPI antibodies. In three independent experiments on different HUVEC preparations, cells were incubated for four hours with either anti-β_2GPI antibodies (P1, P2, P3, P4) or normal control IgG (N1, N2, N3, N4). mRNA was isolated and processed for microarray hybridization and analysis. A genetree and condition tree have been created in GeneSpring software by average linkage hierarchical clustering using the Pearson Correlation. Each column represents results from an individual microarray chip (n=22), each horizontal row represents a gene. Genes have been clustered according to similarities in patterns of expression (vertical axis) as well as per condition (horizontal axis). Branches are color-coded for anti-β_2GPI antibody treated (blue) and control IgG (green) treated samples. Colored bars below figure also indicate location of results from the three independent experiments. Differences in expression level between anti-β_2GPI antibody treated HUVEC and those incubated with normal control IgG are clearly distinguishable on the heat map. Genes with high expression levels in red, intermediate level expression black and low level expression green.

4.2.3 Genespring GO-ontology functional classification

There are a number of ways to classify those genes differentially expressed in microarray analysis. For Experiments 1 and 2, genes were manually assigned to a functional category as an exercise in order to become familiar with those genes identified in the analysis. Clustering techniques group genes that share common patterns of expression together. Gene ontologies (GO) on the other hand, group genes together based on gene attributes such as biological process or function, cellular components, or molecular function for genes and their protein products (http://www.silcongenetics.com).

The 101 genes ($p<0.05$) induced in expression in Experiment 2 were also classified using the Genespring GO-ontology program. In this program, differentially expressed genes are classified according to known functions or predicted functions based on patterns in expression similar to genes of known function (Figure 4.3).

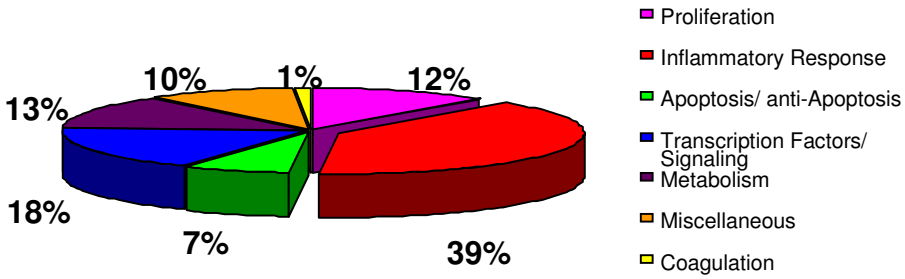


Figure 4.3 Pie Chart of 101 up-regulated genes in Experiment 2. The 101 genes up-regulated in Experiment 2 were divided into functional groups using the GO-ontology program in Genespring software. The number of genes in each functional category was given as a percentage. The largest group of genes accounted for 39% of the total and these genes were associated with an inflammatory response (seen in red). The second largest functional group of 18% of the total could be attributed to transcription factors and signaling. Intriguingly, the smallest functional group was attributed to coagulation.

This Genespring ontology program segregated the 101 2 fold or greater induced genes ($p<0.05$) into 7 functional groups: inflammatory response (39% of total), transcription factors/signalling (18%), metabolism (13%), proliferation (12%), miscellaneous (10%), apoptosis (7%), and coagulation (1%).

Table 4.4 GO ontology- genes up-regulated two fold or more with functions associated with an inflammatory response.

Gene	Fold change	
CCL20	26.95	chemokine (C-C motif) ligand 20
TNFAIP6	21.32	tumor necrosis factor, alpha-induced protein 6
CX3CL1	10.52	chemokine (C-X3-C motif) ligand 1
IL1B	5.115	interleukin 1, beta
BDKRB2	4.729	bradykinin receptor B2
IL1B	4.353	interleukin 1, beta
CXCL2	4.14	chemokine (C-X-C motif) ligand 2
CXCL1	4.0	chemokine (C-X-C motif) ligand 1
SELE	3.77	selectin E (endothelial adhesion molecule 1)
CXCL10	3.337	chemokine (C-X-C motif) ligand 10
APOL3	2.753	apolipoprotein L, 3
RIPK2	2.504	receptor-interacting serine-threonine kinase 2
CXCL11	2.333	chemokine (C-X-C motif) ligand 11
CXCL11	2.261	chemokine (C-X-C motif) ligand 11
CCL5	2.121	chemokine (C-C motif) ligand 5
HRH1	2.075	histamine receptor H1
RIPK2	2.068	receptor-interacting serine-threonine kinase 2
IL1R1	2.046	interleukin 1 receptor, type I

Table 4.5 GO ontology genes up-regulated two fold or more with function associated with cell proliferation.

Gene	Fold change	
IL6	6.0	interleukin 6 (interferon, beta 2)
IL 1B	5.1	interleukin 1, beta
TNFRSF9	4.4	tumor necrosis factor receptor superfamily, member 9
PTHLH	2.2	parathyroid hormone-like hormone
FGF18	4.1	fibroblast growth factor 18
PBEF1	2.0	pre-B-cell colony enhancing factor 1
CSF1	2.0	colony-stimulating factor (CSF-1) Human macrophage specific
CXCL10	3.3	chemokine (C-X-C motif) ligand 10
ISG20	2.2	interferon stimulated gene 20kDa
PDGFRA	2.1	platelet-derived growth factor receptor, alpha polypeptide
CSF3	6.9	colony stimulating factor 3 (granulocyte)
LIF	3.6	leukemia inhibitory factor (cholinergic differentiation factor)
CXCL1	4.0	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
JAG1	2.3	jagged 1 (Alagille syndrome)
CXCL5	5.6	chemokine (C-X-C motif) ligand 5

Table 4.6 GO ontology- genes upregulated two fold or more with function associated with chemotaxis.

Gene	Fold change	
CCL20	26.95	chemokine (C-C motif) ligand 20
CX3CL1	10.52	chemokine (C-X3-C motif) ligand 1
CXCL2	4.14	chemokine (C-X-C motif) ligand 2
CXCL1	4.105	chemokine (C-X-C motif) ligand 1
CXCL10	3.337	chemokine (C-X-C motif) ligand 10
CXCL11	2.333	chemokine (C-X-C motif) ligand 11
CCL5	2.121	chemokine (C-C motif) ligand 5

GO-ontology grouped the largest number of genes into those involved in the inflammatory response (Table 4.4). The second largest functional group included transcription factors and those genes involved in cell signaling.

Of interest, genes involved in cell proliferation were designated as a separate functional category (Table 4.5). Of the 101 genes, those involved in chemotaxis were further segregated by GO-ontology (Table 4.6). Neither of these two functional groups has been generally recognized as making a significant contribution to the pathology of PAPS or indeed APS.

4.2.4 Common genes found to be upregulated by anti-β_2GPI antibodies in Experiments 1 and 2.

Those genes common to both Experiments 1 and 2 ($p<0.05$) and changed in expression by two fold or more are listed in Table 4.7. Fold changes given are those that were found in Experiment 2. Collectively, these genes are representative of those significant genes induced in expression two fold or more in response to five independent polyclonal anti-β_2GPI antibody preparations. A total of 6 independent HUVEC donors were used in the experiments. Such biological replicates are necessary to compensate for variable expression between cells of the same type from different individuals. It has been recommended that each microarray experiment should be repeated three times (as in Experiment 2) in order to increase the reliability of data and compensate for both biological and technical variability (Leung & Calvalieri, 2003).

Importantly, VCAM-1 and ICAM-1 long recognized as aPL mediated proteins are present in the list. NF-κB is conspicuously present as the key transcription factor in inflammatory responses. Genes not previously associated with PAPS included CX3CL1, DSCR1 and RND1. Transcription factors included SOD2, CEBPD, HIVEP2, RAPGFF5 and RND1.

Table 4.7 Differentially expressed and statistically significant (p<0.05) genes common to Experiments 1 and 2 (n=22).

Fold Change/	Gene	
10.52	CX3CL1	Fractalkine
9.667	TNFAIP2	tumor necrosis factor, alpha-induced protein 2
6.323	NKX3-1	NK3 transcription factor related, locus 1 (Drosophila)
6.017	IL6	interleukin 6 (interferon, beta 2)
5.583	CEBPD	Human NF-IL6-beta protein mRNA, complete cds.
4.306	IL18R1	interleukin 18 receptor 1
4.14	CXCL2	chemokine (C-X-C motif) ligand 2
3.519	ASNS	asparagine synthetase
3.415	RAPGEF5	Rap guanine nucleotide exchange factor (GEF) 5
3.408	HIVEP2	Human DNA sequence from clone RP1-67K17 on chromosome 6q24.1-24.3.
3.339	TNFAIP3	tumor necrosis factor, alpha-induced protein 3
3.322	DSCR1	Down syndrome critical region gene 1
3.197	ICAM1	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor
2.894	PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1
2.785	DDIT4	DNA-damage-inducible transcript 4
2.692	SOD2	superoxide dismutase 2, mitochondrial
2.591	RND1	Rho family GTPase 1
2.565	SDC4	syndecan 4 (amphiglycan, ryudocan)
2.504	RIPK2	receptor-interacting serine-threonine kinase 2
2.301	TRIB3	chromosome 20 open reading frame 97
2.21	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
2.164	VCAM1	vascular cell adhesion molecule 1

All genes differentially expressed and changed two fold in expression common to Experiments 1 and 2 are listed in Table 4.8 (n=63). Statistically significant genes found in both experiments (from Table 4.7) are designated in red bold script. Statistically significant genes from Experiment 2 also changed in expression two-fold or more in Experiment 1 but not statistically significant in Experiment 1 are designated in bold blue. Black represents two fold in both, not statistically significant in either experiment.

Table 4.8 Genes common to Experiments 1 and 2 showing two-fold differential expression (n=63).

CCL20	chemokine (C-C motif) ligand 20
TNFAIP6	tumor necrosis factor, alpha-induced protein 6
CXCL3	chemokine (C-X-C motif) ligand 3
CSF2	colony stimulating factor 2 (granulocyte-macrophage)
BCL2A1	BCL2-related protein A1
TNFAIP2	tumor necrosis factor, alpha-induced protein 2
CX3CL1	chemokine (C-X3-C motif) ligand 1
SOD2	superoxide dismutase 2, mitochondrial
CXCL5	chemokine (C-X-C motif) ligand 5
IL6	interleukin 6 (interferon, beta 2)
CEBPB	AL564683 Homo sapiens FETAL LIVER Homo sapiens cDNA clone
CXCL2	chemokine (C-X-C motif) ligand 2
KIAA0146	KIAA0146 protein
CXCL1	chemokine (C-X-C motif) ligand 1
NKX3-1	NK3 transcription factor related, locus 1 (Drosophila)
IL18R1	interleukin 18 receptor 1
SELE	selectin E (endothelial adhesion molecule 1)
MGC5618	Homo sapiens hypothetical protein MGC5618
MGC4504	hypothetical protein MGC4504
CCL8	IL-8
MSC	musculin (activated B-cell factor-1)
SERPINB2	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2
TRAF1	TNF receptor-associated factor 1
CCL3	chemokine (C-C motif) ligand 3
TNFAIP3	tumor necrosis factor, alpha-induced protein 3
ICAM1	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor
BIRC3	IAP homolog C; interacts with TRAF1 and TRAF2 in yeast two hybrid system
RND1	Rho family GTPase 1
HIVEP2	Human DNA sequence from clone RP1-67K17 on chromosome 6q24.1-24.3,
F3	coagulation factor III (thromboplastin, tissue factor)
IL1B	interleukin 1, beta
TNFRSF9	tumor necrosis factor receptor superfamily, member 9
TNFAIP8	tumor necrosis factor, alpha-induced protein 8
DSCR1	Down syndrome critical region gene 1
TNFAIP3	tumor necrosis factor, alpha-induced protein 3
PLN	phospholamban
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1
RIPK2	receptor-interacting serine-threonine kinase 2
STC2	stanniocalcin 2
CCRL2	chemokine (C-C motif) receptor-like 2
RAPGEF5	Rap guanine nucleotide exchange factor (GEF) 5
SOCS3	suppressor of cytokine signaling 3
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1
SDC4	syndecan 4 (amphiglycan, ryudocan)
ASNS	asparagine synthetase
ISG20	interferon stimulated gene 20kDa
LTB	lymphotoxin beta (TNF superfamily, member 3)
PDE5A	phosphodiesterase 5A, cGMP-specific

CSF1	(CSF-1) precursor; Human macrophage-specific colony-stimulating factor
CDH19	cadherin 19, type 2
PAX4	paired box gene 4
GLRA1	glycine receptor, alpha 1 (startle disease/hyperekplexia, stiff man syndrome)
IRF1	interferon regulatory factor 1
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)
DDIT4	DNA-damage-inducible transcript 4
PDZK3	Homo sapiens PDZ domain-containing protein AIPC (AIPC)
FLJ13236	Homo sapiens mRNA; cDNA DKFZp564D042 (from clone DKFZp564D042).
CD69	CD69 antigen (p60, early T-cell activation antigen)
COL10A1	H.sapiens type X collagen gene.
TRIB3	chromosome 20 open reading frame 97
VCAM1	vascular cell adhesion molecule 1
GPR88	G-protein coupled receptor 88
IL1A	interleukin 1, alpha

It was interesting to note the large number of genes differentially induced 2 fold or greater common to both experiments (n=63). The experimental protocol for both was identical, but the statistical analysis applied each set of data was different. Genes common to both potentially previously associated with APS included IL-6, IL-8, E-selectin, ICAM-1, VCAM-1 and TF.

The genes in Table 4.7 are listed in descending magnitude of fold change. The most highly upregulated gene in either experiment was CCL20. Importantly, E-selectin is included, since it is not constitutively expressed and functions as an indicator of EC activation (Cines et al., 1998). BCL2A1 and BIRC3 each have anti-apoptotic function and both are induced by NF-κB in response to inflammatory mediators. TF (F3), the one gene with the functional signature of coagulation is also present. CD 69 is a leukocyte receptor induced on subsets of T and B cells when they are activated. It is highly expressed in leukocyte infiltrates in various chronic inflammatory diseases and has been recently shown to down-regulate autoimmune reactivity by TGF-β induction in collagen mediated RA (Sancho et al., 2003).

4.2.4 Genes different between antibody preparations

Genespring software was used to determine if there were any genes that were statistically different between antibody preparations (P1-P4). The analysis was applied to the genes that were differentially expressed and statistically significant between treated and untreated samples (n=1792). The control samples were

removed from the analysis at this point so that only the differences in antibody preparations would be identified.

Of the 1792 genes tested, the one way ANOVA identified 131 genes statistically different between the four antibody preparations ($p<0.05$). Table 4.9 lists the number of genes out of the total of 131 that were the same or different between preparations. Anti-β_2GPI from Patient 1 showed the greatest variation when compared to the other three preparations (181 in red).

Figures 4.1 and 4.2 show hierarchical cluster analysis of upregulated and downregulated genes in Experiments 1 and 2. Genes were clustered according to their patterns of expression (vertical axis) and also per condition on the horizontal axis (shows similarities between total gene expression profiles in different samples). It is important to note that in the dendrogram similarities in the level of gene expression are grouped (see branching) according to independent experiment (that is per separate HUVEC population rather than per antibody preparation. This implies that the greatest source of variation, in terms of genes regulated by anti-β_2GPI antibody, is determined by individual HUVEC populations rather than between individual anti-β_2GPI antibody preparations. Since only 131 genes were found to be statistically different between the four anti-β_2GPI, inter-experiment variation probably masked more subtle differences in the upregulation/downregulation of genes by anti-β_2GPI antibody from different patients.

Table 4.9 Genes statistically different ($p<0.05$) between the four anti-β_2GPI antibody preparations in Experiment 2 (n=131).

			Different	The same	Total
P1	P1	P2	80	51	131
	P1	P3	37	94	131
	P1	P4	64	67	131
			181		
P2	P1	P2	80	51	131
	P3	P2	52	79	131
	P4	P2	39	92	131
			171		
P3	P1	P3	37	94	131
	P2	P3	52	79	131
	P4	P3	23	108	131
			112		
P4	P1	P4	64	67	131
	P2	P4	39	92	131
	P3	P4	23	108	131
			126		

Table 4.9 shows the variation between antibody preparations (P1-P4) taking into account the number of genes common and different between preparations out of a total of 131 statistically different ($p<0.05$) between the four preparations.

4.3 Confirmation of Microarray Data by Quantitative Real-time PCR.

4.3.1 Background for qRT-PCR

Quantitative RT-PCR (reverse transcription- polymerase chain reaction, qRT-PCR) is the most sensitive method for detection and quantitation of mRNA currently available. qRT-PCR has become the method of choice for validating changes in gene expression determined in array analysis. A study by Dallas et al., (2005) indicated the strong correlation between qRT-PCR and microarray data ($r=0.89$) when identical transcripts were targeted by the two methods. (The two alternative methods used are Northern blot analysis and RNase protection assay that measure end product.) There are four different real time PCR chemistries available but SYBR Green is the simplest and most economical format for use in real time reactions and can be used for microarray validation (Bustin 2000). SYBR Green is

incorporated into double stranded DNA and when this occurs light is emitted. Fluorescence increases as the amount of PCR product (amplicon) accumulates. At the beginning of a PCR reaction DNA amplification proceeds at a constant exponential rate (amplicon should double in every cycle) and in qRT-PCR the logarithmic phase is where measurements of fluorescence are taken. Later in the cycle the rate of reaction plateaus and very little product is formed. The disadvantage of SYBR Green is that the dye can be incorporated into any dsDNA including primer dimers and any contaminating genomic DNA resulting in over-estimation of product. In order to reduce false increases in fluorescence, the qRT-PCR master mix incorporates hot start Taq polymerase which has no activity at RT. This prevents formation of double stranded products during setting up of the assay which would cause a false increase in fluorescence. C_T threshold cycle is the point where Fluorescence is first detected above background noise and the threshold should occur around the 25^{th} PCR cycle.

The 7 RNA samples used for qRT-PCR analysis were; P1 (sample 1), P2 (sample 2), P4 (sample 3), N3 (sample 4), N4 (sample 5), P5 (sample 6) and TNF (sample 7) (P1- P5, Table 2.2). cDNA was synthesized from each RNA sample. PCR using primers for β-Actin was carried out on cDNA samples after RT-PCR to ensure successful cDNA synthesis and the PCR products were run on a 1% agarose gel in parallel to 100 bp ladder in order to visualize the reaction (Figure 4.4). DNA standards for selected genes were prepared as described (Chapter 2, 2.16.1). Standards were run on a 1% gel to demonstrate fragment sized relative to the 100bp ladder (Figure 4.5).

We used the standard curve or absolute method for quantitation of mRNA. Using PCR, a DNA standard was prepared for each gene of interest using cDNA in which the target genes were expressed. All dilutions of standards were run in duplicate and tested on two different occasions. All samples were run in duplicate. β-actin copy number was determined for each sample three times and the mean was established. For each gene the copy number per µl produced in each sample was established twice (on two occasions). The mean and SD were determined for each determination. Each value was then normalized to β-actin, (divided by the copy number for β-actin). This assumes that the levels of β-actin in the cells was

constant (microarray data did not show change in expression in response to anti-β_2GPI antibody). This is relating the value of the unknown back to a constant in order to compensate for the fact that slightly different amounts of RNA may have been added in different tests or runs. Sample 5 (normal control) was arbitrarily given a fold change of 1 as a reference (in order to compare). In order to calculate fold change for the samples, the copy number/µl for the unknown was taken and divided by the copy number of the reference normal (given copy number 1). Our qRT-PCR was an independent validation using RNA from different cells than the microarray. P5 was the exception to this and cDNA was prepared form the RNA used for the microarray analysis.

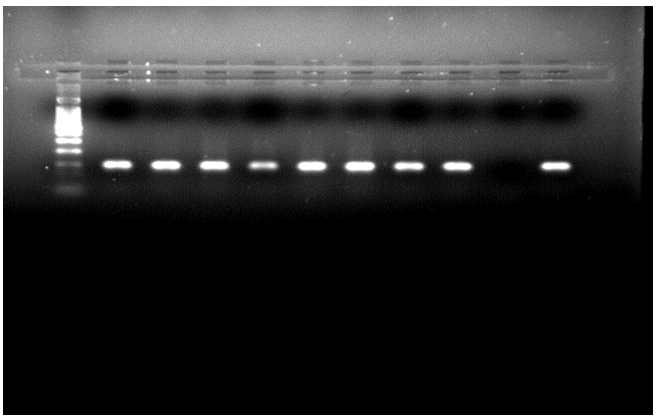
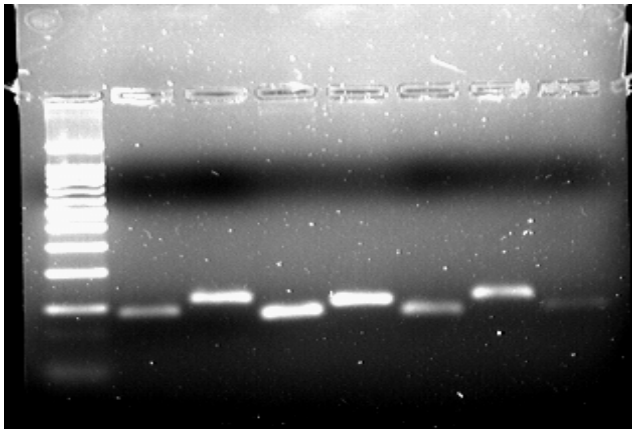


Figure 4.4 cDNA derived β-Actin PCR products. β-actin primers were used for PCR on all cDNA synthesis reactions to ensure that cDNA synthesis was successful before proceeding to PCR for gene standards. All show that the cDNA synthesis has been successful since β-actin primers produced a PCR product (100bp product). Lane 1 shows 100 base pair ladder. cDNAs are; Lanes 2 and 3 are TNF induced RNA, Lane 4 is P1, Lanes 5 and 6 are P2, Lane 7 is P3, lane 8 is P5, Lane 9 is N3, Lane 10 is the reagent blank and lane 11 is N4.



1 2 3 4 5 6 7 8

Figure 4.5 Standards for quantitative RT-PCR. Standards for quantitative RT-PCR were run on a 1% agarose gel in parallel with a 100 bp standard (Lane 1). The standards on the gel are as follows; Lane 2- SOD2 (85bp), Lane 3- FGF18 (113 bp), Lane 4 -CX3CL1 (76bp), Lane 5- ID2 (bp), Lane 6- CSF3 (75 bp), Lane 7- TNC (100 bp), and Lane 8- E-selectin (77 bp) showing faint expression due to small amount of sample applied to the gel.

The efficiencies of the PCR reactions were calculated for each gene where 100 % efficiency was doubling of PCR product in each consecutive cycle. Efficiency was calculated from the slope of the standard curve using the equation Efficiency = $[10^{(-1/slope)}]$ -1 (Bustin 2000). PCR efficiencies for all genes were between 90 and 100 % (data not shown).

Table 4.10 gives the fold changes in expression corresponding to the genes of interest, comparing microarray values and those obtained using qRT-PCR. It is not unusual for levels of fold induction to be higher for qRT-PCR than those determined in microarray analysis (Dallas et al., 2005). Quantitative PCR is able to detect larger differences in fold change due to the high sensitivity of the technology to small changes in copy number whereas microarray is restricted by the limited ability of the software to measure differences in fluorescence. However, the correlation between qRT-PCR and microarray was confirmed by Dallas et al. and found to be very strong (r=0.89) (Dallas et al., 2005).

Of those genes selected for qRT-PCR analysis, CX3CL1 was the most highly induced gene in microarray. This result was duplicated by qRT-PCR with all 4 preparations measuring the highest fold induction for CX3CL1 of those genes

tested. Anti-β_2GPI from Patient 5 appeared to induce substantially larger amounts of mRNA for CX3CL1 and E-selectin than the other 3. It would be tempting to speculate that this patient's antibodies may be more potent in their ability to activate EC as reflected in her considerable clinical history associated with PAPS (Table 2.2).Overall, P1, P2 and P3 appear to have a similar impact on transcription levels for all genes with the possible exception of P2 (CSF3 and TNC) when measured by qRT-PCR. TNF induced high levels of induction of all genes but FGF18 (Table 4.10). ID2 was down-regulated 2.3 fold by microarray analysis and this was very similar to the level of down-regulation by qRT-PCR (Table 4.1 and Fig. 4.7b). ID2 was down-regulated by three of the four anti-β_2GPI (Table 4.10) (Raw real time PCR data is found in the Appendix 5.0).

Table 4.10 qRT-PCR derived fold changes for genes of interest compared to values in microarray in response to patient derived anti-β_2GPI, normal control IgG and TNF positive control.

Gene	Exp. 2, fold change in Micro-array	P1 *	P2 *	P4 *	P5 *	N 3 *	N 4 *	TNF *
CX3CL1	10.5	86	97	93	255	3	1.0	9940
CSF3	6.9	15	5	15	6	2	1.0	21
SOD2	5.7	29	22	18	11	1.9	1.0	412
E-selectin	3.8	28	23	29	68	.3	1.0	852
FGF18	4.1	7	8	4	3	.74	1.0	1.0
TNC	4.5	7	15	7	2	1.0	1.0	16.0
ID2	2.3 ↓	1.04	2.6 ↓	2.6 ↓	3.1 ↓	1.14	1.0	1.2

* Fold change in qRT-PCR in response to four anti-β_2GPI.

The microarray data was confirmed by qRT-PCR for all genes tested (Figure 4.7). In summary, qRT-PCR analysis was carried out for selected novel anti-β_2GPI antibody regulated genes, covering a range of different fold levels of regulation. Genes included in this analysis were; CSF3, CX3CL1, FGF18, SOD2 and Tenascin C plus E-selectin as a positive control gene. We also included the down-

regulated gene ID2 in these experiments. All six upregulated genes (CSF3, CX3CL1, E-Selectin, FGF18, Tenascin C and SOD2) were also found to be upregulated by RT-PCR analysis. Levels of fold upregulation were variable but highest fold upregulation was found for CX3CL1 which was the highest fold upregulated gene of the 6 selected genes by microarray analysis (Figure 4.6a). ID2 was downregulated 2.3 fold by microarray analysis and this was very similar to the level of downregulation by real-time PCR analysis (Figure 4.6b).

In addition, increased mRNA levels of E-selectin and IL-8 following anti-β_2GPI antibody treatment detected by microarray analysis were consistent with increased protein levels following antibody treatment as measured by ELISA (Figures 3.11 and 3.12).

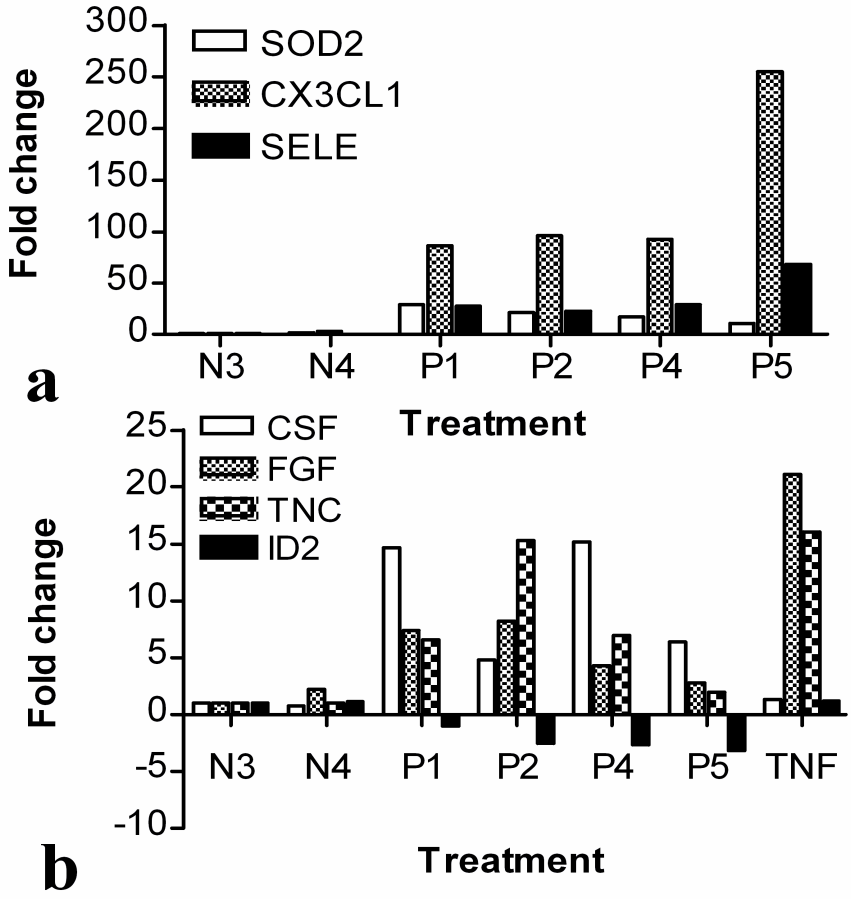


Figure 4.6 Anti-β2GPI antibodies induce HUVEC activation. Cells were incubated with either control normal IgG (N3, N4) or different anti-β2GPI antibody preparations (P1, P2, P4, P5), or TNFα for four hours and total RNA isolated and processed for real-time PCR analysis. Antibody preparations N3, N4, P1, P2 and P4 were previously used in the microarray experiments. Gene expression levels were normalised to the β-actin mRNA level. The results show fold change in expression level relative to control normal IgG (N3) level and represent the mean of duplicate samples from 2 independent experiments. (a) shows data for SOD2, CX3CL1 and E-selectin. TNFα induced expression of these genes but fold induction levels were off scale and omitted from the figure, (b) shows data for CSF, FGF, Tenascin C (TNC) and ID2. TNFα-regulated changes in levels of expression are included for comparison.

4.4 Preliminary Data- Anti-β_2GPI effect on DNA synthesis in HUVEC

Since a number of genes identified in this study were angiogenic, it was decided to see if anti-β_2GPI had a proliferative effect on HUVEC. Proliferation assays were performed in two ways, propidium iodide staining followed by FACS analysis to determine the proportion of cells in different stages of the cell cycle (Figure 4.7) and incorporation of ^{3}H-thymidine (Figure 4.8). The propidium iodide experiment was done twice and the ^{3}H-thymidine incorporation experiment was done once. Approximately 6% more of the total cells were detected in S and G_2 when cells were incubated with anti-β_2GPI compared to normal control IgG. Results were consistent for the three experiments performed. Preliminary data suggested that anti-β_2GPI may stimulate DNA synthesis in EC when compared to normal control IgG and AECA control antibody. Results were not statistically significant but suggest a trend towards an increase in anti-β_2GPI treated cells. Further experiments are needed to determine if anti-β_2GPI does indeed significantly induce EC proliferation *in vitro.*

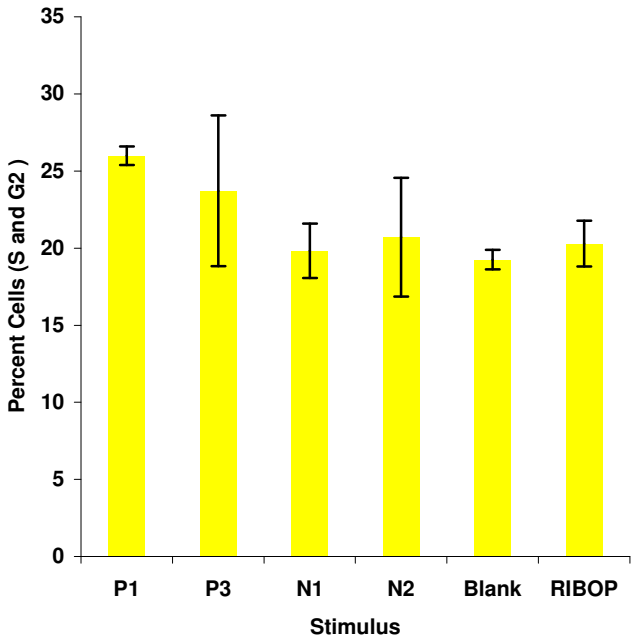


Figure 4.7 Propidium iodide staining and FACS analysis of HUVEC after incubation with anti-β_2GPI. Passage 3 HUVEC were grown to 70-80% confluence in complete media and incubated for 18 hours (37°C with 5% CO_2) with anti-β_2GPI from P1, P3, N1 and N2 as well as AECA control antibody anti-Ribosomal P (all at 50µg/ml). Cells were trypsinized and

stained with propidium iodide (50µg/ml) followed by FACS analysis. Percent of total HUVEC between S and G_2 in cell cycle gated as M2 and M3 combined (M2+M3) are shown in Figure 4.8. Data from two experiments was combined, each column represents the mean of four determinations with error bars for SEM.

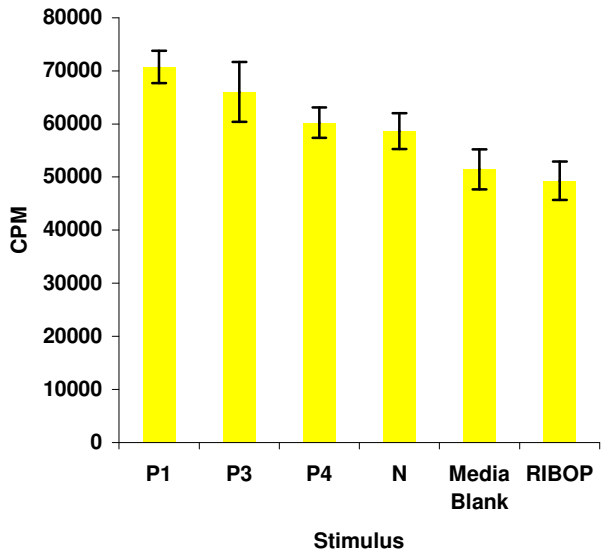


Figure 4.8 Incorporation of ^{3}H-thymidine in HUVEC in the presence of anti-β_2GPI. Passage 3 HUVEC were grown to 70-80% confluence in complete media and incubated for 18 hours (37°C with 5% CO_2) with anti-β_2GPI from P1, P3, P4 and N as well as control antibody anti-Ribosomal P (all at 50µg/ml). Each sample was tested in quadruplicate and the results represent the mean of four values (CPM). N is combined data for 2 normal control IgG (N1 and N2). Error bars represent SEM. The experiment was done once.

4.5 Discussion

Experiment 1 and 2 used the same experimental design and protocol, the difference being experiment 1 tested the effect of affinity purified polyclonal anti-β_2GPI from 1 patient and 1 normal control IgG on global gene expression in HUVEC. Experiment 2 tested and compared anti-β_2GPI induced expression in HUVEC in response to four additional affinity purified polyclonal anti-β_2GPI and four normal control IgG preparations. In total, 5 anti-β_2GPI preparations were tested and HUVEC from 6 different donors were used in the experiments.

In Experiment 2, following gene chip hybridization and scanning, HUVEC were found to express 13,727 out of 18,400 transcripts (approximately 58% of genes in the array). This observation was consistent across all samples in the three separate experiments within Experiment 2 and arrays in Experiment 1. This observation is important if gene expression between biological replicates is to be meaningful. The number of genes expressed in a cell type should be consistent. Genes that were significantly changed ($p<0.05$, up or down) two fold or more were filtered and categorized as anti-β_2GPI antibody-regulated genes. Importantly, the majority of genes represent those not previously reported in association with anti-β_2GPI.

Genes were divided into APS relevant categories; apoptosis/ anti-apoptosis, chemokines, metabolism, transcription factors/ signalling, adhesion molecules/ receptors and miscellaneous and classified using the information provided by Affymetrix annotation on line (www.Affymetrix.com). Gene tables within the chapter replace the Affymetrix accession numbers with the corresponding identification number assigned to each gene by the NCBI.

Experiment 1 confirmed the anti-β_2GPI induced expression of ICAM-1 and VCAM-1 in response to anti-β_2GPI using microarray technology supporting the findings other *in vitro* studies (Simantov et al., 1995; Del Papa et al., 1997; Pierangeli et al., 1999). Up-regulated chemokines and cytokines included CXCL2, Fractalkine (CX3CL1), CCL7 and IL-6. Genes that were induced two fold or greater in expression, previously associated with APS, but that did not pass the stringency of the paired t-test included E-selectin, TF and IL-8. A total of 231

genes showed differential expression of two fold or more in Experiment 1. Expression of vascular endothelial cell growth factor (VEGF) was statistically significant and induced 3 fold. VEGF is one of the most potent inducers of EC proliferation and angiogenesis (Ferrara & vis-Smyth 1997). Upregulation of this gene in response to anti-β_2GPI was of interest because VEGF was found to be present in increased concentrations in the plasma of APS patients (Williams et al., 2000). A recent paper by Cuadrado et al. (2006) showed that monocytes from PAPS patients express more VEGF and VEGF receptor flt-1 when compared to normal control monocytes. IgG from PAPS patients induced monocyte VEGF expression in a dose dependent fashion through the p38MAPK activation pathway (Cuadrado et al., 2006).

The majority of genes common to both experiments 1 and 2 ($p<0.05$, $n=25$) were transcription factors and signalling molecules (Table 4.7). Recent studies have identified several important signaling cascades that may be activated in response to anti-β_2GPI binding. They are the NF-κB pathway (Dunoyer-Geindre et al., 2002) and MyD88 signaling through TLR4 followed by subsequent NF-κB pathway activation (Raschi et al., 2003a). The p38 MAPK pathway has been implicated in the regulation of TF in EC and monocytes and may be activated in response to anti-β_2GPI (Branger et al., 2003; Bohgaki et al., 2004). NF-κB was up in Exp. 2 ($p<0.05$) and Exp. 1(2 fold criteria only). Transcription factor SOD2 was induced ($p<0.05$) in both experiments and it is known to be activated, at least in part, through NF-κB via the Protein Kinase C pathway (Rakkola, Matikainen & Nyman, 2007). RIPK2 (a serine threonine kinase) was up in both experiments ($p<0.05$) and is known to be a potent activator of NF-κB as well as an inducer of apoptosis (Munz et al., 2002).

Of novel interest is the induction of Down Syndrome Critical Region 1 (DSCR1) ($p<0.05$) in both Experiments 1 and 2. The VEGF-regulated expression of DSCR1 expression has recently been described (Hesser et al., 2004). Increased expression of VEGF was statistically significant in Experiment 1. VEGF was also induced in Experiment 2 but did not meet the two-fold up selection criteria. DSCR1 regulates expression of inflammatory markers on EC by blocking calcineurin (CnA)

signaling which in turn blocks the de-phosphorylation of nuclear factor of activated T cell transcription factor. Expression of DSCR1 reduces the expression of TF, E-selectin and COX-2 and may provide a negative feedback loop to reduce expression of inflammatory genes following EC activation. Other CnA target genes include IL-8 and GM-CSF (Hesser et al., 2004).

Rho GTPase 1 was increased in both experiments ($p<0.05$). It is one of a family of 23 signaling proteins that control the polymerization, branching and bundling of actin filaments in the cell cytoskeleton. This in turn directs cellular processes of motility and phagocytosis and Rho GTPase binding (Jaffe & Hall 2005). Induction of Rho GTPase results in the activation of the MAPK signaling pathway and a number of transcription factors. In turn, this triggers production of pro-inflammatory cytokines (Jaffe & Hall 2005). Increased expression of this enzyme may contribute to changes in the EC membrane, increased vascular permeability and increased targeting of inflammatory cells.

CCL20 (macrophage inflammatory protein-3α or MIP-3α) was the most highly induced gene in Experiment 2, up 27 fold in expression. Its function is thought to involve the recruitment of memory T cells and dendritic cells to sites of inflammation in diseases such as dermatitis and arthritis. CCL20 is highly expressed in colonic tissue from patients with inflammatory bowel disease and evidence suggests that it acts as an antimicrobial peptide (Yang et al., 2003). Its expression can be strongly induced by proinflammatory cytokines such as TNF-α, IL-1β or *Escherichia coli* LPS. This chemokine is a specific ligand for the CCR6 chemokine receptor expressed by most B cells, subsets of T cells and dendritic cells (Perez-Canadillas et al., 2001). Its production has been associated with a number of different epithelial cells types, (inflammation of intestinal epithelial cells), and fibroblasts but interestingly, not EC. CCL20 induced VEGF expression in human gingival fibroblasts (Hosokawa et al., 2005). EC induced anti-β_2GPI expression of CCL20 may be partially responsible for perpetuating the recruitment of cells involved in the autoimmune response to the EC surface.

Fractalkine (CX3CL1) is the only CX3 chemokine to have been described in the literature and its expression was upregulated 10.5 fold in response to anti-β_2GPI. CX3CL1 acts as an adhesion molecule and chemoattractant and is expressed on endothelial cells that have been activated by inflammatory cytokines such as TNF-α. Its receptor, CX3CR1 is expressed on vascular smooth muscle cells (SMC), natural killer cells (NK), and macrophages. Soluble CX3CL1 causes migration of these cells while the membrane bound protein binds cells bearing CX3CR1 with high affinity, even under shear stress. CX3CL1 allows adhering leukocytes to bypass the integrins ICAM-1 and VCAM-1, potentially facilitating the extravasation of circulating leukocytes (Umehara et al., 2004). Interestingly, CX3CL1 has been shown to enhance the integrin-dependent adhesion and transmigration of CX3CR1+ monocytes. In addition, samples from human coronary atherosclerotic plaques contain CX3CL1 mainly expressed by mononuclear cells (differentiating monocytes). CX3CL1 has been also been shown to mediate chemotaxis of vascular smooth muscle cells *in vitro* via its receptor CX3CR1 (Lucas et al., 2003).

Surprisingly, but perhaps reassuringly, TF is the only gene directly involved in coagulation that has been shown to be upregulated in this study (Table 4.1). This result implicitly reinforces the longstanding belief that TF is a major contributing factor to thrombosis in APS. TF was the only gene directly involved in coagulation found to be upregulated ($p<0.05$) in Experiment 2. Numerous studies have reported increased TF expression on monocytes and EC in the presence of aPL (anti-β_2GPI) and this has been considered to be directly relevant to disease pathology in APS (Branch et al., 1993; Cuadrado et al., 1997; Kornberg et al., 1997). Indeed PAPS patients with thrombosis express increased TF on their monocytes (Dobado-Berrios et al., 1999). Plasma levels of TF are increased in patients with APS (Cuadrado et al., 1997; Amengual et al., 1998). A recent study by Szotowski *et al.* (2005) has identified a soluble isoform of alternatively spliced human (asHTF) responsible for procoagulant activity in cell supernatants which is released from EC in response to TNFα and IL-6 (Szotowski et al., 2005).

As in Experiment 1, the list of up-regulated genes includes some other previously identified anti-β_2GPI-induced genes such as E-selectin, ICAM-1, and VCAM-1 but

the majority of the genes we have identified represent anti-β_2GPI-induced genes not previously reported.

Adhesion molecules and receptors common to Experiments 1 and 2, ($p<0.05$) included ICAM-1, VCAM-1 and IL18R1. Chemokines included CX3CL1, CXCL1 and 2. A number of inflammatory genes activated by NF-κB pathway included IL-1β, TNF-α, IL-6, and IL-8 (Tak & Firestein 2001). A total of 63 genes were changed two fold or greater in expression and common to both Experiments 1 and 2 (Table 4.8).

While the GO-ontology program in Genespring designated a number of anti-β_2GPI-regulated genes a proliferative function, a literature search for the association of aPL with vascular EC and myeloid cell lines yields very few papers. Isolated case reports present evidence of mitogenic activity in smooth muscle cells, fibroblasts and EC as part of the pathology in APS associated hypertension and intra-renal vascular disease (Durand et al., 1994). A woman with SLE and aPL presented with reactive angioendotheliomatosis (RAE) and histopathology of a skin lesion revealed marked expansion of the dermal microvasculature with intravascular EC proliferation and thrombosis (Creamer, Black, & Calonje, 2000). The possible contribution of selected genes identified in this study to cellular proliferation in PAPS is further addressed in the general discussion. GO-ontolgy was used to further discriminate those genes involved in chemotaxis of leukocytes (Table 4.5).

qRT-PCR analysis was carried out for selected novel anti-β_2GPI antibody regulated genes, covering a range of different fold levels of regulation. Genes included in this analysis were; CSF3, CX3CL1, FGF18, SOD2 and Tenascin C plus E-selectin as a positive control gene. The down-regulated gene ID2 was also incuded in these experiments. The results of these experiments are shown in Figure 2. All six up-regulated genes (CSF3, CX3CL1, E-Selectin, FGF18, Tenascin C and SOD2) were also found to be upregulated by qRT-PCR. Levels of fold upregulation were variable, but highest fold upregulation was found for CX3CL1 that was the highest fold upregulated gene of the 6 selected genes selected for

microarray analysis (Figure 4.5a). Levels of fold induction for microarray Experiment 2 and corresponding qRT-PCR fold changes are given in Tables 4.6 and 4.7). In experiments 1 and 2 the largest group of up-regulated genes were transcription factors, and those genes involved in cell signaling. Ideally, our experimental protocol would have incorporated different time points to look at changes in gene expression over time. When a ligand makes contact with its receptor, the resulting signal can be amplified many times and a number of different and/or interacting signaling pathways may be activated. We chose the four hour time point to look at early gene expression in HUVEC in response to anti-β_2GPI. This was early gene expression in the sense that *in vivo,* the EC are continually exposed to circulating anti-β_2GPI. By looking at the four hour time point, activation of numerous genes would have already occurred and declined resulting in a number of autocrine mechanisms of gene regulation. Of interest, Simantov et al. (1995) reported a 4 hour time lag before increased evidence of monocyte adhesion in the presence of aPL (APS IgG). In conclusion, our qRT-PCR analysis confirmed those changes in gene expression identified by microarray analysis.

Combined results of Experiments 1 and 2 identified a number of novel anti-β_2GPI induced genes. Of particular interest were high levels of chemokines and growth factors that could potentially contribute to the diverse spectrum of clinical phenomenon and systemic effects seen in APS and PAPS. Upregulation of large numbers of transcription factors and signaling molecules indicate a complex activation mechanism in EC in response to these antibodies. Many of these may contribute to further autocrine meachanisms of activation and regulatory mechanisms.

In this study, microarray results revealed a transcription profile largely predominated by pro-inflammatory genes. Past opinion has defined the thrombosis in PAPS as being typified by non-inflammatory lesions (Lie et al., 1996) and such a description has been included in the diagnostic criteria of the disease (Wilson et al., 1999). Reassuringly, a number of genes previously associated with APS were identified and TF upregulation was confirmed. It is interesting to note that results

of another microarray experiment designed to identify differential PBMC genomic markers in APS patients with and without thrombosis in association with aPL did not in fact identify genes involved in coagulation (Potti et al., 2006). This may imply that the pro-inflammatory genes induced by anti-β_2GPI are in fact responsible for the development of the pro-thrombotic EC phenotype.

In conclusion, combined results of Experiments 1 and 2 identified a number of novel anti-β_2GPI induced genes. Of particular interest were high levels of chemokines and growth factors that could potentially contribute to the diverse spectrum of clinical phenomenon and systemic effects seen in APS and PAPS. Upregulation of large numbers of transcription factors and signaling molecules indicate a complex activation mechanism in EC in response to these antibodies. Many of these may contribute to further autocrine mechanisms of activation and regulatory mechanisms.

Chapter 5

General Discussion

5.1 Introduction

The most striking feature of this study was the extent and diversity of anti-β_2GPI antibody regulated genes in EC. The results revealed induction of a complex pro-inflammatory as well as a pro-adhesive and pro-coagulant milieu by these antibodies, which potentially could be responsible, at least in part, for inducing and/or sustaining the vasculopathy of the syndrome. A number of angiogenic chemokines not previously associated with the syndrome were differentially expressed as well as several growth factors necessary for recruitment and proliferation of a number of cells involved in the inflammatory response. The relatively large number of anti-β_2GPI antibody regulated genes identified also suggested activation of a signaling transduction cascade(s) as well as autocrine routes of activation following anti-β_2GPI antibody binding to HUVEC. The possible contributions that some of the most highly upregulated genes may make to the pathology of PAPS are discussed in this chapter.

5.2 Adhesion molecules and inflammation

The endothelium normally acts as a selective barrier between blood and underlying tissue. It maintains blood flow, vascular integrity and supports normal hemostasis. However, the endothelium can be damaged by substances in the blood or physical stresses which result in changes in EC function. EC activation may be the result of turbulent blood flow, nitric oxide synthase (NOS), induced expression of inflammatory cytokines such as IL-1β and TNFα or infection. The first *in vivo* evidence of EC activation is the upregulation of selectins (P-selectin and E-selectin in that order) and integrins (ICAM-1 and VCAM-1) on activated EC (Cines et al., 1998).

In vivo, expression of these molecules enables the adhesion of leukocytes to the EC surface. After EC activation, newly expressed E-selectin loosely binds neutrophils as they roll along the endothelium carried by the flowing blood. The subsequent expression of ICAM-1 in combination with chemokine IL-8 on the extracellular matrix results in their firm adhesion. In addition, IL-8 activates surface receptors LFA-1 and Mac-1 on the neutrophil surface, and their expression results in firm

adhesion (tethering) to the EC surface. The chemokine IL-8 was upregulated 2 fold in Experiment 2 (Table 4.4). The recruitment and activation of monocytes succeeds that of neutrophils. In the presence of inflammatory cytokines such as TNF-α and IL1-β, monocytes migrate through the endothelium into the intima of the vessel wall where they differentiate into macrophages. This study has confirmed previously reported anti-β_2GPI mediated upregulation of adhesion molecules E-selectin, ICAM-1 and VCAM-1 using microarray (Simantov et al., 1995; George et al., 1998a). It is possible that persistent exposure of the endothelium to anti-β_2GPI *in vivo* may result in the upregulation of adhesion molecules, subsequent recruitment and differentiation of monocytes into macrophages and chronic inflammation. This ongoing process may contribute to the proadhesive and prothrombotic EC phenotype associated with PAPS.

5.3 Accelerated atherosclerosis

Roughly one third of PAPS patients have atherosclerosis and a direct association of aPL with the pathogenesis of accelerated atherosclerosis in APS patients has been reported (Pierangeli et al., 2000; George, Harats & Shoenfeld, 2001). Immunoperoxidase staining has identified high concentrations of β2-GPI localised within atherosclerotic plaques, especially in subendothelial regions (George et al., 1998b). It has been suggested that anti-β_2GPI could contribute to the accelerated arterial disease seen in PAPS patients by stimulating upregulation of leukocyte adhesion molecules (Simantov et al., 1995; George et al., 1998a). Other cytokines and adhesion molecules found to be upregulated by anti-β_2GPI antibodies may also have a role in the development of atherosclerosis. Monocytes have been shown to strongly express IL-18 in atheromatous lesions *in situ* (Mallat et al., 2001) and EC expression of IL-18R was increased 4.3 fold in this study. Gerdes *et al*. suggested an IL-18 mediated paracrine proinflammatory pathway involving monocytes, EC and smooth muscle cells in association with atherogenesis (Gerdes et al., 2002). CX3CL1 and its receptor CX3CR1 are expressed in the atherosclerotic lesions of humans and mice (Lucas et al., 2003) and in CX3CL1-deficient mice there is a major reduction of atherosclerosis (Teupser et al., 2004).

Clot formation and atherogenesis involve the sustained recruitment of mononuclear cells to the endothelial surface. Atherosclerotic lesion formation is initiated by the adherence of monocytes to adhesion molecules on the surface of activated EC. These monocytes differentiate into scavenger receptor bearing macrophages that absorb oxidized low density lipoprotein (oxLDL) forming foam cells characteristic of the lesions. These cells migrate into the sub-endothelium and contribute to plaque formation. Anti-β_2GPI bind to β_2GPI-oxLDL complexes and have been shown *in vitro* to enhance oxLDL uptake into monocytes/macrophages potentially accelerating the lesion formation (Hasunuma et al., 1997). ACL were reported to cross-react with anti-oxLDL antibodies and Vaarala and co-workers suggested that there may be a connection between this finding and the accelerated arterial disease observed in APS patients (Vaarala et al., 1993). It has been reported recently that β_2-GPI inhibits LDL oxidation and cholesterol accumulation by macrophages *in vitro* and may therefore confer a protective effect by preventing the uptake of cholesterol (Hasunuma et al., 1997). If β_2GPI confers a protective effect against atherosclerosis, antibodies to β_2GPI may indeed interfere with this protective process.

OxLDL is a risk factor for the development and progression of atherosclerosis. Initially it impairs endothelial relaxation (vasodilation) by reducing the expression of NOS and induces generation of reactive oxygen species (Morawietz et al., 2001). It also increases adhesion molecule expression and endothelial apoptosis (Morawietz et al., 2001). Expression of oxLDL receptor OLR1 was upregulated over four fold in this study (Table 4.1). Human OLR1 gene encodes the endothelium derived lectin-like oxidized low density lipoprotein receptor involved in the binding, internalisation and proteolytic degradation of oxLDL suggesting that it may play a significant role in atherogenesis and acute myocardial infarction (Kume et al., 1998; Mango et al., 2003). OLR1 expression is induced *in vitro* by inflammatory cytokines such as TNF-α and *in vivo* by pro-atherogenic conditions like hypertension, hyperlipidaemia, and diabetes mellitus (Kume et al., 1998). It acts as a mediator of endothelial dysfunction favouring superoxide generation, inhibiting nitric oxide production, and enhancing endothelial adhesiveness for monocytes. An increase in OLR1 expression could result in the increase in binding

of oxLDL and subsequent foam cell development and migration and would clearly contribute to atherosclerosis.

Endothelin-1 (ET-1) is increased in cardiovascular diseases and increased levels are found in the plasma of patients with APS (Atsumi et al., 1998). ET-1 is a potent vaso-constrictor, expression is induced by aPL and ET-1 induces expression of OLR-1 (LOX-1) in EC (Morawietz et al., 2001). One downregulated gene GJA4 (connexin 37) is an EC specific gap junction protein and interestingly polymorphisms in this protein have been associated with the development of arteriosclerotic plaques in human subjects. One allelic form of the gene (codon 1019, C to T substitution) is significantly associated with smooth muscle cell proliferation and thickening of the carotid intima (Boerma et al., 1999). It has been proposed that GJA4 polymorphism may provide a link between chronic inflammation and development of atherosclerosis. Down-regulation of the gene in response to anti-β_2GPI may mirror this effect although the mechanism is unclear (Boerma et al., 1999).

5.4 Proliferation

The GO-ontology program in Genespring classified a number of anti-β2GPI regulated genes into the functional category of proliferation**.** A number of chemokines such as IL-8 and growth factors such as FGF18 (fibroblast growth factor) and VEGF were upregulated by anti-β_2GPI antibodies in the present study and may contribute to the hyperplasia associated with PAPS. APS is associated with EC proliferation and fibrosis characterized by intimal hyperplasia within the lumen of micro-capillaries typically within the kidney (Frampton et al., 1991). It is also associated with cardiac lesions involving thickening of heart valves with deposition of aPL in the subendothelial layers (Nesher et al., 1997). Histologic examination of renal biopsies from 16 patients with PAPs showed small vessel vaso-occlusive lesions associated with myofibroblastic intimal cellular proliferation and thrombosis, five patients showed endothelialized channels indicating recanalizing thrombosis and EC proliferation (Nochy et al., 1999). In addition to intrarenal arteries and arterioles, intimal hyperplasia has been noted in PAPS patients in lung, skin and brain (Amigo & Garcia-Torres 2000).

Chemokines induced by anti-β_2GPI in this study included CCL20, CXCL3, CX3CL1 (fractalkine), CXCL5, CXCL2 and CXCL1. All are involved in recruitment, chemotaxis and proliferation of mononuclear cells and/or granulocytes. These findings are consistent with a number of *in vitro* and *in vivo* studies reporting that anti-β_2GPI antibodies increased monocyte adhesion to EC (Simantov et al., 1995; Del Papa et al., 1995; Pierangeli et al., 1999). Moreover, placental biopsies from APS patients had a higher concentration of inflammatory cells particularly macrophages (Stone et al., 2006) and an association has been found between neutrophil recruitment and fetal loss in APS (Girardi et al., 2003). Other growth related genes of interest with increased expression included growth factors CSF2 (GM-CSF, up 12 fold) and CSF3 (G-CSF, up 7 fold). These factors may also contribute to proliferation, concentration and homing of white blood cells (Barreda, Hanington & Belosevic, 2004).

5.5 Angiogenesis

A number of genes identified in this study are associated with angiogenesis and this process involves the proliferation of vascular EC. Development of new vessels (neovascularization) may contribute to plaque formation and plaque growth. It is more common at sites where there has been infiltration of inflammatory cells such as macrophages and lymphocytes (Khurana et al., 2005). Angiogenesis is evident in renal lesions in APS patients. This is associated with renal artery lesions, stenosis, thrombi, microagiopathy and hypercellularity of EC and mononuclear cells. Intriguingly all five of the CXC chemokines upregulated in response to anti-β_2GPI in this study are proangiogenic in function (CXCL3, CXCL2, CXCL1, CXCL5, CXCL8) (Streiter et al., 2005).

VEGF is the most potent stimulator of angiogenesis. VEGF acts in combination with G-CSF to mobilize EC progenitor cells from the bone marrow (Ferrara et al., 1997). Recently described, DSCR1 is a calcineurin inhibitor and its expression is induced by VEGF (Hesser et al., 2004). Induced genes with inhibitory functions

are of interest because they may represent endogenous feedback mechanisms that shut down the inflammatory response. DSCR1 is an example of one such gene.

5.6 Signaling Pathways

Regulation of many of the anti-β_2GPI antibody induced genes occurs through NF-κB signaling and this pathway has been shown previously to be involved in anti-β_2GPI antibody mediated effects in EC (Dunoyer-Geindre et al., 2002). Of note, both NFκB1 and one of its inhibitors NFκBIA were induced in this study. To date the mechanisms of signal transduction upstream of NFκB1 not been characterized. Anti-β_2GPI - induced translocation of NF-κB to the cell nucleus results in a pro-inflammatory and pro-thrombotic EC phenotype similar to that seen in response to LPS and pro-inflammatory cytokines IL1-β and TNF-α. This observation is supported by the large number of pro-inflammatory genes upregulated in this study. LPS, IL1-β and TNF-α induced activation pathways are mediated by the TNF-receptor associated factor (TRAF) adapter family (Meroni et al., 2000). LPS and IL-1 interact with toll-like receptor 4 (TLR4) and IL1 receptor (IL-1R) respectively and both of these receptors share a common cytoplasmic signaling domain, the toll/IL-1R receptor domain (TIR).

Investigations by Raschi et al. (2003a) indicated that anti-β_2GPI - mediated EC activation potentially involves a member of the TLR/IL-1 receptor family and activation occurs through a mechanism different from that induced by TNF-α (Raschi et al., 2003). Their group made the association between the TLR/myeloid differentiation protein (MyD88) dependent signaling pathway and anti-β_2GPI-dependent NF-κB induced gene expression. Dominant negatives of MYD88 and TRAF6 completely abrogated anti-β_2GPI–induced NF-κB activation in a microvascular EC line (Raschi et al., 2003a).

Thrombin is a serine protease whose functions include involvement in coagulation, cell proliferation and inflammation. Thrombin acts through a specific thrombin receptor on the EC surface. A recent microarray study has identified a number of thrombin-induced genes in HUVEC. These included ICAM-1, IL-8, CXCL3,

CXCL1, and BIRC3. VCAM-1, CXCL2, CCL20, CSF2 and CD69 were up-regulated but did not reach statistical significance in that study (Okada et al., 2006). Interestingly, all of these genes were induced by anti-β_2GPI in this study. CCL20 was one of the more highly induced genes in response to thrombin. CCL20 (macrophage inflammatory protein-3α or MIP-3α) was the most highly induced gene in Experiment 2: up 27 fold in expression in this study. Since the expression of CCL20 can be strongly induced by pro-inflammatory cytokines such as TNF-α, IL-1β or *Escherichia coli* LPS (Perez-Canadillas et al., 2001), this observation indicates some common elements of activation in EC in response to a number of pro-inflammatory mediators and these should include anti-β_2GPI antibodies.

However, the anti-β_2GPI antibody induced gene panel described in this study is largely distinct to those described in gene profiling studies on HUVEC with different cytokines or LPS (Zhao et al., 2001; Mayer et al., 2004). Nonetheless, a small subset of anti-β_2GPI antibody-induced genes (for example, E-selectin, IL-8, VCAM-1), have been shown to be induced by cytokine and/or LPS-induced gene profiling of HUVEC (Zhao et al., 2001; Mayer et al., 2004). Genes induced by LPS include plasminogen activator inhibitor and monocyte chemotractant protein. Both of these genes exert a procoagulant effect on EC and interestingly neither of these genes was up-regulated by anti-β_2GPI (Zhao et al., 2001). In addition, phosphorylation of p38MAPK is known to be increased in the presence of aPL (Vega-Ostertag et al., 2005) and is involved in the transcription and expression of TF, IL-6 and IL-8, and all three of which genes were induced in this study. The p38MAPK signaling pathway has been shown to have a role in anti-β_2GPI mediated signaling in HUVEC and monocytes, and this pathway may also contribute to the regulation of some of the genes identified in our study (Raschi et al., 2003a; Bohgaki et al., 2004).

5.7 Possible receptors for β_2GPI

β_2GPI has been reported to bind to cells in a number of different ways. For example, it can bind to anionic membrane molecules such as heparan sulphate and it has also been reported to bind as a ligand to the annexin II receptor (Meroni et

al., 2005; Zhang et al., 2005). Annexin II receptor is the receptor for plasminogen /plasminogen activator inhibitor. Annexin II receptor is not a trans-membrane protein so must therefore be associated with an adaptor protein in order to convey signal to the cells. It has been proposed that similar sequence homology between bacterial/viral peptides (natural ligands for TLRs) and β_2GPI may be responsible for the binding of β_2GPI to TLR4 alone or in combination with another receptor. It has been postulated that anti-β_2GPI may bind to Annexin II in combination with TLR co-receptor, crosslinking the TLRs via $F(ab)_2$ binding and resulting in cellular activation (Zhang et al., 2005).

In support of this hypothesis, an *in vivo* study by Pierangeli et al. (2007) looked at the effects of aPL on thrombus formation and white cell adhesion in a mouse model with a polymorphism in TLR4 gene. This polymorphism is associated with impaired response to LPS (Shoenfeld et al., 2006). Injection of APS IgG induced formation of significantly larger thrombi and induced more white cell adhesion in wild type mice when compared to those with the TLR4 mutation or injection of normal control IgG (Pierangeli et al., 2007). These results suggest a possible association between β_2GPI and the TLR family. Since β_2GPI displays molecular mimicry with microbial pathogens, it seems possible that β_2GPI may associate with TLRs (Shoenfeld et al., 2006).

Annexin II is also a high affinity receptor for Tenascin C, a component of the extracellular matrix that functions as an adhesion molecule, shown in this study to be upregulated by anti-β_2GPI antibody (Chung et al., 1996).

An important question in relation to the findings in this study is their relevance to the *in vivo* situation. Is there evidence *in vivo* for the presence of increased expression of any or all of these genes in APS patients? aPL activate endothelium both *in vitro* and in *in vivo* experimental models by inducing a pro-inflammatory/coagulant phenotype. A recent study, measuring a limited number of parameters of EC function, concluded that aPL antibodies were unable to support a full-blown endothelial perturbation *in vivo* (Meroni et al., 2004). There is evidence however, from other studies, for increased circulating levels of TF, IL-6, TNFα

(Forastiero, Martinuzzo, & de Larranaga 2005) and VCAM-1 in APS patients (Kaplanski et al., 2000). This study suggests that increased levels of some cytokines might, at least in part, be EC derived and therefore evidence of endothelial perturbation *in vivo*. It will be interesting to address whether there are increased plasma levels of some of the cytokines/chemokines newly identified in this study as anti-β_2GPI antibody EC regulated genes in APS patients.

In order to determine an association between aPL and β_2GPI *in vivo*, it was necessary to establish whether β_2GPI was expressed on EC throughout the vasculature. Indeed, preliminary studies revealed that β_2GPI was expressed on HUVEC (Del Papa et al., 1995) while both human monoclonal and affinity purified polyclonal IgG anti-β_2GPI antibody preparations recognized β_2GPI adherent to EC in placental vessels (La Rosa et al., 1994). In order to explain the wide range of clinical associations between anti-β_2GPI and vasculopathy associated with both macro and microvasculature, it was necessary to determine whether or not β_2GPI is expressed on EC of different anatomical origin. Expression of β_2GPI on both human brain microvascular EC (HBMEC) and dermal EC (HDMEC) was confirmed and it seems likely that β_2GPI is constitutively expressed (Meroni et al., 2003).

EC are recognized for their heterogeneous phenotypes within different parts of the vasculature and within organ systems (Cines et al., 1998). Decrease in blood flow, injury to the vessel wall (EC damage) and a change in pro-coagulant/anticoagulant balance create a pro-thrombotic EC phenotype. Differences in EC phenotype and signaling pathways unique to portions of the vascular bed give rise to focal thrombotic lesions such as those seen in APS (Rosenberg & Aird 1999). An extensive and interesting study by Chi et al. (2003) used global gene expression profiling in order to determine differences in gene expression between EC from a variety of anatomical sites. Not surprisingly, their results confirmed distinct differences in EC gene expression patterns between large and microvascular vessels, between arterial and venous EC and identified organ specific differences as well. The heterogeneity of EC phenotype within the vasculature combined with different binding and activation characteristics of patient derived polyclonal anti-

β_2GPI antibodies could help to explain the diverse clinical pathology observed in patients with PAPS.

The largest subset of differentially expressed genes that were identified in this study were those genes involved in the inflammatory response. Vascular thrombosis is a common feature of pathologic inflammation in the vasculature and numerous *in vitro* experiments have linked coagulation with inflammatory processes (Kaplanski et al., 1997). It is tempting to speculate that a combination of increased adhesion molecules, pro-inflammatory cytokines and chemokines in addition to increased TF expression could strongly support development of thrombosis and contribute to the advancement of atherosclerotic lesions in response to anti-β_2GPI antibodies. For example, circulating anti-β_2GPI in susceptible individuals may stimulate an on-going chronic activation of EC, in the cerebral circulation for example, or in conjunction with impaired circulation in a leg vein or capillaries in the extremities. Up-regulation of chemotactic chemokines and adhesion molecules on the EC surface will encourage recruitment and activation of leukocytes and platelets. Monocytes and EC, already primed to produce TF will produce even more. This would result in more platelet activation, thrombin generation and localized clot formation within either the venous and/or arterial circulation. This scenario presents a very plausible explanation for the increased incidence of thrombosis in PAPS patients.

It is not uncommon for the value of microarray analysis to be diminished by being defined as a "fishing expedition". It is in fact just that, but its value lies in its ability to screen for significant changes in expression in vast numbers of genes in the human genome in relation to different biological samples and variables. In the present report, rigorous experimental design and replication, access to well-defined primary tissues/clinical sources and robust statistical analysis have contributed to the generation of meaningful and reliable data. This observation was further strengthened by our validation of previous findings by other investigators. The microarray approach interrogates the expression levels of a large number of known genes so there is no bias for or against those genes thought to be involved in EC biology and PAPS.

5.8 Limitations of the study

- This study was restricted to the analysis of gene expression in response to anti-β_2GPI after a four hour incubation time. Analysis of differential gene expression at a number of time points would be potentially more informative. However, the four hour data provides a reference point on which to base future studies.
- Well-characterized, readily attainable, macrovascular HUVEC were used as the source of EC in this study. In addition, it would have been interesting and useful to use cells such as microvascular EC that would be more biologically appropriate to the pathology of APS.
- Normal control IgG was used as negative control antibody throughout the study. Another specific AECA could have been used in addition to normal control.
- An additional control that would have been valuable in this investigation would be IgG from patients with history of thrombosis and no aPL.

5.9 Future perspectives

Further experiments, utilizing our panel of anti-β_2GPI antibody-regulated genes and specific inhibitors and/or dominant negatives of important signaling components will map particular signal transduction pathways to induction of specific genes. One possibility however that has to be considered is that our regulated genes may include some or many genes not regulated by anti-β_2GPI antibody directly but rather indirectly by autocrine cytokine/chemokine production produced by EC cells shortly after anti-β_2GPI antibody exposure. Clearly, many cytokines/chemokines are induced and they could themselves then induce gene expression in EC by binding to high affinity receptors on the EC. This possibility should be addressed in future studies aimed at investigating the signal transduction mechanisms responsible for anti-β_2GPI antibody mediated gene regulation in EC. Future studies should aim to identify specific genes responsible for the functional effects of anti-β_2GPI antibodies on EC.

It would be valuable to map the specific signal transduction pathways responsible for mediating gene expression, by using specific inhibitors of the different pathways. Mapping of the signaling pathways could identify potential molecular targets for future therapeutic approaches in APS. Potential cell receptors such as Annexin II or TLR4 could be targeted with their specific ligands and the resultant gene expression could be compared with that obtained with anti-β_2GPI antibodies. Specific genes within the panel could be targeted using siRNA. Down regulation of a specific gene may be seen to inhibit anti-β_2GPI mediated monocyte adhesion or other EC functions including angiogenesis and apoptosis.

EC from different vascular beds (microvascular) could be used to see if there are differences in antibody-mediated gene expression in EC that may be more relevant to disease pathology. It may be possible to obtain samples of relevant tissue from APS patients such as placental tissue (trophoblasts), kidney biopsies, heart valves or skin biopsies. RT-PCR or fluorescent microscopy could be used to compare gene expression in these tissues compared to normal tissue.

This study did not identify large differences in gene expression profiles between the different anti-β_2GPI antibody preparations. As previously discussed, the largest differences in gene expression were between EC from different donors and not due to the individual antibody preparations. Even though the corresponding patients had differences in their clinical histories, there were also similarities. It would be interesting to look at gene expression profiles in response to anti-β_2GPI in patients with aPL and a history of purely venous or arterial thrombosis or recurrent pregnancy loss as well as SLE patients to see if there were differences in antibody induced gene expression. Also, anti-β_2GPI antibodies from patients with aPL but no history of APS symptoms would be a useful source for comparison in gene expression studies.

Fitting with disease criteria, many of the genes identified in this study are involved in the mechanisms of inflammation, proliferation and angiogenesis. New evidence suggests that aPL-mediated pregnancy loss and IUGR may be the result of complement activation and recruitment of inflammatory cells. Subsequent increase

in expression of TNFα and other inflammatory mediators shifts the cytokine balance from TH2 associated with normal pregnancy outcome to TH1 and the innate immune response (Berman et al., 2005). In the present study however, anti-β_2GPI were able to activate EC in the absence of complement *in vitro* and induce expression of a number of proinflammatory genes. Taken together, these findings suggest a more significant inflammatory component in the etiology of PAPS (and APS) than previously thought.

In conclusion, by using microarray gene profiling we have confirmed and extended the analysis of anti-β_2GPI antibody regulated genes and identified a panel of genes that may be responsible for EC phenotype in PAPS. Characterization of the expression levels *in vivo* in APS patients and the signal transduction cascades responsible for induction or downregulation of this gene panel should provide important new information relevant to understanding the role of anti-β_2GPI antibodies in APS pathology. Most importantly, therapeutic targeting of one or more pathological induced genes could contribute to lessening of disease severity. It may also be possible to link upregulation of one or more gene- regulated plasma markers in APS to patients at greater risk of developing thrombosis or accelerated arterial disease or at the very least gene(s) products may act as important biomarkers of the disease. Moreover, these findings could have wider implications for other diseases, such as SLE, where anti-β_2GPI have been described.

Chapter 6

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Appendix

1.0 Appendix: Buffers and Formulations

1.1 Reagents for aCL ELISA

1. PBS

    3 PBS tablets

    600 ml deionized water

    pH 7.4

2. 10% ABS-PBS solution

    20 ml adult bovine serum (Sigma)

    180 ml PBS

3. Substrate Buffer- Magnesium Carbonate pH 9.8

    0.6g $NaHC0_3$

    0.025g $MgCl_2.6H_20$

    100 ml dH_20

1.2 Coating buffer for anti-β_2GPI ELISA

Coating Buffer- 0.05M Carbonate Buffer pH 9.6

1.59g Na_2C0_3 (anhydrous) MW 105.89

2.93g $NaHC0_3$ MW 84.01

Dissolved in 1l of deinoised water.

1.3 Storage solution for umbilical cords

For 8 pots;

1) 360ml H20 (sterile water)
2) 40ml Hanks buffer (Sterile, Sigma)
3) 15ml bicarb/phenol red (Bicarb: 44g/l, add 0.1% phenol red (Sigma)
4) 10ml 1M HEPES
5) 5ml gentamycin (7.5mg/ml in PBS)

Cord pots can be obtained from Scientific Labs (www.scientificlabs.com)
Cat; SL57588 (250ml, metal cap plain label)

1.4 Reagents for IgG and anti-β_2GPI purification

1. Binding Buffer

   20 mM $NaP0_4$, pH 7.0

   2.76 g $NaP0_4$ FW = 138.0

   Dissolved in 1L dH_20.

2. Elution Buffer

   0.1M Glycine-HCl, pH 2.7

   1.115g Glycine-HCl FW=111.5

   Dissolved in 100 ml dH_20

3. Neutrallization Buffer

   1 M Tris-HCl, pH 9.0

   15.76g Tris-HCl FW=157.6

   Dissolve in 100 ml dH_20.

1.5 Reagents for preparation of β_2GPI affinity purification columns

1. Coupling Buffer

   0.2M $NaHC0_3$ FW= 84.0 13.36g dissolved in 200 ml dH_20.

   0.5M NaCl FW=58.44 5.844g dissolved in 200 ml dH_20.

   pH 8.3

2. Inactivation of HP (blocking of free binding sites)

Buffer A

0.5M ethanolamine (.054 ml))

0.5 M NaCl (2.922 g).

in 100 ml dH_20 pH 8.3

Buffer B

0.1M acetate (0.6 ml acetic acid)

0.5M NaCl (2.922 g)

in 100 ml dH_20 pH 4.0

3. Storage Buffer

   20 mM NaH_2P0_4 FW=120.0 (0.12 g in 50 ml dH_20)

   0.1% NaN_3 0.05g

2.0 Reagents and equipment required for HUVEC isolation and culture

- Collagenase Enzyme- working concentration 100mg/ml (Sigma 500 mg reconstituted in 5.0 ml sterile deionized water, aliquoted and frozen at –70°C.
- Fetal Calf Serum- Endotoxin tested (Biowest)
- 0.1% gelatin (Sigma 2% stock, diluted in sterile deionized water, store at 4°C.)
- DMEM- Dulbecco's Eagle's Medium- Sigma (contains 1000 mg glucose/l, L-glutamine, NaHCO3, pyridoxine HCl)
- DMEM + 10% Fetal Calf Serum (Biowest)
- Fungizone (Gibco- 250 μg/ml stock- working concentration 2.5 μg/ml, add 5 ml of stock to 500 ml of media)
- L-Glutamine (Sigma- 200 mM stock- working concentration 2mM- add 5 ml to 500 ml)
- Penicillin and Streptomycin (Sigma P4333- 5mLs stock in 500 ml media- final concentration 100 u/ml Penicillin/ 100 μg/ml Streptomycin)
- Medium 199- Gibco BRL- 25mM Hepes, 500 ml.
- Medium 199 +10% FCS + Pen/Strep- complete media
- Hanks Balanced Salt Solution (Sigma)
- 0.5 %Trypsin-EDTA (Sigma- T3924)
- Large and small tissue culture flasks (75 and 250 ml)
- Sterile 50ml Falcon tubes
- Sterile 20ml syringes
- Sterile 20ml universals
- Sterile 5ml and 10ml pipettes
- Bone marrow needles (2 per cord)
- String, gloves
- Scissors and clamps
- 0.22μm Millipore filters
- Class 2 tissue culture hood for human work

3.0 Contents of Qiagen RNeasy Mini Kit (50 columns)

Catalog no. 74104 74106

Number of preps 50

RNeasy Mini Spin Columns (pink) 50
Collection Tubes (1.5 ml) 50
Collection Tubes (2 ml)* 50
Buffer RLT*† 45 ml
Buffer RW1† 45 ml
Buffer RPE‡ (concentrate) 11 ml
RNase-Free Water 10 ml

Two-Cycle Target Labeling
■ Two-Cycle Target Labeling and Control Reagents, Affymetrix, P/N 900494
A convenient package containing required labeling and control reagents to perform 30 two-cycle labeling reactions. Each of these components may be ordered individually (described below) as well as in this complete kit.

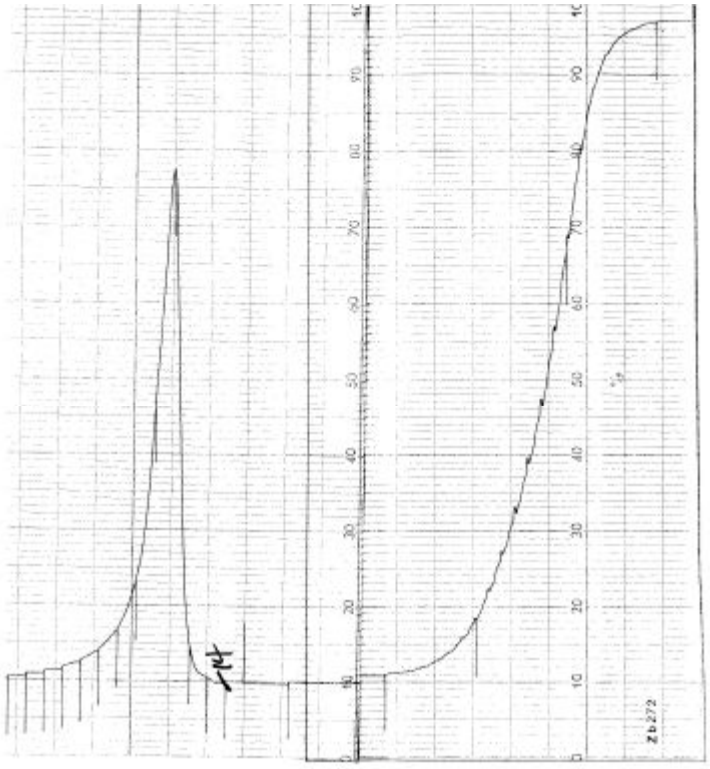
Contains:
■ 1 IVT Labeling Kit (Affymetrix, P/N 900449)
■ 1 Two-Cycle cDNA Synthesis Kit (Affymetrix, P/N 900432)
■ 2 Sample Cleanup Modules (Affymetrix, P/N 900371)
■ 1 Poly-A RNA Control Kit (Affymetrix, P/N 900433)
■ 1 Hybridization Control Kit (Affymetrix, P/N 900454)
■ MEGAscript® High Yield Transcription Kit, Ambion Inc, P/N 1334

4.0 Sterilization

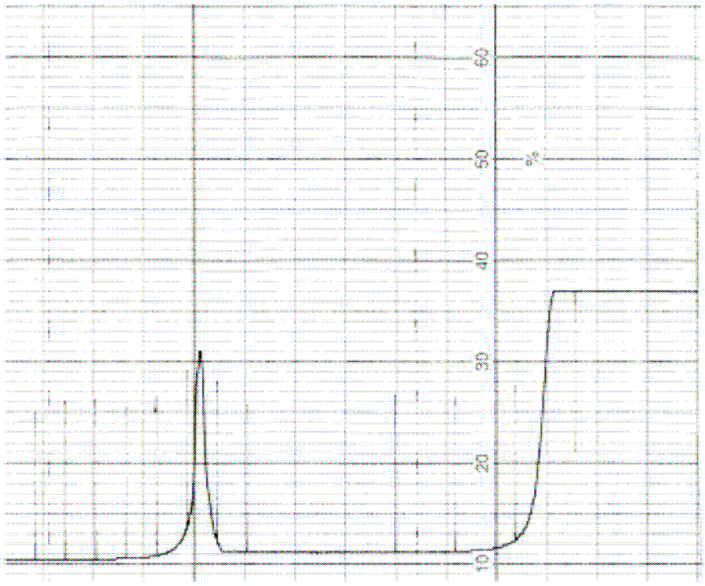
All sterilization procedures were carried out by autoclaving at 15lb/in^2 for 20 minutes at 121ºC using a Rodwell Series 32 autoclave.

Appendix Table 1 Activation of E-selectin and ICAM-1 in HUVEC induced by incubation with IgG from APS patients.

			Ratio	Ratio
Sample	**E-selectin**	**ICAM**	**Pt IgG to**	**Pt IgG to**
	OD	**OD**	**N Cont IgG**	**N Cont IgG**
			E-Selectin	**ICAM-1**
1	0.683	0.794	3.15	4.08
2	0.593	0.786	2.34	3.97
3	0.65	0.858	2.84	4.96
4	0.564	0.681	2.09	2.53
5	0.589	0.654	2.31	2.16
6	0.469	0.679	1.28	2.51
7	0.538	0.734	1.87	3.26
8	0.516	0.676	1.68	2.46
9	0.514	0.66	1.66	2.25
10	0.561	0.682	2.07	2.55
11	0.531	0.675	1.81	2.35
12	0.449	0.589	1.1	1.27
13	0.432	0.595	0.957	1.36
NIgG 14	0.437	0.569	1.0	1.0
Negative control	0.321	0.496		
TNF Positive control	0.836	0.879		



A

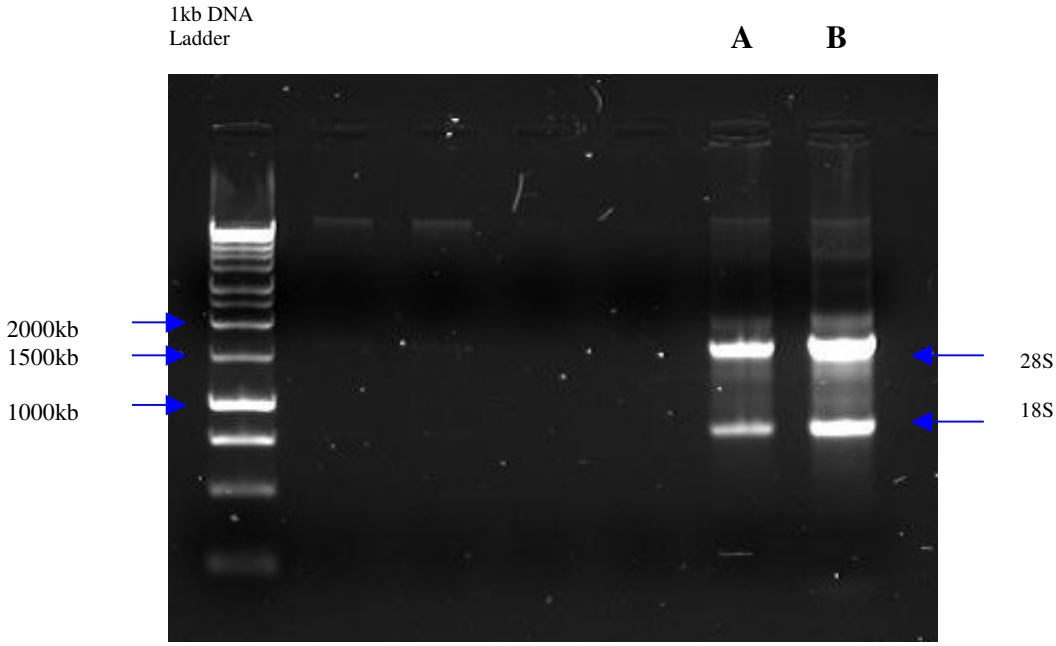


B

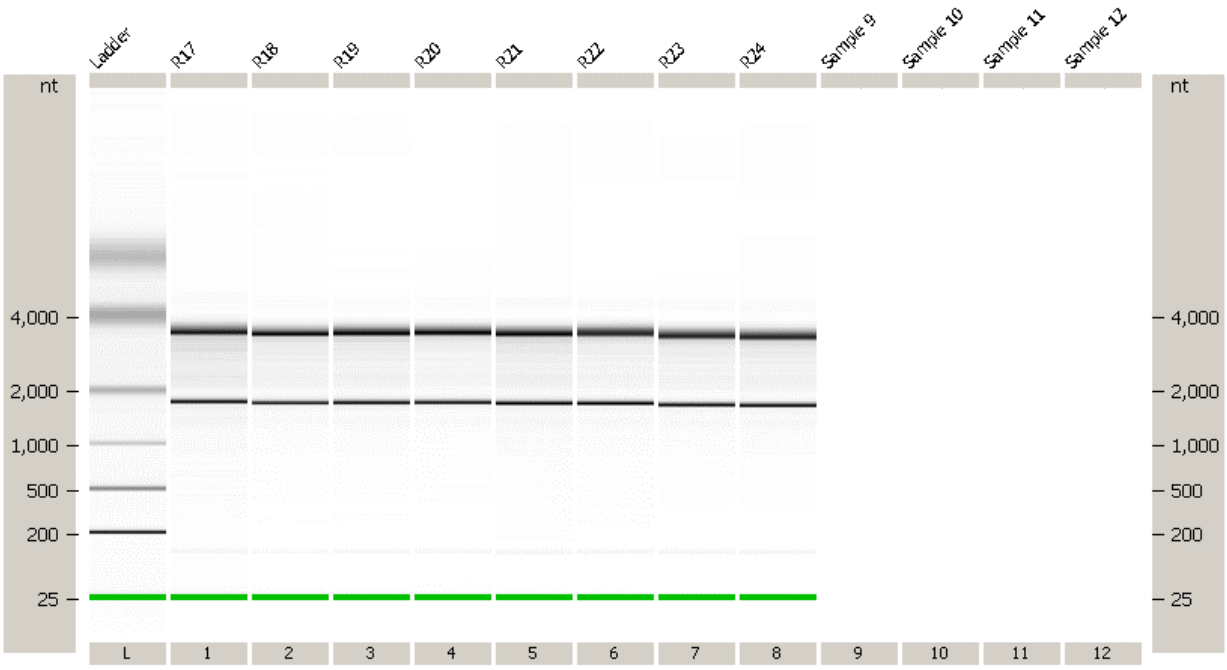
Appendix Figure 1A and 1B Elution of IgG and anti-β_2GPI using AKTAprime. Plot of elution (acid pH 2.7) of PAPS patient (A) IgG from Sepharose HiTrap G Protein S column and (B) anti-β_2GPI from NHS activated sepaharose column coupled to β_2GPI (B) using the UV recorder (AKTAprime). The x axis represents time in minutes, the y axis represents OD at

Appendix Table 2 Determination of RNA content in confluent HUVEC. RNA concentration in HUVEC was determined. HUVEC were checked for viability using trypan blue exclusion test. One well of confluent HUVEC (six well tissue culture plate) yielded enough RNA for an Affymetrix chip (approximately 5 µg).

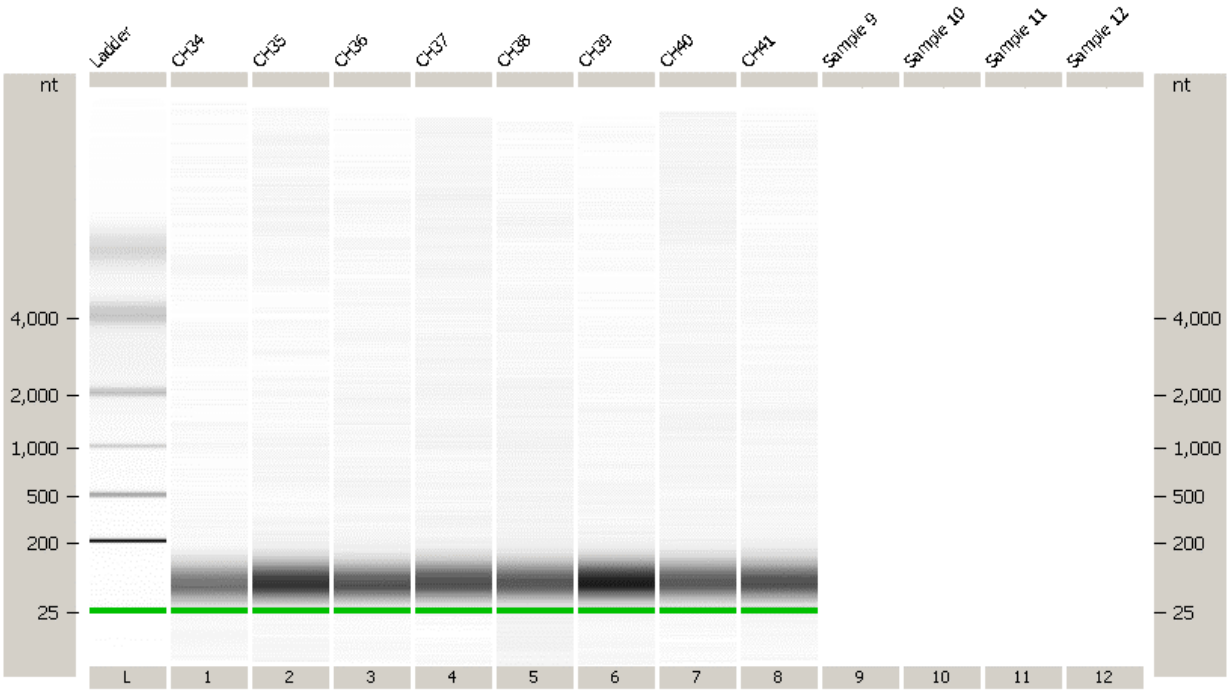
	Ratio of Purity	Total RNA In µg	Viability
1 well			
810,000	2.44	4.7	98%
2 wells			
1,600,000	2.273	12	99%



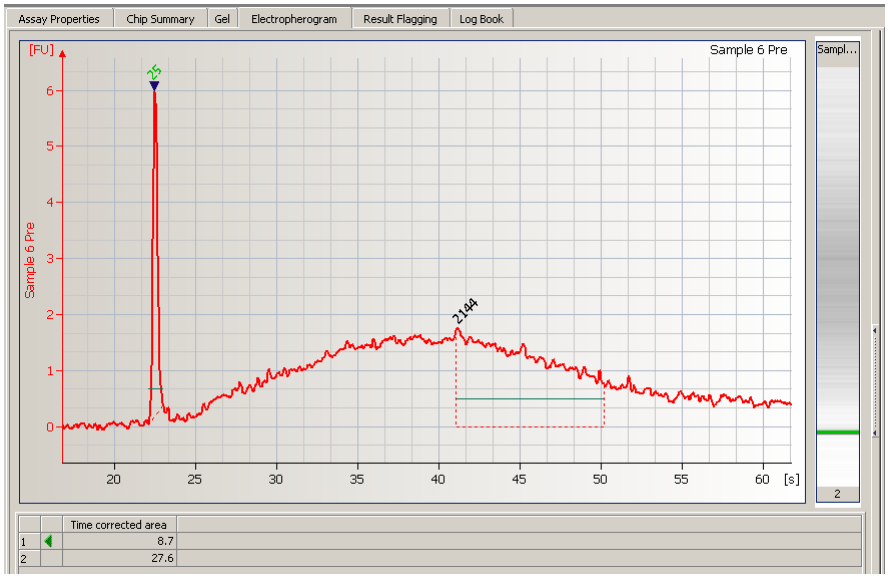
Appendix Figure 2 Gel electrophoresis of HUVEC RNA. RNA was extracted from confluent HUVEC grown in a six well tissue culture Plate. A: RNA was extracted from one well (approximately 1 x 10^6 cells) B: RNA was extracted from two wells. Both A and B show two ribosomal bands, 28S and 18S. DNA ladder was included to indicate proper running of gel, not for sizing.



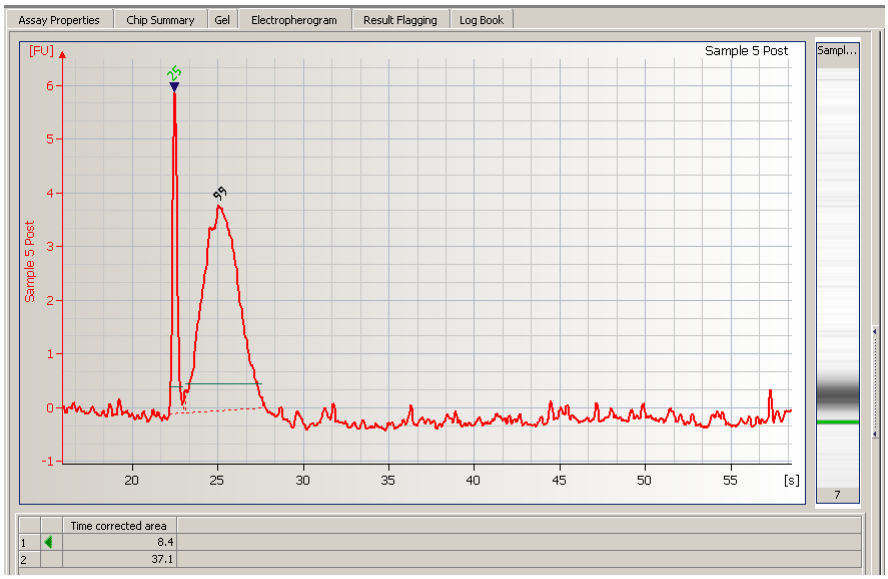
Appendix Figure 3 RNA for Affymetrix microarray analysis. Quality of Qiagen purified RNA was checked on a RNA 6000 Nano Assay gel chip before proceeding with protocol for preparation of fragmented cRNA. Samples shown are 8 samples processed in the first part of Experiment 1.



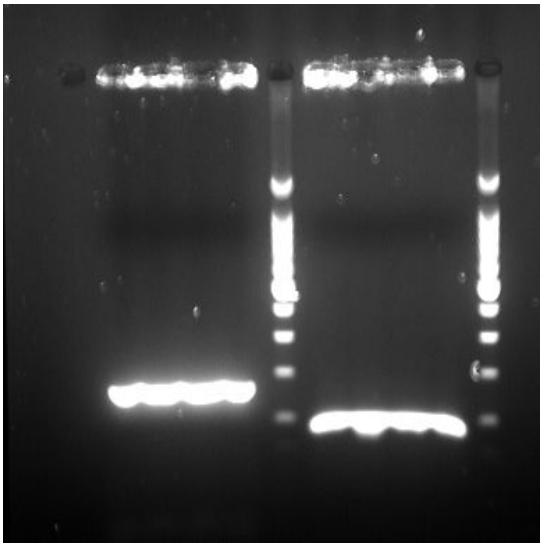
Appendix Figure 4 Fragmented cRNA for hybridization to Affymetrix chips. Successful fragmentation of cRNA was checked on a RNA 6000 Nano Assay gel chip before proceeding to preparation of the hybridization cocktail. Fragmented cRNA should range from 35-200 bp in length as seen in this figure.



Appendix Figure 5 Scan of cRNA prepared for Affymetrix run on gel. Scan shows marker on left and the distribution cRNA transcripts for one sample determined by their size. The right of the figure shows a smear of cRNA ranging from 50 to 3000 bp long.

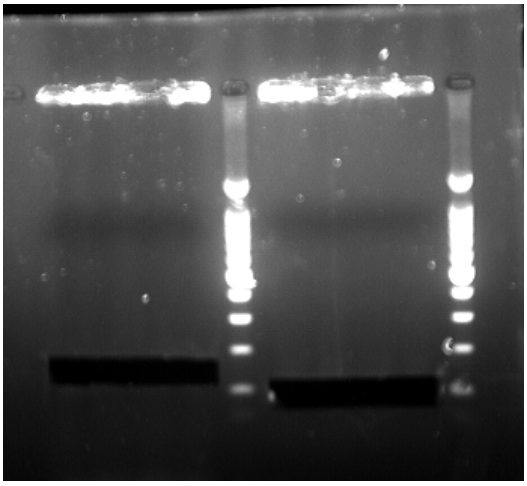


Appendix Figure 6 Scan of fragmented cRNA. Scan shows marker on left and distribution of cRNA transcripts after fragmentation for one sample. Fragment sizes range from 35-200 bp in length.



A

1 L 2 L



B

L L

Appendix Figure 7 Gel purification of cDNA standards for qRT-PCR. A. cDNA standards for SOD2 (1) and ID2 (2) were run on a 2% agarose gel containing EtBR for visualization. 100 bp ladder (L) was run in parallel. B. Bands of gel containing standards were carefully cut out and placed in dialysis tubing.

5.0 qRT-PCR Raw Data

SOD2

	Mean copy no /µl	SD	SEM
Sample 1	7502000000	7.75E+09	3872790747
Sample 2	5927250000	5.81E+09	2906988286
Sample 3	1469650000	1.59E+09	794830918.6
Sample 4	552400000	5.57E+08	278685417
Sample 5	49267500	50106585	25053292.32
Sample 6	1338675000	1.48E+09	738974157.6
Sample 7	25413500000	2.85E+10	14239950453

Normalised to β-actin

	B-actin	SOD2	normalised values	fold change
Sample 1	97200000	7.5E+09	77.18106996	29.4515475
Sample 2	103840000	5.93E+09	57.08060478	21.781405
Sample 3	31680000	1.47E+09	46.39046717	17.7021522
Sample 4	108200000	5.52E+08	5.105360444	1.94815601
Sample 5	18800000	49267500	2.620611702	1
Sample 6	44860000	1.34E+09	29.84117254	11.3871019
Sample 7	23566666.67	2.54E+10	1078.366337	411.494132

CX3CL1

	Mean copy no/ul	SD	SEM
Sample 1	9280000	6393762.586	3196881.293
Sample 2	11080000	5610805.052	2805402.526
Sample 3	3255000	1099014.71	549507.3551
Sample 4	396450.8425	584553.6704	292276.8352
Sample 5	20787.015	16128.51314	8064.256572
Sample 6	12630000	5817806.001	2908903.001
Sample 7	259000000	55506756.35	32046840.72

Normalised to β-actin

	β-actin	CX3CL1	normalised values	fold change
Sample 1	97200000	9280000	0.095473251	86
Sample 2	103840000	11080000	0.106702619	97
Sample 3	31680000	3255000	0.102746212	93
Sample 4	108200000	396450.8425	0.003664056	3
Sample 5	18800000	20787.015	0.001105692	1
Sample 6	44860000	12630000	0.281542577	255
Sample 7	23566666.67	259000000	10.99009901	9940

ID2

	Mean copy no/ul	SD	SEM
Sample 1	201411.78	24493.5	12246.7485
Sample 2	88383.0675	13331.07	6665.535831
Sample 3	25691.43	13055.9	6527.949307
Sample 4	267034.885	54205.99	27102.99708
Sample 5	40423.0875	12571.49	6285.742562
Sample 6	30678.1875	22661.23	11330.6127
Sample 7	59304.93	45800.91	22900.45681

	β-actin	ID2	normalised values	fold change
Sample 1	97200000	201411.8	0.002072138	0.963711342
Sample 2	103840000	88383.07	0.000851147	0.395851923
Sample 3	31680000	25691.43	0.000810967	0.377165077
Sample 4	108200000	267034.9	0.002467975	1.14780763
Sample 5	18800000	40423.09	0.002150164	1
Sample 6	44860000	30678.19	0.000683865	0.318052487
Sample 7	23566666.67	59304.93	0.002516475	1.170364139

E-selectin

	Mean copy no/ul	SD	SEM
Sample 1	17100000	2412468	1206233.808
Sample 2	15150000	2595509	1297754.471
Sample 3	5947500	1844639	922319.3138
Sample 4	213473.7	68326.23	34163.11395
Sample 5	119067.9	61973.79	30986.89255
Sample 6	19450000	6605301	3302650.451
Sample 7	1.27E+08	41119501	23740354.77

	β-actin	SELE	normalised values	fold change
Sample 1	97200000	17100000	0.176	27.777498
Sample 2	1.04E+08	15150000	0.146	23.036221
Sample 3	31680000	5947500	0.188	29.642345
Sample 4	1.08E+08	213473.7	0.002	0.3115161
Sample 5	18800000	119067.9	0.006	1
Sample 6	44860000	19450000	0.434	68.457907
Sample 7	23566667	1.27E+08	5.400	852.66766

TNC

	Mean copy no/ul	SD	SEM
Sample 1	88723	19442	9721
Sample 2	219358	67154	33577
Sample 3	30406	16975	8488
Sample 4	15517.00333	4275	2468
Sample 5	2598	384	192
Sample 6	12053.5	3574	2063
Sample 7	52299	29900	14950

	β-actin	TNC	normalised values	fold change
Sample 1	97200000	88723	0.000912783	6.6045402
Sample 2	103840000	219358	0.002112463	15.284946
Sample 3	31680000	30406	0.000959782	6.9446039
Sample 4	108200000	15517	0.00014341	1.0376608
Sample 5	18800000	2598	0.000138205	1
Sample 6	44860000	12053.5	0.000268691	1.9441453
Sample 7	23566666.67	52299	0.002219195	16.057214

FGF18

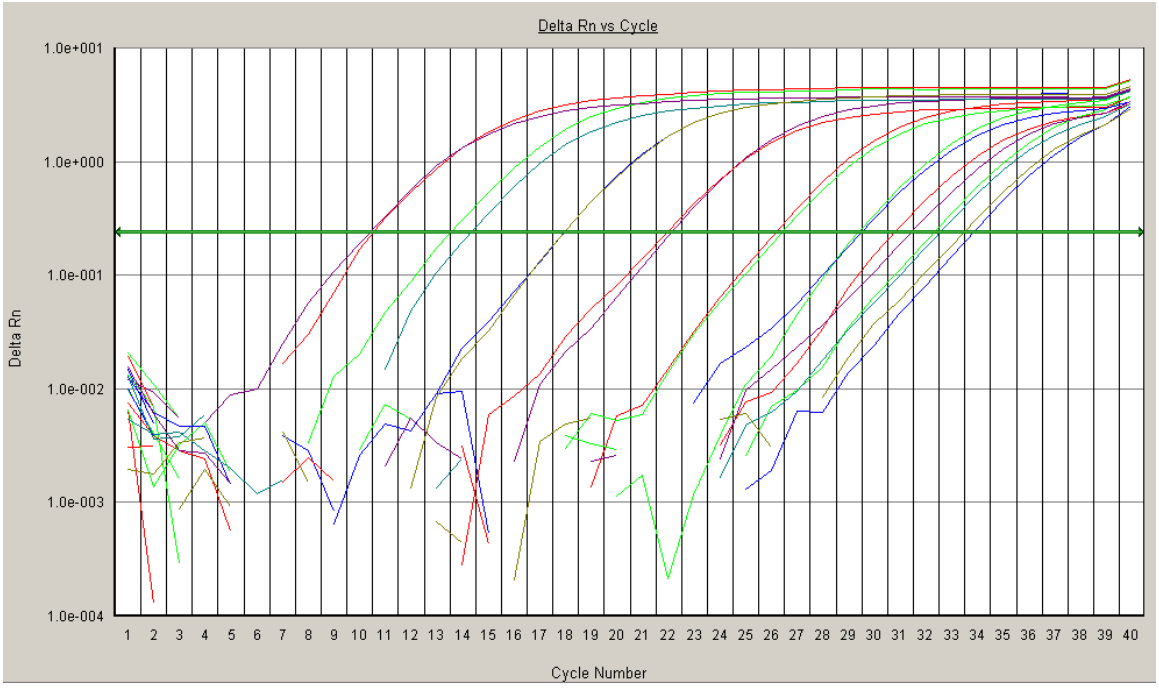
	Mean copy no/ul	SD	SEM
Sample 1	41685	7531	3766
Sample 2	49754	7102	3551
Sample 3	7964	406	203
Sample 4	13838	3549	1775
Sample 5	1093	328	164
Sample 6	7201	2107	1054
Sample 7	28949	10834	5417

	β-actin	FGF	normalised values	fold change
Sample 1	97200000	41685	0.000428857	7.4
Sample 2	103840000	49754	0.000479138	8.2
Sample 3	31680000	7964	0.000251383	4.3
Sample 4	108200000	13838	0.000127893	2.2
Sample 5	18800000	1093	5.81328E-05	1.0
Sample 6	44860000	7201	0.000160514	2.8
Sample 7	23566666.67	28949	0.001228392	21.1

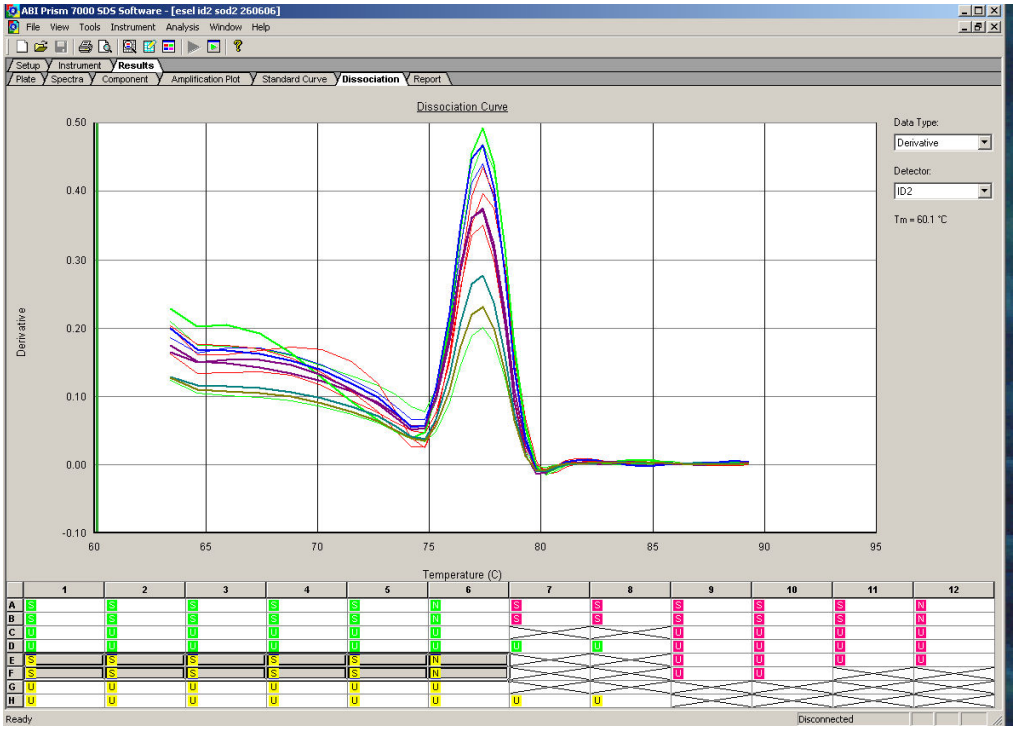
CSF3

	Mean copy no/ul	SD	SEM
Sample 1	749421	114924	57462
Sample 2	261875	25089	12544
Sample 3	252702	33728	16864
Sample 4	42088	5766	2883
Sample 5	9884	3179	1590
Sample 6	149550	96403	48202
Sample 7	16280	12637	6318

	β-actin	CSF	normalised values	fold change
Sample 1	97200000	749421	0.00771	14.67
Sample 2	103840000	261875	0.00252	4.80
Sample 3	31680000	252702	0.00798	15.17
Sample 4	108200000	42088	0.00039	0.74
Sample 5	18800000	9884	0.00053	1.00
Sample 6	44860000	149550	0.00333	6.34
Sample 7	23566666.67	16280	0.00069	1.31



Appendix Figure 8 qRT-PCR standard curve for β-actin. Standard was run in duplicate for 1 x 10^8 – 1x 10^0 copy number.



Appendix Figure 9 Dissociation curve for ID2.