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The Role of B2-glycoprotein I in Homeostatis

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The role of β_2-glycoprotein I in homeostasis

A thesis submitted for the degree of
Doctor of Philosophy

by

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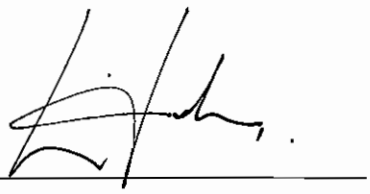

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Abstract

β_2-glycoprotein I (β2GPI) is a phospholipid-binding protein of 326 amino acids found in plasma at a concentration of approximately 180 μg/mL. It has a sequence of positively charged amino acids located at the carboxy terminus that mediates anionic phospholipid binding. β2GPI is suspected to have a role in inhibition of thrombosis. This suspicion is reinforced by the observation that β2GPI is the major target for autoantibodies in the antiphospholipid syndrome. However, little is known about circulating levels of the protein in common thrombotic diseases or inflammation. In the first part of this thesis, we developed a sensitive sandwich-direct ELISA for β2GPI quantification and measured serum β2GPI level in 344 healthy controls, 73 women with uncomplicated pregnancies, 102 patients with non-haemorrhagic stroke, 121 patients with acute coronary syndrome and 200 patients with elevated C-reactive protein (CRP) suffering from a variety of inflammatory disorders. In healthy individuals, we found a strong positive correlation between age and β2GPI concentration (r=0.274, P<0.001) and that β2GPI level gradually decreases over the first 36 weeks of pregnancy (P=0.002). We also found a significantly reduced level of β2GPI in patients with stroke (mean ± SD: 170.2 ± 48.4 μg/mL versus 187.5 ± 47.5 μg/mL in age-matched controls, P=0.013) and in elderly patients with myocardial syndrome (mean ± SD: 167.9 ± 50.7 μg/mL versus 189.3 ± 45.8 μg/mL in age-matched controls, P=0.046). However, in neither group did β2GPI level change in the following six months, suggesting that the reduced level was not a transient post-event phenomenon. In patients with inflammation, β2GPI level was reduced and showed a significant negative correlation with CRP level (r=-0.284, P<0.001) and positively correlated with albumin and transferrin levels (r=0.372 and 0.453, respectively with P<0.001 for both). Furthermore, the largest reduction in β2GPI level occurred in patients with the highest CRP values (i.e. above 105.1 mg/L; mean β2GPI concentration ± SD: 133.1 ± 57.7 μg/mL with P<0.001). These observed reductions in serum β2GPI level may be important in the pathogenesis of the prothrombotic diathesis observed in many of these conditions.

Because little is known about a possible association between polymorphic variants of β2GPI and common thrombotic disorders, in the second part of this study, we determined the incidence of four point mutations in the β2GPI (APOH) gene at codon positions 88, 247, 306 and 316 in 323 healthy individuals, 113 individuals with non-haemorrhagic stroke, 360 patients with acute coronary syndrome, and 47 females with recurrent miscarriage. We also correlated circulating level of β2GPI

with these polymorphisms in normal controls and patients with stroke. The incidence of each of the point mutations in the healthy individuals was similar to previously reported findings. Healthy controls with point mutations at positions 306 and 316 had a significantly reduced circulating level of β2GPI (mean ± SD: 81.9 ± 31.8 μg/mL in heterozygotes versus 182.1 ± 44.9 μg/mL in non-carriers at position 306, and 112.0 ± 32.1 μg/mL in heterozygotes versus 183.2 ± 46.1 μg/mL in non-carriers at position 316, P<0.0001 for both). A comparable reduction was found in patients with stroke (mean ± SD: 109.5 ± 54.3 μg/mL in heterozygotes versus 175.0 ± 41.6 μg/mL in non-carriers with P<0.0001 at position 306, and 128.4 ± 30.4 μg/mL in heterozygotes versus 172.6 ± 46.4 μg/mL in non-carriers with P=0.0061 at position 316). Variation at codons 88 and 247 had no effect on circulating level of β2GPI. A significant genotypic association was found between heterozygosity at positions 247 and 306 and acute coronary syndrome (*P*=0.009 and *P*=0.047 respectively). No association with other disorders or mutations examined was found. This is the first study to find an association between inherited variants of β2GPI and a common thrombotic disorder. These results also suggest that the pattern of thrombosis-related risk conferred by genetic variants of β2GPI is not simply due to their effect on circulating level of the protein.

Lastly, we examined the synthesis of β2GPI by the liver in an inflammation-type mouse model. Although this part of the work was not fully brought to completion, we could clearly observe a difference between the synthesis of haptoglobin and that of β2GPI. In this model, the synthesis of haptoglobin increased and the synthesis of β2GPI decreased following an inflammatory insult. This supports our observation made in the first part of this study, which showed a negative correlation between β2GPI level and the degree of inflammation and suggests that at least some of the reduction in the observed level of β2GPI is due to diminished synthesis.

This thesis contributes to our understanding of both the metabolism of β2GPI and the polymorphic variants associated with a variety of prothrombotic conditions. A better understanding of these factors may ultimately contribute to novel therapeutic strategies to decrease the morbidity and mortality of thrombotic disorders.

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Publications and presentations

Papers

Lin F, Ryan F, Shields DC, Vaughan J, Livingstone W, Smith O, Feighery C, Jackson J. 2007. β_2-glycoprotein I polymorphisms effect on serum levels and the incidence of common thrombotic disorders (manuscript in preparation).

Lin F, Murphy R, White B, Kelly J, Feighery C, Doyle R, Pittock S, Moroney J, Smith O, Livingstone W, Keenan C, Jackson J. 2006. Circulating levels of β_2-glycoprotein I in thrombotic disorders and in inflammation. *Lupus*. 15: 87-93.

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Lin F, Guerin J, Smith O, White B, Feighery C, Jackson J. 2000. Increased β_2-glycoprotein I (β2GPI) levels in patients with stroke. *J Autoimmunity*. 15: A44. *9th International Congress on Antiphospholipid Antibodies. Tours, France, poster presentation.*

Abbreviations

β2GPI	β_2-glycoprotein I (apolipoprotein H)
95%CI	95% confidence interval
ACLA	Anticardiolipin antibody(ies)
aPL	Antiphospholipid antibody(ies)
apo	Apolipoprotein
APOH	Apolipoprotein H (β_2-glycoprotein I)
APS	Antiphospholipid syndrome
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CL	Cardiolipin
CP	Crossing point
CRP	C-reactive protein (expressed in mg/L)
C_T	Threshold cycle
CV	Coefficient of variation
DEPC	Diethylpyrocarbonate
DIC	Disseminated intravascular coagulation
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
ELISA(s)	Enzyme-linked immunosorbent assay(s)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
ICAM-1	Intracellular adhesion molecule-1
IEF	Isoelectric focusing
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IU	International unit
Kb	Kilobase
K_d	Dissociation constant
LA	Lupus anticoagulant
LDL	Low density lipoprotein
mM	Millimole/litre
Mol	Mole
mRNA	Messenger ribonucleic acid
OD	Optical density
OPD	Orthophenylenediamine dihydrochloride
OR	Odds ratio
PAGE	Polyacryl amide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween®20
PCR	Polymerase chain reaction
PS	Phosphatidylserine
rβ2GPI	Recombinant β_2-glycoprotein I
RNA	Ribonucleic acid
RT	Reverse transcriptase
SAA	Serum amyloid A
SCR	Short consensus repeat
SD	Standard deviation error
SDS	Sodium dodecyl sulfate
SLE	Systemic lupus erythematosus

	Nucleotide base	1-letter code
Purines:	**Adenine**	A
	Guanine	G
Pyrimidines:	**Cytosine**	C
	Thymine	T
	Uracil	U

Amino acid	3-letter abbreviation	1-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonin	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

1 General introduction

1.1 Overview

Cardiovascular and cerebrovascular diseases represent two of the most common causes of morbidity and mortality in humans worldwide (Balter, 1999) particularly in the Western world (Hoyert *et al*, 2005) (figure 1.1). Nearly half of all deaths are from cardiovascular diseases, which account each year for over 4,000,000 deaths in Europe, 900,000 in the U.S.A., and 17,000 in Ireland (Irish Heart Foundation, 2004; Hoyert *et al*, 2005). More specifically, coronary heart disease and cerebrovascular diseases are the first and third most common causes of death in Europe, the U.S.A. and much of Asia, with around 40% and 30% of all deaths from vascular diseases respectively (Breslow, 1997; Braunwald, 1997; Hoyert *et al*, 2005).

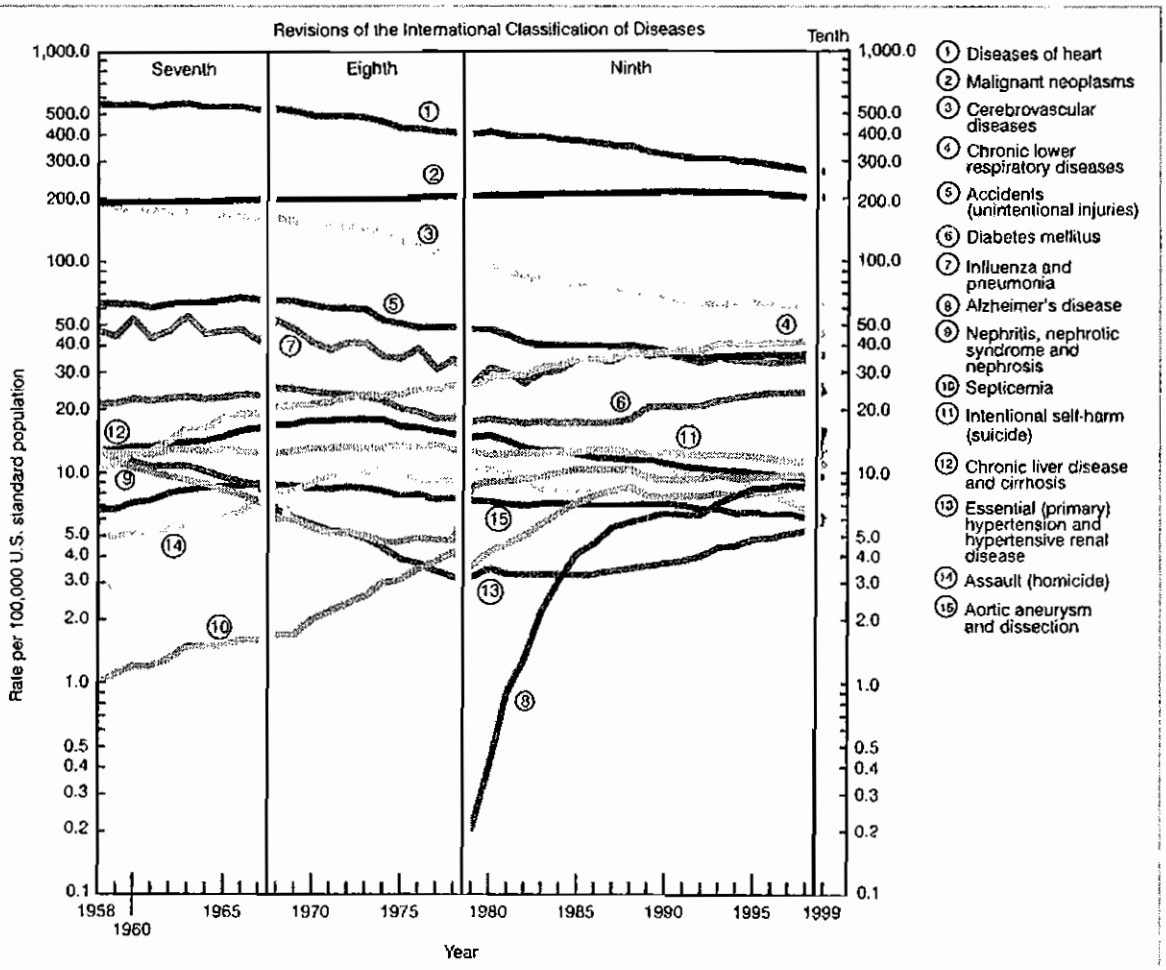


Figure 1.1: Age-adjusted death rates for the 15 leading causes of death in United States, 1958-1999 (per 100,000 U.S. standard population based on year 2000 standard). From Hoyert *et al.* (2001).

1.2 Thrombotic disorders

Alteration of haemostasis is central in the pathogenesis of thrombotic events even though its causes may differ depending on the location. Thrombi can arise in any part of the circulatory system, but tend to occur more frequently in large vessels (arteries or veins) and in cardiac cavities and valves. Typically, arterial and venous thromboembolic disorders form two relatively different and characteristic spectra of conditions: the former are characterized by long-term atheromatous plaque formation culminating in atherothrombotic obstructive lesions that lead to tissue damage, and the latter by *in situ* thrombus formation and the variable presence of embolic manifestations (Wheater *et al*, 1986; Majno *et al*, 1996).[1]

Arterial thrombosis usually occurs at sites of vascular damage caused by superficial intimal injury or disruption of an atherosclerotic plaque after the exposure of adhesive proteins and other prothrombotic moieties (such as von Willebrand factor, collagen, fibronectin, laminin, vitronectin or thrombospondin) (Magliano *et al*, 2003). The underlying molecular processes that lead to atheroma formation are not clearly understood. They are complex and involve multiple interrelated systems that regulate vasoactivity, adhesion molecules and their ligands, the immunoreactivity of macrophages and lymphocytes, lipid metabolism, and the coagulation and fibrinolytic pathways (Ross, 1993 & 1999). Clinical presentation can vary considerably according to the vessel that is predominantly affected. The two most important clinical foci of arterial thrombotic events are coronary artery disease and ischaemic stroke.

Coronary artery disease and its thrombotic complications, unstable angina and myocardial infarction, are the clinical manifestations of the chronic development of coronary artery atheroma that can deteriorate and lead to a pathologic process of plaque rupture and coronary thrombosis. The consequence is an insufficient delivery of oxygenated blood to the myocardium, which becomes ischaemic when metabolic demand for oxygen exceeds supply. Ischaemia leads to cardiac pumping dysfunction, which predisposes to abnormal heart rhythms, and, if prolonged, to myocardial infarction (Copstead *et al*, 2000).

Ischaemic strokes are sudden neurologic disturbances due to diseased brain blood vessels caused by the occlusion of a cerebral artery due to thrombi or emboli. Thrombotic ischaemic strokes, which represent about 85% of all

[1] Even though these distinctions are not absolute.

cerebrovascular diseases[2] (Underwood, 1992), are associated with atherosclerosis and hypercoagulable states, while embolic strokes are most often associated with thrombi of cardiac origin (Zazulia, 2002; Roldan *et al*, 2005).

Venous thrombosis is a frequently occurring disorder with an incidence of one per 1,000 people per year (Anderson *et al*, 1991; Nordström *et al*, 1992). It can be characterized according to the site and the extent of thrombus formation or to the underlying cause (inherited or acquired). Familial venous thrombosis is associated with younger age of onset, lower influence of environmental stimuli, and thrombus formation often in unusual sites (including the brain (Coull *et al*, 1993)). In the absence of a genetic basis, acquired venous thrombosis occurs predominantly in the older population, most commonly in the veins of the lower limbs and largely in the context of marked environmental influences such as surgery, immobilization, obesity, trauma, plaster casts, lupus anticoagulant, underlying malignancy and the use of estrogen. The formation of most venous thrombi seems to begin at valves, which protrude into the vessel lumen and naturally produce turbulence in blood circulation. In cases of reduction in blood pressure (due to for example surgery or myocardial infarction), stasis of the blood (following e.g. prolonged immobilisation) or increased alteration of the normal laminar blood flow (by e.g. damaged valves due to trauma or occlusion), the clotting cascade can be activated to produce thrombi (Majno *et al*, 1996; Kumar *et al*, 2005).

[2] The remainder being intracerebral (~10%) and subarachnoid (~5%) haemorrhagic strokes (Underwood, 1992).

1.3 Predisposing factors

From a modern perspective, prothrombotic states are considered as multi-causal diseases that may be caused by heritable (genetic) or classical (also known as conventional, environmental or acquired) risk factors. The distinction between the two types of risk factors is not clear-cut. A combination of them is often involved; for instance, because of the late onset of most thrombotic events, it is unlikely that genetic changes are the unique determinants of thrombosis.

Besides the classical versus genetic categorisation, risk factors can also be classified according to the three main conditions - known as Virchow's triad - which are considered, alone or in association, to predispose to thrombosis. The elements are: (1) alterations in vascular wall integrity (for instance, atheromatous plaques in arteries, inflammation in veins (phlebitis) or necrotic myocardial infarction), (2) alterations in the normal haemodynamic laminar flow of blood (slow or absent flow - blood stasis - or abnormal turbulences), and (3) alterations in blood composition (e.g. increased number or adhesiveness of platelets, increased concentrations of procoagulant and/or anti-fibrinolytic factors) (Wheater *et al*, 1986). Thrombin is the key regulator of thrombosis. It activates platelet aggregation and cleaves fibrinogen to fibrin, which then cross-links to form the basis for the clot matrix (figure 1.8). Thrombi can either occlude the vessel at the site of endothelial injury or embolise distally through a combination of shear stress and the action of intrinsic antithrombotic mechanisms. With time, thromboemboli lyse spontaneously, but this can occur too late to prevent permanent infarction.

1.3.1 Classical risk factors

Overall, classical risk factors can be divided into three general categories, which are personal, lifestyle and physiological characteristics (appendix A, table A1). Two other terms that are often used when referring to these classical risk factors are environmental or acquired risk factors. These terms have a broader sense as they embrace changes induced by diverse "external" influences such as pregnancy and childbirth, surgery, diet and smoking, hormone ingestion, as well as inter-current disorders such as diabetes mellitus, hypertension and dyslipidaemia, and hyperhomocyst(e)inaemia, etc. Numerous epidemiological studies have shown that many of these factors, identified in individuals free of vascular disease, are predictors of the subsequent development of atherosclerotic and thromboembolic vascular diseases. A number of underlying diseases have

been shown to predispose to thrombosis. The following table lists some of these diseases.

Table 1.1: Non-exhaustive list of underlying conditions that can represent a risk factor for thrombosis[3&4].

	Arterial thrombosis	Venous thrombosis
Peripheral vascular diseases	Trauma, dissection, spasm, atherosclerosis, vasculitis (e.g. polyarteritis nodosa, Takayasu's arteritis, temporal arteritis, thromboangitis obliterans), etc.	Trauma, dissection, spasm, vasculitis (venulitis), etc.
Heart diseases	Atrial fibrillation, dilated cardiomyopathy, infective and non-infective endocarditis, myocardial infarction, valvular heart disease, ventricular aneurysm, etc.	Congestive heart failure, etc.
Connective tissue diseases	Rheumatoid arthritis, scleroderma, systemic lupus erythematosus (SLE), etc.	Collagen vascular disorders, SLE, etc.
Hypercoagulable / Hyperviscosity states[5]	Dysproteinaemia, myeloproliferative syndrome (e.g. polycythemia vera, thrombocytosis), thrombophilia (e.g. dysfibrinogenaemia, elevated level of antiphospholipid -aPL- and anti-β_2-glycoprotein I antibodies), antiphospholipid syndrome (APS), etc.	Chronic venous stasis, myeloproliferative conditions, sickle cell anaemia, thrombophilia (e.g. antithrombin III deficiency, protein C deficiency, protein S deficiency, elevated level of fibrinogen, elevated level of aPL and anti-β_2-glycoprotein I antibodies), APS, etc.
Other	Diabetes, dyslipidaemia, hyperhomocyst(e)inaemia[6], infections, etc.	Infection, neoplasm, etc.

Atherosclerosis is not only a disease in its own right, but it is also probably, of all risk factors, the principal contributor to the pathogenesis of cardiovascular diseases. It is a multifactorial process that may present itself in different ways depending to a large extent on its location and complications. Of these, the most important include acute occlusion, chronic narrowing of the vessel lumen, aneurysm formation and embolisation.

1.3.2 Genetic risk factors

Functional variants of a multitude of different genes have been considered as potential risk factors for atherosclerosis and thrombosis (Bertina, 1997 & 1999; Rosendaal, 1999; Franco *et al*, 2001). Table A2 in appendix A lists a number of these genetic risk factors. These variants modulate the expression of biologically active products that play a role in coagulation, vascular homeostasis, or lipid metabolism, such as platelet and endothelial surface proteins, pro- and anti-coagulation as well as pro- and anti-fibrinolytic factors, angiotensin-1-converting enzyme,

[3] Distinctions between arterial and venous are not as clear-cut as pictured in table 1.1 Underlying mechanisms are sometimes common to thrombosis occurring in both circulatory systems.

[4] Compiled data from Benditt *et al*, 1994; Creager, 1994; Carter, 1996; Millenson *et al*, 1996; Rabkin, 1996; Acevedo *et al*, 2001.

[5] Hypercoagulable / Hyperviscosity states are more convincingly related to venous than arterial thrombosis. Patients harbouring these defects will often have a history of peripheral venous thrombosis, pulmonary embolism, or recurrent foetal loss.

[6] Like hypercholesterolaemia, hyperhomocyst(e)inaemia is caused by both genetic and dietary factors (Boushey *et al*, 1995; Stampfer *et al*, 1995).

methylenetetrahydrofolate reductase, or apolipoproteins. For many of these genetic risk factors, the association of genotype with disease is not fully established (due to inconsistency in, or lack of confirmation of results), although they are highly suggested especially in selected patients and when in combination with acquired risk factors. Some genetic risk factors (such as factor V Leiden mutation or the G20210A mutation in the prothrombin gene) have however been proven to be independent risk factors for arterial and venous thromboembolism (Lane *et al*, 1996 & 2000).

1.3.3 Combination and interrelationship of risk factors

It is a well-known fact that the co-occurrence of several classical risk factors statistically increases the risk of thrombotic disorders (Rabkin, 1996; Cattaneo *et al*, 1997). Similarly, the co-inheritance of more than one relatively mild thrombophilic genetic risk factor causes more severe clinical expression. Reports in the literature support the hypothesis that familial thrombophilia is a multiple gene disorder and that the penetrance of thrombotic disease is higher in carriers of multiple gene defects (Lane *et al*, 1996). On the other hand, gene-environment interactions most probably have crucial importance in disease, with haemostatic gene polymorphisms influencing atherosclerosis or thrombotic risks by interacting with other established acquired risk factors. For instance, genetic factors regulating cytokine secretion and the plasma lipoprotein profile together with environmental factors, like infections and dietary factors, are important determinants of the inflammatory reaction in atherosclerotic vessels (Ross, 1993; Libby *et al*, 2002a&b; Hansson, 2005). As another example, neither factor V mutation nor 20210A prothrombin mutation are major risk factors for myocardial infarction or stroke, unless accompanied by other classical risk factors, including diabetes mellitus, hypertension and smoking (Rosendaal *et al*, 1997a&b). Similarly, the presence of deficiencies in antithrombin III, protein C or protein S seems to magnify the risk for thrombosis when taking oral contraceptives (Pabinger *et al*, 1994; Longmore *et al*, 2001), during pregnancy (Demers *et al*, 1992; De Stefano *et al*, 1994a&b), during surgery (De Stefano *et al*, 1994b) or when concomitantly present with activated protein C resistance (Zöller *et al*, 1994 & 1995; van Boven *et al*, 1996).

Thus, the combination of risk factors produces a more than additive increase in incidence of vascular disease. Subjects with both inherited defects - with profound life-long consequences - and exposure to transient or long-lasting environmental/acquired risk factors are at very high risk of thrombosis.

1.4 Autoimmune diseases and autoantibodies in the context of coagulopathies

Among the potential risk factors for thrombosis are several autoimmune disorders and the presence of antibodies specific to self-antigens that often characterise these disorders. Various autoantibodies have been implicated in the pathogenesis of acquired thrombotic complications (Salonen *et al*, 1992; Puurunen *et al*, 1994; Roubey *et al*, 1994; Vermylen *et al*, 1997). Among these autoantibodies are antibodies to phospholipids (aPL), the two most prominent representatives of which being anticardiolipin antibodies (ACLA) and lupus anticoagulant (LA) (appendices B & C).

Serum-derived aPL have been commonly identified in a variety of clinical settings including autoimmune and non-autoimmune diseases. In systemic lupus erythematosus (SLE) and the primary antiphospholipid syndrome (APS), ACLA alone or co-existing with LA (Galli *et al*, 1992b) have frequently been associated with a number of clinical manifestations, including recurrent arterial and venous thrombotic events, obstetrical complications, thrombocytopenia, cardiac abnormalities, a plethora of neurological syndromes, and osteoarticular and cutaneous manifestations (appendix D). However, an association in apparently healthy people has not been clearly established and comparatively little or non-convincing information is available on the prevalence of aPL in unselected patient populations and in prospective studies (Sletnes *et al*, 1992; Tsakiris *et al*, 1992; Galli *et al*, 2003). One should note here that detection of aPL is subject to considerable inter-laboratory variation (Peaceman *et al*, 1992) in part because of the lack of standardization of laboratory protocols. It is conceivable that the presence of other autoantibodies in serum may represent a source of confusion and that the antibodies binding to cardiolipin in solid-phase immunoassay may also target other antigenic structures available in the assay. Some may recognize phospholipids, others be directed against cross-reactive epitopes commonly found with oxidized LDL (Vaarala *et al*, 1993 & 1996b; Hörkkö *et al*, 1996 & 2000) or bind to plasma-phospholipid proteins such as β2GPI, an antigenic target of aPL that is often present among assay reagents (see next chapter).

The question of whether these autoantibodies actually play a true causal role in the development of atherothrombosis and obstetrical complications or whether they are mere epiphenomena is still open. However, a number of observations support the pathogenic role of aPL. For instance, the passive immunization of mice with human ACLA from patients with the APS promotes thrombosis (Pierangeli *et*

al, 1995 & 1996; Olee *et al*, 1996), results in accelerated atherosclerosis (George *et al*, 1997a), and induces murine ACLA as well as foetal loss and growth retardation (Branch *et al*, 1990; Harris *et al*, 1990a; Blank *et al*, 1991; Bakimer *et al*, 1992). Moreover, *in vitro* studies also suggest that aPL may contribute to the development of the atherosclerotic process in the APS by enhancing lipid accumulation and inflammation in the arterial vessel wall (Sailer *et al*, 2005). In addition, SLE patients, significant portions of whom have aPL, possess humoral markers associated with atherosclerosis (George *et al*, 1999d) and suffer from premature atherosclerosis and thrombotic disorders that are not adequately explained by a deranged lipid profile or medication (Jonsson *et al*, 1989; Bruce *et al*, 2000; Asanuma *et al*, 2003). As will be described below, several mechanisms by which aPL might predispose to thrombosis have been proposed. They revolve around the interference with different types of cells involved in haemostasis and the interference with different steps of the coagulation cascade.

1.5 β_2-glycoprotein I (β2GPI)

1.5.1 β2GPI as a cofactor for antiphospholipid antibodies

Antiphospholipid antibodies (aPL) associated with the APS and SLE were originally thought to be directed exclusively against anionic phospholipids. Over the past two decades, however, evidence from a number of laboratories has shown that they also tend to be directed against an array of plasma protein cofactors (listed in appendix B). Much attention has been given to β2GPI since it was identified as a cofactor for LA activity (Galli *et al*, 1992b; Oosting *et al*, 1992) and for the binding to cardiolipin of some aPL purified from patients with autoimmune disorders (Galli *et al*, 1990; McNeil *et al*, 1990; Verrier *et al*, 1992). As will be described below, the initial hypothesis was that β2GPI possessed anticoagulant functions *in vivo* that were somehow negated by the binding of β2GPI-specific aPL. The latter are indeed strongly associated with thrombosis and other features of the APS (Matsuda *et al*, 1993c; Martinuzzo *et al*, 1995; Pierangeli *et al*, 1996; Guerin *et al*, 1997; Carreras *et al*, 2000), and experiments with animal models support the pathogenic role of anti-β2GPI antibodies. In fact, injections of non-homologous β2GPI in mice have been shown to cause the appearance of aPL and of anti-β2GPI antibodies, as well as accelerated atherosclerosis (George *et al*, 1998b; Afek *et al*, 1999) and manifestations of the APS (Blank *et al*, 1994; Aron *et al*, 1995; Garcia *et al*, 1997). Nevertheless, the presence of β2GPI-specific aPL is not currently included in the criteria for the APS (Wilson *et al*, 1999) (listed in appendix C). Interestingly, β2GPI dependence seems to be useful in distinguishing aPL associated with autoimmune diseases from those associated with infection (Loizou *et al*, 1990; Vaarala, 1991; Hunt *et al*, 1992; Forastiero *et al*, 1996), even though this characterization is not absolute (Hojnik *et al*, 1994).

1.5.2 β2GPI structure

β2GPI is a soluble 50 kDa plasma glycoprotein composed of 326 amino acids arranged in five repeating domains (figure 1.2) which characterise the members of the complement control protein or short consensus repeats (SCR) superfamily (appendix E).

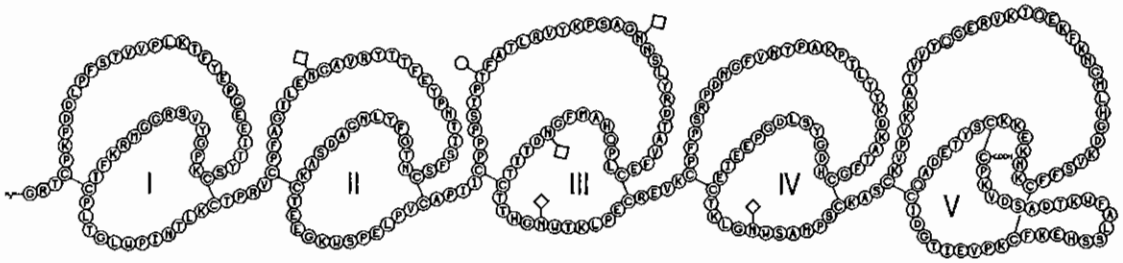
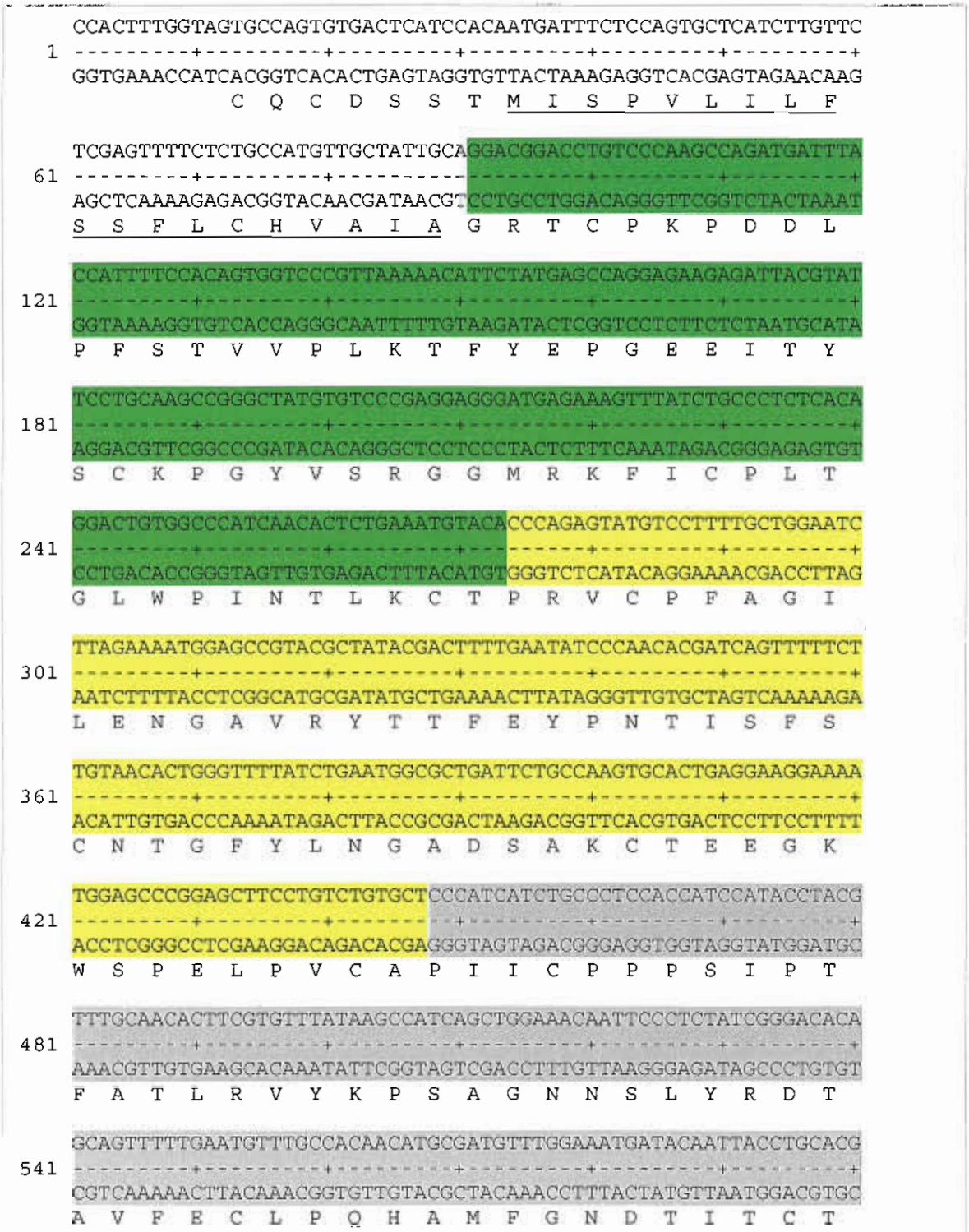


Figure 1.2: Schematic representation of human β2GPI showing the organisation of the molecule into five short consensus repeats, each with two intra-domain disulphide bridges except for the C-terminal domain which contains three intra-domain disulphide bridges. The *N*-linked and *O*-linked glycosylation positions are indicated with squares (◊) and circles (o) respectively. Adapted from Bendixen *et al.* ([1992]).

It was first isolated as a perchloric acid-soluble glycoprotein in human serum by Schultze in 1961 ([Schultze *et al*, 1961]). Its unique nucleotide sequence has been established by peptide and cDNA sequencing methods ([Kristensen *et al*, 1991; Steinkasserer *et al*, 1991; Lozier *et al*, 1984]) (figure 1.3).



Continued on next page

Figure 1.3: Nucleotide and deduced amino acid sequences of human β2GPI (see figures 1.5 & 4.2). Domains are colour-differentiated. An asterisk marks the stop codon that ends β2GPI. The amino acid sequence is presented in the standard one-letter code. The putative 19-amino acid-residue leader sequence is underlined. It starts with a methionine residue (-19) and ends with an alanine residue (-1).
Source: GenBank accession N° NM_000042.

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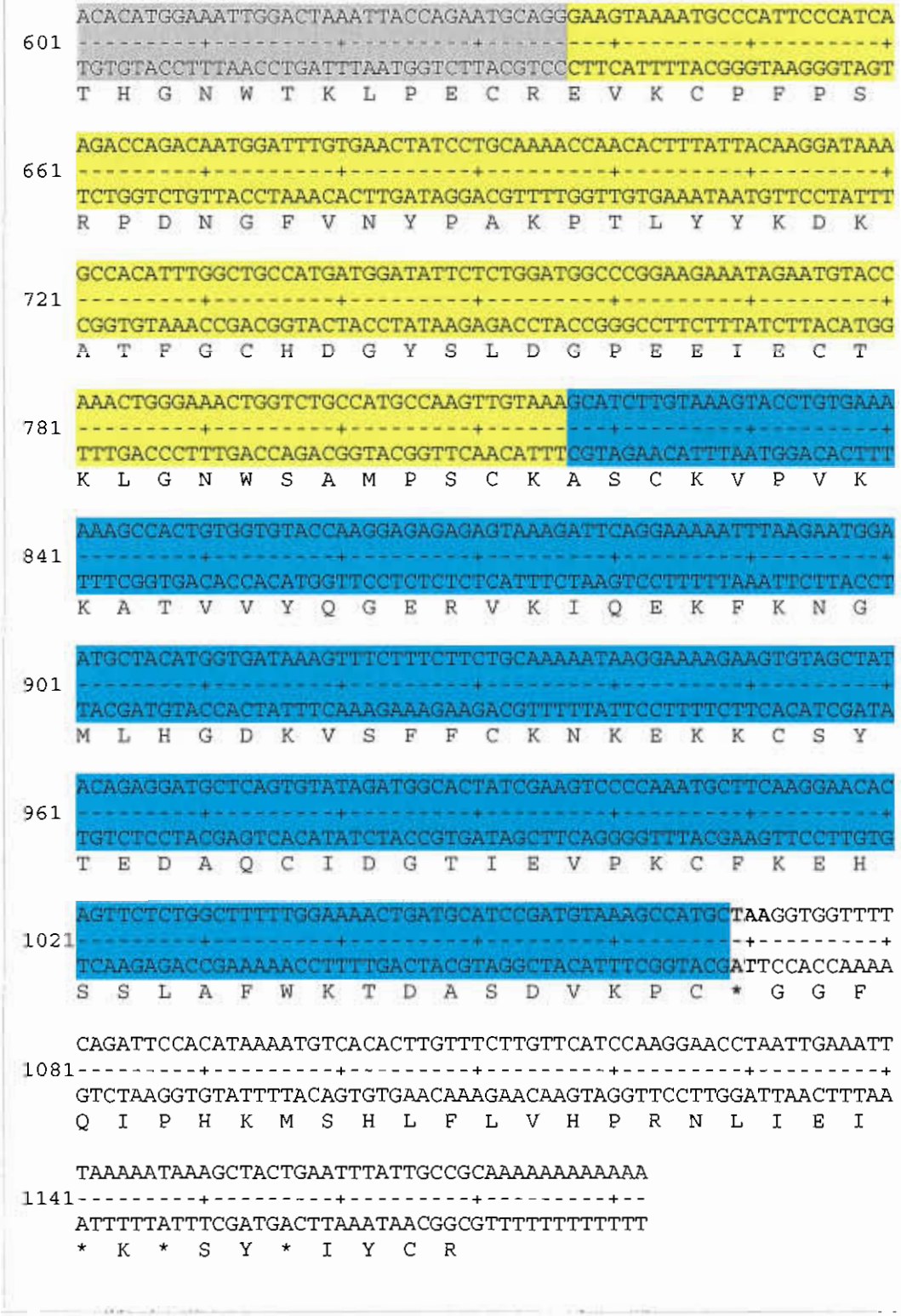

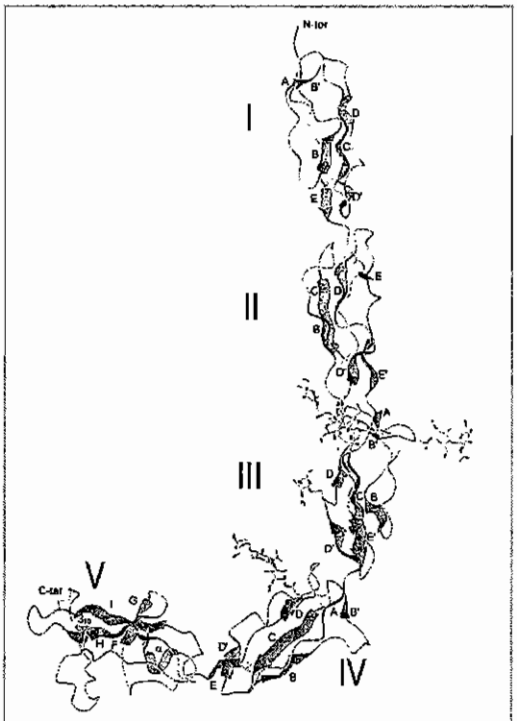

Figure 1.4: Fish-hook appearance of β2GPI. Ribbon drawing of β2GPI with consecutive domains labelled CCP 1 to 5. The strands are shown as arrowed bands and helices as strands. From Schwarzenbacher *et al.* ([1999]).

Proline is the most abundant amino acid (31 per β2GPI molecule), which makes β2GPI one of the most proline-rich eukaryotic proteins together with collagen and related molecules. The second most abundant amino acid is lysine with a total of 30 residues per molecule, half of which being in the fifth domain. Crystal structure analysis has shown that β2GPI has an elongated J-shaped or fish-hook-like arrangement with overall dimensions of 130 x 85 Å, with β2GPI domains put together like beads on a string and the C-terminal fifth domain deviating strongly from the standard fold ([Bouma *et al*, 1999; Schwarzenbacher *et al*, 1999]) (figure 1.4).

Unlike other apolipoproteins, β2GPI does not self-associate in aqueous solution and its secondary and tertiary organisations are not affected by the presence or absence of lipids at neutral pH ([Lee *et al*, 1983]). Moreover, β2GPI associates with plasma lipoproteins through protein-protein interactions rather than protein-lipid interactions ([Lee *et al*, 1983]). Its circular dichroic spectrum in the far-ultraviolet is unusual with a weak maximum at 235 nm and a relatively weak minimum at 205 nm, suggesting that β2GPI's secondary structure is different from that of other apolipoproteins ([Lee *et al*, 1983]). It consists mainly of β-sheets and random coils, with little α-helixes ([Lozier *et al*, 1984; Bouma *et al*, 1999; Schwarzenbacher *et al*, 1999]).

β2GPI has been extensively characterised at both peptide and nucleotide levels from a number of other mammalian species, including cow ([Kato *et al*, 1991]), dog ([Sellar *et al*, 1993]), mouse ([Nonaka *et al*, 1992]), and rat ([Aoyama *et al*, 1989]). As can be seen in figure 1.5, peptide and nucleotide sequences in these species are identical to those of humans for about 83%, 80%, 78% and 76% respectively. The most striking homologous characteristic across species is the highly-conserved disulphide bond organisation (see below).

Leader sequence:

```
-19.....-10.....-1
1>MISPVLILFSSFLCHVAIA
2>-PP-A-V-LLG--------
3>---LG------V-----T-
4>-V----A---A--------
5>
```

Domain I:

```
  1.....10.....20.....30.....40.....50.....60..
1>GRTCPKPDDLPFSTVVPLKTFYEPGEEITYSCKPGYVSRGG_MRKFICPLTGLWPINTLKCT
2>--------E----------RT-----Q-VF--Q--------_I-R-T--------------M
3>---------I--A---------D---Q-A-T-Q----F--L_T-R-T-----V--T--VR-I
4>--I---------A-------S-D---Q-V-----------_----------M------R-V
5>                              MIS-ALIFFSAFLCHVAIAGRRM---------
```

Domain II:

```
  .....70.....80.....90.....100.....110.....120
1>PRVCPFAGILENGAVRYTTFEYPNTISFSCNTGFYLNGADSAKCTEEGKWSPELPVCA
2>-------------T----------------H-----K--S-----------D-----
3>----------------------------A---------SS----------VD----T
4>-------------I----S----KN---A--P--F---TS-S----------DI-A--
5>-------------V-----------G-A--P-Y----TS-S----------------
```

Domain III:

```
  121.....130.....140.....150.....160.....170.....180...
1>PIICPPPSIPTFATLRVYKPSAGNNSLYRDTAVFECLPQHAMFGNDTITCTTHGNWTKLPECR
2>--T----P--K--S-S----L-----F-GSK---K---H--------V---E-----Q-----
3>RVT-----V-K----S-F--L-T-----GNK-------HY----------A-----T-----
4>R-T----PV-K--L-KD-R---------Q--V--K---HF--I----VM--EQ----R----L
5>R-T----P--K--A-KE--T-V--S-F-Q--V--K---HF-------V---A-----Q-----
```

Domain IV:

```
  ....190.....200.....210.....220.....230.....240...
1>EVKCPFPSRPDNGFVNYPAKPTLYYKDKATFGCHDGYSLDGPEEIECTKLGNWSAMPSCK
2>--R-------------H--N-V-----T------ET--------V--S-F-----Q----
3>--------------------QI-------MY----T-T-----VV--N-F-----Q----
4>-------P--E--Y-------V-L----------ET-K------A----T-T--FL-T-R
5>----------------------V-S-----V----ET-K------V----T-----L----
```

Domain V:

```
....250.....260.....270.....280.....290.....300.....310
1>ASCKVPVKKATVVYQGERVKIQEKFKNGMLHGDKVSFFCKNKEKKCSYTEDAQCIDGTIEVPKCFKE
2>----LSI-R---I-E----A--N---------Q-------H----------E-------I------
3>----LS------L-------L-----D-----Q-IH-Y----------VE-H-R-----I------
4>E---L-------L---M------Q-----M-----H-Y----------E---------I-S----
5>----LS------L---Q-----DQ-----M-----H-Y----------E---------I------

  .....320....327
1>HSSLAFWKTDASDVKPC
2>-----------------
3>-----------------
4>------------ELT--
5>--------------T--
```

Figure 1.5: Deduced amino acid sequences of (1>) human, (2>) bovine, (3>) dog, (4>) mouse and (5>) rat β2GPI (structured in domains). The complete sequence is shown for human β2GPI (see figure 1.3). For β2GPI of other mammals, only residues differing from human β2GPI are shown. Gaps, represented by underscore bars, have been inserted to maximise homology. Sources: GenBank accession N° NM_000042, Sellar *et al.* ([1994]) and Steinkasserer *et al.* ([1991 & 1992a]).

The first four domains of β2GPI are the typical highly conserved SCR (also called "sushi" domains) of the complement control protein superfamily. They are highly homologous to one another with a consensus pattern of four invariant cysteine residues and a small number of additional conserved residues ([Bork *et al*, 1996]) (figure 1.6).

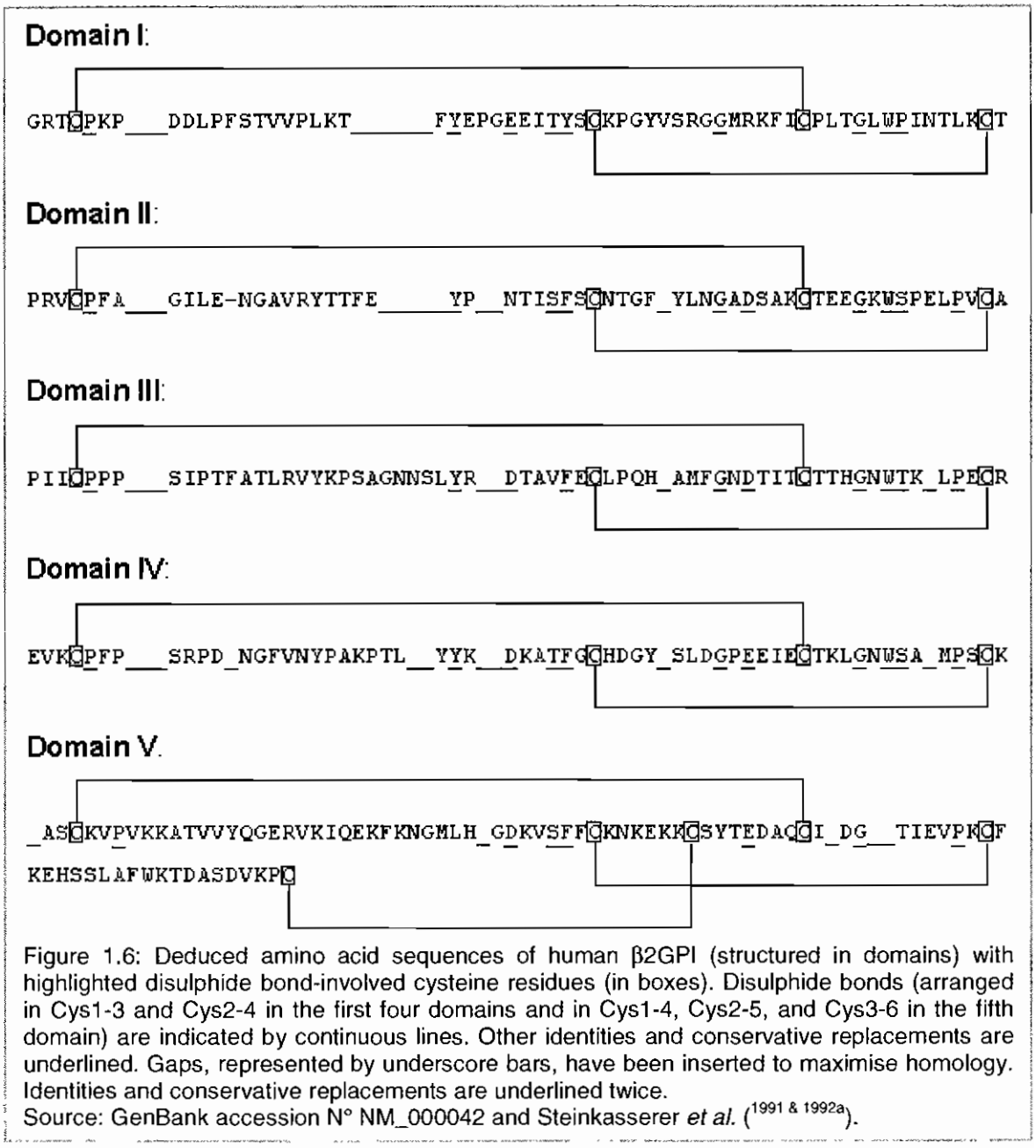

Figure 1.6: Deduced amino acid sequences of human β2GPI (structured in domains) with highlighted disulphide bond-involved cysteine residues (in boxes). Disulphide bonds (arranged in Cys1-3 and Cys2-4 in the first four domains and in Cys1-4, Cys2-5, and Cys3-6 in the fifth domain) are indicated by continuous lines. Other identities and conservative replacements are underlined. Gaps, represented by underscore bars, have been inserted to maximise homology. Identities and conservative replacements are underlined twice.
Source: GenBank accession N° NM_000042 and Steinkasserer *et al.* ([1991 & 1992a]).

Each domain is composed of some 60 amino acids each, with the cysteine residues arranged in a characteristic Cys1-3, Cys2-4 disulphide bonding pattern ([Janatova *et al*, 1989; Kato *et al*, 1991; Steinkasserer *et al*, 1992a]). The fifth domain is aberrant in that it contains 82 amino acids, two extra cysteine residues (with the cysteine residues arranged in a Cys1-4, Cys2-5, and Cys3-6 disulphide bonding pattern), a high proportion of lysine residues (18%), and a 19-residue long C-terminal tail ([Steinkasserer *et al*, 1992a; Hunt *et al*, 1994]). Of particular interest in the fifth domain is its central β-spiral core of four anti-parallel β-sheets with two small helixes that fold in such a way that the highly positively charged sequence KNKEKK (residues 282 to 287), constrained by a disulphide bridge, is likely to be present as a surface-exposed turn that is ideally positioned for interaction with negatively charged substances ([Barlow *et al*, 1991; Steinkasserer *et al*, 1991; Schwarzenbacher *et al*, 1999]). Three-dimensional models have also suggested that the adjacent sequence SSLAFWKT (residues 311 to 318) in the C-terminal tail forms a hydrophobic loop that can readily insert into phospholipid bilayers ([Bouma *et al*, 1999; Schwarzenbacher *et al*, 1999]) (figure 1.7).

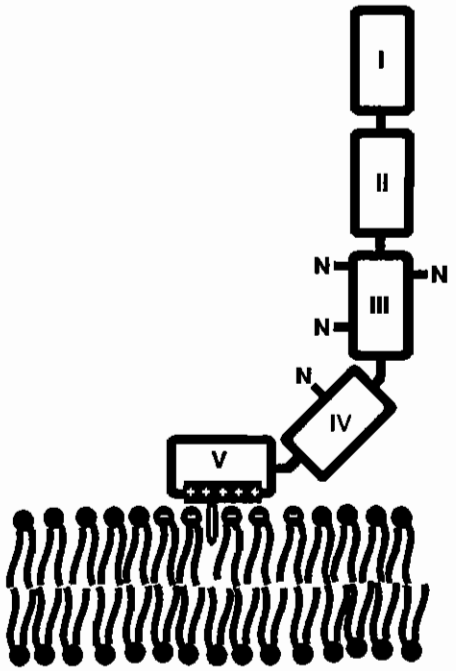

Figure 1.7: Mechanism of binding of β2GPI to phospholipid surface. Adapted from Bouma *et al.* ([1999]).

1.5.3 Interaction of β2GPI with macromolecules

Numerous observations have been reported as regards the binding capacity of β2GPI to negatively charged macromolecules and surfaces. β2GPI has long been known to bind to anionic phospholipids (Polz *et al*, 1979b; Schousboe, 1979 & 1983b; Wurm, 1984) that are largely present in the plasma in the form of lipoproteins (Valet *et al*, 1997), of which β2GPI is thought to be a component (see below). However, β2GPI has also been shown to bind to a number of other ligands:

- dextran sulfate (Schousboe *et al*, 1988a),
- heparin (Polz *et al*, 1979b & 1980; Wurm, 1984; McNally *et al*, 1994a),
- factor XI (Shi *et al*, 2004 & 2005),
- megalin (Moestrup *et al*, 1998),
- calmodulin (Klaerke *et al*, 1997; Rojkajer *et al*, 1997),
- annexin II (Ma *et al*, 2000),
- oxidatively modified LDL (Hasunuma *et al*, 1997; Kobayashi *et al*, 2003; Matsuura *et al*, 2003),
- apolipoprotein (a) (Köchl *et al*, 1997),
- apolipoprotein E receptor 2' (van Lummel *et al*, 2005),
- DNA (Krøll *et al*, 1976),
- ATP (Chapman *et al*, 2005), and
- pathogenic proteins such as hepatitis B surface antigen (Mehdi *et al*, 1994; Gao *et al*, 2003) and proteins p18, p26 and gp160 of HIV (Stefas *et al*, 1997).

Macroscopically, β2GPI has also been shown to bind to cell membranes such as those of:

- endothelial cells (Le Tonqueze *et al*, 1995; George *et al*, 1999b),
- platelets (Schousboe, 1980 & 1983b; Nimpf *et al*, 1985; Vázquez-Mellado *et al*, 1994) and
- platelet-derived microparticles (Nomura *et al*, 1993 & 1994),
- macrophages (Balasubramanian *et al*, 1998),
- astrocytes and neurones (Caronti *et al*, 1998),
- trophoblast cells (Chamley *et al*, 1993b & 1997; La Rosa *et al*, 1994),
- mitochondria (Schousboe, 1983b), and to
- membranes of senescent/apoptotic cells (Price *et al*, 1996; Levine *et al*, 1998; Pittoni *et al*, 2000).

The physiological importance of most of these interactions is still unknown.

1.5.3.I β2GPI binding to phospholipids and phospholipid-binding sites

The avidity with which β2GPI binds to phospholipids ([Polz *et al*, 1979b; Schousboe, 1979 & 1983b; Wurm, 1984]) is highly dependent on their nature. On the one hand, β2GPI binds to purified anionic phospholipids such as phosphatidylserine and phosphatidylinositol ([Gharavi *et al*, 1987]).[7] However, this affinity is much weaker than previously thought ([Long *et al*, 1995; Willems *et al*, 1996; Arnout *et al*, 1998]), especially when compared to that of clotting factors like prothrombin, protein C, protein S, factor X/Xa, factor Va, and annexin V ([Harper *et al*, 1998]). On the other hand, β2GPI does not bind, or if so with a very low affinity, to zwitterionic phospholipids such as phosphatidylcholine, phosphatidylethanolamine and sphingomyelin ([Polz *et al*, 1979b; Wurm, 1984; Gharavi *et al*, 1987; Willems *et al*, 1996; Harper *et al*, 1998]). This has relevance since cells such as platelets or neutrophils usually display cell membrane asymmetry with a minimal amount of anionic phospholipids on the outer leaflet ([Chap *et al*, 1977; Bevers *et al*, 1983; Zwaal *et al*, 1997a&b]) and lose this asymmetry and expose anionic phospholipids in certain situations such as cell activation ([Bevers *et al*, 1982, 1983 & 1993]), senescence ([Connor *et al*, 1994; Schroit *et al*, 1985]), or apoptosis ([Fadok *et al*, 1992; Savill *et al*, 1993; Price *et al*, 1996]). Because β2GPI's capacity to bind to cardiolipin remains unaffected by heat inactivation and reduction/alkylation, it was suggested that this capacity relies on a stable domain, present as a linear sequence motif ([Kertesz *et al*, 1995]). At a molecular level, it is now generally accepted that domain V contains the main binding site of β2GPI to phospholipids ([Hunt *et al*, 1994]) (figure 1.7). It has been proposed that this binding occurs through a simple membrane binding mechanism via a large positively charged lysine-enriched patch (that includes lysine residues from the Cys^{281}-Cys^{288} loop and Lys^{308} and Lys^{324}). This patch interacts with the negatively charged head end of phospholipids and a flexible hydrophobic loop (at Ser^{311}-Lys^{317}) on the surface of the aberrant half of domain V that inserts itself and anchors into the phospholipid layer ([Hunt *et al*, 1993 & 1994; Kertesz *et al*, 1995; Sheng *et al*, 1996; Del Papa *et al*, 1998]). The latter statement was partly supported by the observations that mutations in the fifth domain (as detailed in chapter 4) ([Sanghera *et al*, 1997a&b; Mehdi *et al*, 2000a]) and a cleavage of the peptide bond between Lys^{317} and Thr^{318} ([Hagihara *et al*, 1997; Ohkura *et al*, 1998; Matsuura *et al*, 2000]) resulted in a decrease or loss of β2GPI binding to phospholipid layers.

---

[7] β2GPI even has the ability to insert itself into phospholipid monolayers. Such binding is stronger when a higher content of negatively charged lipids is present in the membrane ([Balasubramanian *et al*, 1997; Wang *et al*, 1998a]).

1.5.3.II Binding of aPL/anti-β2GPI antibodies to β2GPI

Because a significant relationship was reported between the presence of β2GPI-dependent ACLA and a history of thrombosis (Roubey, 1994; Martinuzzo *et al*, 1995), it has been proposed that some aPL/anti-β2GPI antibodies exert their pathogenic effect by binding not directly to phospholipids but rather to β2GPI, associated or not with phospholipids (Matsuura *et al*, 1992; Triplett, 1993; Roubey, 1994). In other words, β2GPI may be the required cofactor for some LA and ACLA (Galli *et al*, 1992b; Oosting *et al*, 1992; Roubey *et al*, 1992). However, whether the target of many aPL is β2GPI alone or a complex between anionic phospholipids and β2GPI (Galli *et al*, 1990; Matsuura *et al*, 1992 & 1994; McNeil *et al*, 1990; Roubey *et al*, 1992) is still a matter of debate, although current thinking seems to favour the former hypothesis. Some authors have found that β2GPI was recognized by ACLA even in the absence of phospholipids in patients with the primary APS (Cabral *et al*, 1992; Keeling *et al*, 1992), but others did not find any particular ACLA-reactivity to β2GPI (Gharavi *et al*, 1993). β2GPI is also considered by some only as an enhancer, not a prerequisite, for ACLA binding to cardiolipin (Sammaritano *et al*, 1992; Pierangeli *et al*, 1992).

Several non-exclusive hypotheses have been proposed to describe the interactions between β2GPI and aPL/anti-β2GPI antibodies. The first hypothesis relies on the evidence that interaction of β2GPI with negatively charged surfaces results in a major conformational change in the three-dimensional structure of the protein (Keeling *et al*, 1992; Borchman *et al*, 1995; Chamley *et al*, 1999; Hammel *et al*, 2001), which could result in the expression of otherwise cryptic epitopes (Wagenknecht *et al*, 1993; Ichikawa *et al*, 1994; Matsuura *et al*, 1994 & 1995; Pengo *et al*, 1995). The revealed epitopes would be targeted by aPL/anti-β2GPI antibodies. The second hypothesis considers binding of aPL/anti-β2GPI antibodies on native β2GPI (Galli *et al*, 1990; Arvieux *et al*, 1991; Tincani *et al*, 1996). Anionic structures would increase the density of β2GPI which would allow these low-affinity antibodies to target the protein (Roubey *et al*, 1995 & 1996; Tincani *et al*, 1996; Sheng *et al*, 1998; Reddel *et al*, 2003). In a third hypothesis, a cross-linking interaction between phospholipid-bound-β2GPI and aPL/anti-β2GPI antibodies may cause an increase in the affinity of β2GPI for phospholipids (Willems *et al*, 1996; Takeya *et al*, 1997; Arnout *et al*, 1998; Harper *et al*, 1998).

At a molecular level, there is no consensus as to which domains of β2GPI are predominantly recognised by aPL/anti-β2GPI antibodies. All domains have been shown to contain epitopes recognised by aPL/anti-β2GPI antibodies (Hunt *et al*, 1994;

George *et al*, 1998a; Iverson *et al*, 1998 & 2002; Reddel *et al*, 2000; McNeeley *et al*, 2001). However, domains III and IV are heavily glycosylated (Hammel *et al*, 2002) and therefore partially shielded from protein-protein interactions. Moreover, anti-β2GPI autoantibodies do not seem to bind directly to solid-phase domain V (Sheng *et al*, 1996; Sorice *et al*, 1996; George *et al*, 1998a), and mouse and human monoclonal ACLA from the APS subjects were found to bind β2GPI mutants with deleted domain V (Igarashi *et al*, 1996). Crystal structure analysis reveals that domain V intimately interacts with phospholipid membranes (Bouma *et al*, 1999; Schwarzenbacher *et al*, 1999), thereby rendering domain V quite unlikely to contain a major epitope for aPL/anti-β2GPI antibody binding under physiological conditions. By contrast, when β2GPI molecules interact with phospholipids, domains I and II are far away from the membrane into the solution, and well positioned for easy interactions with circulating autoantibodies. Interestingly, surface-exposed amino acid residues in domain I constitute a negatively charged region that may be of significance since pathogenic aPL are known to contain clusters of positively charged arginine and lysine residues in their antigen-binding region (Guerin *et al*, 2000; Iverson *et al*, 2002; Giles *et al*, 2003).

1.5.4 Hypothetical roles of β2GPI

Despite numerous *in vitro* studies on the functions of β2GPI, the exact physiological roles of this protein are still unclear. It is thought to be involved in blood coagulation and lipid/lipoprotein metabolism. β2GPI's interaction with lipid membranes containing anionic phospholipids has been considered as the basic mechanism for the biological functions of β2GPI. However, β2GPI could actually have more indirect than direct functions, e.g. as an element of immune-mediated reactions. Anti-β2GPI and/or β2GPI-dependent aPL may indeed target β2GPI epitopes and thereby affect β2GPI-exposing cells or activate the complement pathway at those locations. As no definite metabolic function has yet been attributed to β2GPI through tangible *in vivo* experiments, we must emphasise here that many of the intriguing functional scenarios that are mentioned in this section are currently speculative.

1.5.4.I Effect of β2GPI on blood coagulation

Numerous *in vitro* studies have shown that β2GPI may play several - apparently contradictory - roles in the coagulation cascade owing to its capacity to bind to anionic phospholipids. These phospholipids are essential catalytic surfaces for most coagulation reactions (Schafer, 1994), and the binding of β2GPI to these phospholipids may displace coagulation factors from these surfaces. β2GPI could influence blood coagulation either directly (through direct binding to effective elements of the cascade) or indirectly (when involving aPL/anti-β2GPI antibodies).

1.5.4.I.a Direct effect of β2GPI on blood coagulation

Among the first properties of β2GPI that have been reported is its capacity to bind to activated platelets (Schousboe, 1980; Galli *et al*, 1993; Vázquez-Mellado *et al*, 1994). Such binding was shown to have anticoagulant consequences as it was accompanied *in vitro* by the inhibition of adenosine diphosphate-induced platelet aggregation (Nimpf *et al*, 1985 & 1987) and of the prothrombinase activity of activated platelets (Nimpf *et al*, 1986; Shi *et al*, 1993). Other *in vitro* experiments have also shown that β2GPI can inhibit the contact activation of the intrinsic blood coagulation pathway (Schousboe, 1985) at the level of factor XII activation (Henry *et al*, 1988; Schousboe *et al*, 1988a&b) and auto-activation (Schousboe *et al*, 1995) and factor XI activation (Miyakis *et al*, 2004), and can inhibit factor XIIa-mediated activation of prekallikrein (Schousboe, 1988c). Moreover, β2GPI has been shown to interact with C4b-binding protein (Walker, 1993) and protein S (Atsumi *et al*, 1997; Merrill *et al*, 1999), thereby inhibiting the interaction between protein S and C4b-binding protein.

This could represent a mechanism of modulation of the anticoagulant proteins C/S system.

On the other hand, procoagulant properties have also been reported for β2GPI. For instance, β2GPI would inhibit thrombomodulin-mediated activation of protein C (Keeling *et al*, 1993) as well as factor Va degradation activity of activated protein C (Mori *et al*, 1996). Additionally, β2GPI has been shown to have some inhibitory effect on intrinsic fibrinolysis but only in concentrations exceeding physiological ones (Shiozaki *et al*, 1994).

The fact that *in vitro* coagulation reactions in normal plasma compared to β2GPI-depleted plasma are barely different (Oosting *et al*, 1992; Roubey *et al*, 1992) could be partly explained by the low affinity of β2GPI for coagulation-promoting phospholipid surfaces (Long *et al*, 1995; Willems *et al*, 1996; Arnout *et al*, 1998). The affinity of β2GPI for phospholipid is indeed highly dependent on the composition of the latter (see above) and the binding of β2GPI to phospholipids can easily be prevented by increasing ionic strength (Kertesz *et al*, 1995). Recent papers have identified two mechanisms by which β2GPI seems to bind to endothelial cell membranes: through the putative phospholipid-binding site located in the fifth domain of the molecule as mentioned above, and through annexin II (a receptor of plasminogen/tissue plasminogen activator) (Ma *et al*, 2000). These observations, added to the hypotheses that endothelial cells may synthesise β2GPI (Caronti *et al*, 1999) and that β2GPI might represent a protective and viability-maintaining factor for endotheliocytes and hepatocytes *in vitro* (Cai *et al*, 1995; Averna *et al*, 2004), suggest that β2GPI might have a more important physiological role than initially hypothesized.

Although congenital β2GPI deficiency - a rare inheritable trait (Cleve *et al*, 1968) - has not been identified as an independent risk factor for coagulopathies (Bancsi *et al*, 1992, Takeuchi *et al*, 2000), many *in vivo* observations of β2GPI functions tend to support the idea that β2GPI does play an active role in haemostatic reactions. The most prominent of these observations include the presence of β2GPI on the normal intraluminal surface of placental vessels, the syncytiotrophoblast and extravillous cytotrophoblast (i.e. areas highly susceptible to thrombosis) (Chamley *et al*, 1993b & 1997; La Rosa *et al*, 1994), and the reduction of β2GPI level in patients with disseminated intravascular coagulation (Schousboe, 1985; Matsuda *et al*, 1993a; Brighton *et al*, 1996). Further research is obviously required in order to substantiate this question.

1.5.4.I.b Effect of aPL/anti-β2GPI antibodies on blood coagulation

The association between aPL/anti-β2GPI antibodies and thrombosis in man has been more than extensively documented. However, the mechanisms of action of these autoantibodies are not fully understood yet. aPL/anti-β2GPI antibodies have been proposed to interfere with different components of the coagulation cascade summarized in figure 1.8. Some of these interfering reactions depend on the concurrent presence of β2GPI.

Although many studies have been, and are still performed to determine how aPL might increase thrombus formation *in vitro*, the mechanism of action of these antibodies remains unclear. Studies have proposed that the effect of aPL/anti-β2GPI antibodies on several steps of the coagulation cascade, as well as on the activation of endotheliocytes, platelets, and monocytes, inducing pro-adhesive, pro-inflammatory and pro-coagulant characteristics in these cells, may contribute to the prothrombotic state in the APS.

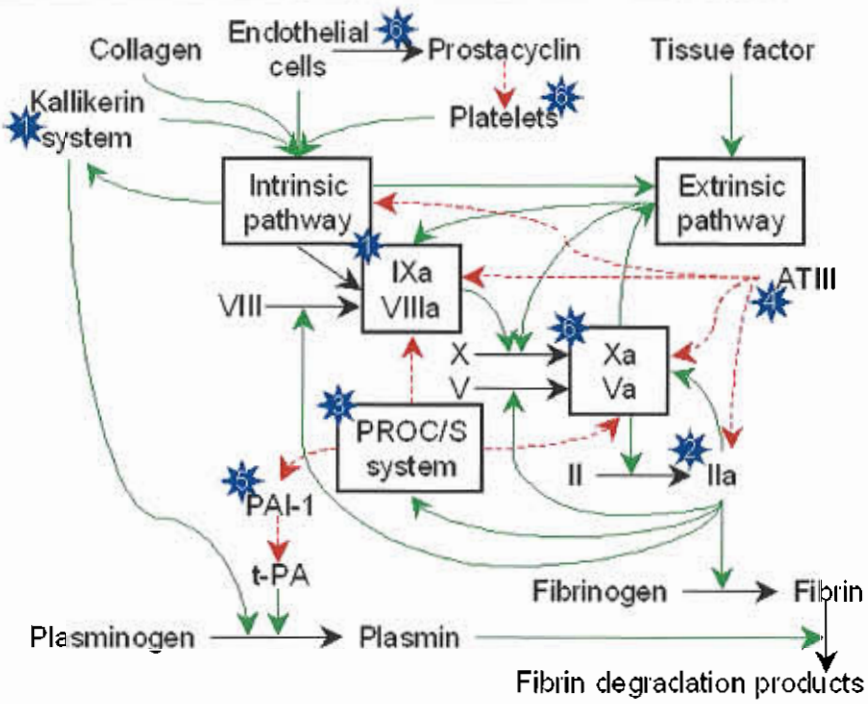

Figure 1.8: Highly simplified diagram illustrating the most commonly proposed sites of interaction (✳) of aPL/anti-β2GPI antibodies in the coagulation process. Injury to the vessel wall results in the exposure of collagen and the release from endothelial cells of a number of specific factors that are able to promote the activity of the intrinsic and extrinsic pathways. Activation and perturbation of cellular membranes result in the exposure of anionic phospholipids, which become available to promote various steps in the clotting process. These, and other points in the process, may be targeted by aPL. In most cases, such interaction results in enhancement of clotting; the contrary happens when aPL target phospholipids in the prothrombinase and factor X-converting complexes, which is a proposed mechanism for LA activity. Arrows indicate promotion (green) or inhibition (red) of pathway (black) or molecule.

1. **Interference with components of the kallikrein system and the intrinsic pathway** (Jones *et al*, 2000 & 2002; Sugi *et al*, 2001)
2. **Modulation of thrombin** (IIa) **generation and activity** (Ginsberg *et al*, 1993, Ferro *et al*, 1994; Musial *et al*, 1997; Field *et al*, 1999)
3. **Interference with components of the proteins C/S** (PROC/S) **pathway** (de Groot *et al*, 1996)
   - Interference with the activation of PROC by the thrombomodulin-thrombin complex (Cariou *et al*, 1986 & 1988; Freyssinet *et al*, 1986a&b; Tsakiris *et al*, 1990)
   - Inhibition of the assembly of the PROC complex (Smirnov *et al*, 1994)
   - Inhibition of the activated PROC pathway, directly (Matsuda *et al*, 1995a; Pötzsch *et al*, 1995; Ieko *et al*, 1999a&b, Nojima *et al*, 2005) or through its cofactor, PROS (Ames *et al*, 1996; Atsumi *et al*, 1997)
   - Binding to the substrates of activated PROC, factor Va and VIIIa, thereby protecting them from inactivation (Matsuda *et al*, 1995b; Pötzsch *et al*, 1995)
4. **Inhibition of antithrombin III** (ATIII) **activity** (Chamley *et al*, 1993a; Shibata *et al*, 1994a&b)
5. **Impairment of fibrinolysis through thrombomodulin-inducible fibrinolysis inhibitor and plasminogen activator inhibitor-1 activity** (PAI-1) (Ieko *et al*, 1999b & 2000; Lopez-Lira *et al*, 2006)
6. **Modulation of the cellular activity of cells involved in haemostasis**
   - Endotheliocytes (Del Papa *et al*, 1997; Simantov *et al*, 1995; Navarro *et al*, 1996; Pierangeli *et al*, 1999; Cho *et al*, 2002)
   - Polymorphonuclear cells (Arvieux *et al*, 1995; Simantov *et al*, 1995; Pierangeli *et al*, 1999; Kaplanski *et al*, 2000)
   - Monocytes (Kornberg *et al*, 1994, Cuadrado *et al*, 1997; Reverter *et al*, 1998; Dobado-Berrios *et al*, 2001)
   - Platelets (Arvieux *et al*, 1993; Martinuzzo *et al*, 1993; Shi *et al*, 1993; Vázquez-Mellado *et al*, 1994; Reverter *et al*, 1998)

1.5.4.II Possible effect of β2GPI on lipid metabolism

Around 65% of total plasma β2GPI is thought to circulate in a free form (Polz *et al*, 1979b&c). The rest is found bound to all the major lipoprotein fractions as one of their regular structural components: 16% of total β2GPI is associated with chylomicrons and very-low-density lipoproteins, 2% with low-density lipoproteins, and 17% with high-density lipoproteins (Polz *et al*, 1979a). Although these data are widely accepted, other researchers have found divergent proportions from only 10% of plasma β2GPI in a free form (McNally *et al*, 1995a) to no more than 5% of plasma β2GPI associated with lipoproteins in normal subjects (Cassader *et al*, 1994; Gambino *et al*, 1999a). However, whatever the distribution between free and bound forms, β2GPI may play an important role in lipid metabolism as a constituent of several lipoprotein particles.

Triglyceride. Studies have reported that β2GPI has a high affinity for triglyceride-rich particles, causing their selective precipitation by sodium lauryl sulfate or sodium dodecyl sulfate (SDS) (Burstein *et al*, 1972), and some authors have described a moderate to strongly positive correlation between β2GPI and triglyceride levels (Ichikawa *et al*, 1992; McNally *et al*, 1994b; Cassader *et al*, 1997; Mehdi *et al*, 1999). Earlier data showed that β2GPI level increases in post-prandial plasma and that the difference between fasting and post-prandial plasma was exclusively due to the amount of β2GPI present in chylomicrons (Polz *et al*, 1979c). β2GPI has also been demonstrated to enhance the clearance of triglycerides in rats (Wurm *et al*, 1982). These observations, together with the proven production of the protein by intestinal cells (Averna *et al*, 1997; Ragusa *et al*, 2006), suggest that β2GPI may be involved in triglyceride metabolism.

Cholesterol. In normal subjects, a direct correlation has been observed between β2GPI and total cholesterol levels (McNally *et al*, 1994b; Crook *et al*, 1999; Mehdi *et al*, 1999). However, some authors have found such correlation only in females (Crook *et al*, 1999; Mehdi *et al*, 1999) and others did not find any correlation at all (Matsuda *et al*, 1993b). More specifically, as regards lipoprotein-specific cholesterol, there has also been a number of conflicting observations. For instance, in healthy subjects, authors have described the correlation between β2GPI and HDL-cholesterol levels to be moderately positive (Mehdi *et al*, 1999), negative (McNally *et al*, 1994b) or non-existent (Crook *et al*, 1999); and that between β2GPI and LDL-cholesterol levels to be positive (McNally *et al*, 1994b) or absent (Crook *et al*, 1999).

Oxidized LDL. β2GPI has been shown to have an anti-oxidant-like effect on LDL oxidation ([Lin *et al*, 2001]),[8] to react with oxidized LDL ([Hasunuma *et al*, 1997; Kobayashi *et al*, 2003; Matsuura *et al*, 2003]), and to prevent its clearance by macrophages via scavenger receptors ([Hasunuma *et al*, 1997; Romero *et al*, 1998]). This feature confers anti-atherogenic properties to β2GPI via the prevention of foam cell formation (more details below).

Lipoprotein (a). ELISA and transfection experiments have shown that there is a direct interaction between β2GPI and apolipoprotein (a) ([Köchl *et al*, 1997]), a major component of lipoprotein (a).[9] This suggests that lipoprotein (a) may be involved in the establishment of β2GPI autoimmunity. Interestingly, the presence aPL/anti-β2GPI antibodies has been associated with an elevated concentration of lipoprotein (a) ([Yamazaki *et al*, 1994; Atsumi *et al*, 1998]). However, even though lipoprotein (a) level has been associated with higher levels of β2GPI-containing immune complexes ([George *et al*, 1999a]), no significant correlation was found between serum β2GPI and lipoprotein (a) levels ([Crook *et al*, 1999]). This suggests that the β2GPI-lipoprotein (a) interaction may simply reflect the affinity of β2GPI for phospholipids in the lipoprotein particles ([Polz *et al*, 1979c]).

Thus, although some of the above observations seem conflicting, their combination with that of the *in vitro* modulating influence of β2GPI on the lipoprotein lipase hydrolytic activity of artificial lipid emulsion ([Nakaya *et al*, 1980]) suggests a possible role for β2GPI in lipid/lipoprotein metabolism. This, together with β2GPI's constitutional presence in lipoproteins, also led to the designation of β2GPI as "apolipoprotein H" ([Nakaya *et al*, 1980]). However, this name and the capacity to interact with lipids are the only common characteristics between β2GPI and other apolipoproteins, as β2GPI is significantly different from other apolipoproteins from a genetic and structural perspective. It must be noted that β2GPI deficiency has not been strictly associated with aberrant lipoprotein metabolism, even though evidence of this was found in clinically normal subjects who presented lower HDL_{2b} and HDL_3 levels and an undetectable level of β2GPI ([Hoeg *et al*, 1985]). In a recent paper, two families with complete β2GPI deficiency were studied. In one family, all affected individuals had an increased serum LDL-cholesterol level, but in

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[8] Oxidized LDL is considered as an auto-antigen candidate implicated in atherosclerosis by influencing foam cell formation and cell cytotoxicity ([Steinberg *et al*, 1989; Yla-Herttuala *et al*, 1989; Witztum *et al*, 1991 & 1994; Aviram *et al*, 1993; Stemme *et al*, 1995]).

[9] Lipoprotein (a) is considered as an independent risk factor for vascular diseases and atherosclerosis when present in increased concentration ([Dahlen *et al*, 1986; Murai *et al*, 1986; Utermann, 1989; Nagayama *et al*, 1994; Kraft *et al*, 1996]).

the other family, no individual had apparent abnormality in lipid metabolism (Yasuda *et al*, 2000b). Accordingly, it was proposed that β2GPI plays little, if any, role in plasma lipoprotein metabolism in man, and could simply be a passive "passenger" on lipoprotein particles (Hoeg *et al*, 1985). More recently, an autosomal genome-wide scan for LDL peak particle diameter was performed with a total of 442 markers being genotyped, and strong evidence of linkage was demonstrated on chromosome 17q21.33 where the β2GPI (APOH) gene is localized (Bosse *et al*, 2003 & 2005). As will be described in chapter 4, the influence of a genetically determined β2GPI structural polymorphism on plasma lipids has also been evaluated.

1.5.4.III Possible involvement of β2GPI in immune-mediated reactions

The interaction of β2GPI with aPL/anti-β2GPI antibodies has been implicated in the perturbation of blood coagulation (see above), but also in other physiological and pathological mechanisms.

β2GPI was found to be associated *in vivo* with anionic phospholipid-containing liposomes (Chonn *et al*, 1995) as well as with the surface of erythrocyte ghosts (Balasubramanian *et al*, 1997) and apoptotic cells (Price *et al*, 1996; Balasubramanian *et al*, 1997; Levine *et al*, 1998; Pittoni *et al*, 2000). This binding was shown to enhance the uptake and phagocytosis of these compounds and cells by macrophages (Chonn *et al*, 1995; Balasubramanian *et al*, 1997 & 1998; Levine *et al*, 1998; Manfredi *et al*, 1998a&b; Rovere *et al*, 1999). This suggests that β2GPI may be a key player - serving as an opsonin - in the immune detection and clearance of these cells and particles. As mentioned earlier, this mechanism could be responsible, when affecting platelets, for the immune-mediated thrombocytopenia that often accompanies aPL/anti-β2GPI antibodies.

The partial cross-reactivity of ACLA with anti-endothelial cell antibodies in patients with SLE (Vismara *et al*, 1988; Cervera *et al*, 1991), related to the cross-reactivity of the latter antibodies with oxidized LDL and β2GPI in SLE (Wu *et al*, 1999), is particularly intriguing, especially as regards the accelerated atherosclerosis that is associated with anti-oxidized LDL (Salonen *et al*, 1992; Puurunen *et al*, 1994; Wu *et al*, 1997) and with aPL (Ames *et al*, 1994 & 2002; Vaarala, 1996a; Roman *et al*, 2001 & 2003). Passive immunization of naive mice with heterologous β2GPI leads to the development of aPL with recurrent pregnancy loss and thromboembolic complications (Blank *et al*, 1994; Aron *et al*, 1995; Garcia *et al*, 1997) and enhanced early atherosclerosis (George *et al*, 1998b). In addition, human atherosclerotic lesions have been shown to contain β2GPI in association with endotheliocytes, macrophages and $CD4^+$ lymphocytes (George *et al*, 1999b). These

observations support the possible role of β2GPI in atherosclerosis. As mentioned earlier, β2GPI has also been shown to react directly with oxidized LDL (Hasunuma *et al*, 1997; Hörkkö *et al*, 1997; Kobayashi *et al*, 2003; Matsuura *et al*, 2003) and to prevent endocytosis of oxidized LDL by macrophages via scavenger receptors (Hasunuma *et al*, 1997), which implies that it may have anti-atherogenic functions. However, this also suggests that β2GPI may serve as a target antigen for an immune-mediated response and as such significantly contribute to the progression of atherosclerosis. This is supported by the existence of β2GPI-reactive aPL that have several pro-atherogenic characteristics. Indeed, some of these antibodies have been shown to cross-react with oxidized LDL (Vaarala *et al*, 1993; Hörkkö *et al*, 1997 & 2000) and to enhance the *in vitro* uptake and accumulation of oxidized lipoprotein by macrophages when in the presence of β2GPI (Hasunuma *et al*, 1997). This phenomenon could stimulate foam cell formation and thereby atherosclerotic plaque formation (Yla-Herttuala *et al*, 1994; Wu *et al*, 1995; George *et al*, 1998b). These antibodies may also cause atherosclerosis by directly activating the vascular endothelium (Del Papa *et al*, 1997; Simantov *et al*, 1995; Pierangeli *et al*, 1999) or by reducing paraoxonase activity (leading to increased oxidation of LDL) (Delgado *et al*, 2002; Ames *et al*, 2005). Interestingly, although antibodies against oxidized LDL have been demonstrated to predict myocardial infarction (Puurunen *et al*, 1994; Wu *et al*, 1997) and progression of carotid atherosclerosis (Salonen *et al*, 1992), they have also been shown to be less strongly associated with the development of arterial thrombosis than aPL (Aho *et al*, 1996) and anti-β2GPI antibodies (Romero *et al*, 1998) in SLE and the secondary APS.

Therefore, the potential involvement of aPL/anti-β2GPI antibodies in atherosclerotic plaque formation may represent a pathogenic mechanism for the accelerated atherosclerosis that is associated with the APS (George *et al*, 1997b & 1998b; Vaarala, 1997), with aPL in general (Ames *et al*, 1994 & 2002; Vaarala, 1996a; Roman *et al*, 2001 & 2003), and with anti-β2GPI antibodies in particular (Cabral *et al*, 1995; McNally *et al*, 1995b; Gómez-Pacheco *et al*, 1999). This may represent a potential link between β2GPI/anti-β2GPI antibodies and atherogenesis. Interestingly, from a clinical point of view, anti-β2GPI antibody testing has appeared to be superior to anticardiolipin assays in identifying patients with the APS (McNally *et al*, 1995b; Guerin *et al*, 1997) and more generally with autoimmune thrombosis (Puurunen *et al*, 1996; Wahl *et al*, 1998; Gómez-Pacheco *et al*, 1999; Zanon *et al*, 1999), although this opinion is not shared by all observers (Detkova *et al*, 1999; Lee *et al*, 1999; Lynch *et al*, 1999; Franklin *et al*, 2000). Unfortunately, the large variety of proposed mechanisms of action of aPL/anti-β2GPI antibodies makes it difficult to identify the

main primary mechanism, and it cannot be excluded that different mechanisms exist or co-exist among patients and even in one single patient.

Just like LDL, β2GPI could also be subject to oxidation, particularly of some amino acids. This phenomenon could alter phospholipid binding, and thereby modulate β2GPI functions and/or autoantibody recognition (Arvieux *et al*, 2001). Interestingly, it was recently shown that covalent modification of β2GPI with oxidation products of cardiolipin made it more antigenic for serum samples, purified IgG and monoclonal ACLA from patients with the APS (Hörkkö *et al*, 2000).

The demonstration that β2GPI and aPL/anti-β2GPI antibodies can bind to a number of different cell types (see above) also raises the possibility that aPL/anti-β2GPI antibodies may have a more important physiological or pathological role to play than originally thought. For instance, the observation that astrocytes and neurones synthesize β2GPI (Caronti *et al*, 1999; Ragusa *et al*, 2006) and that anti-β2GPI antibodies bind to the central nervous system (Caronti *et al*, 1998) and seem to be able to cause permeabilization of synaptoneurosomes (Chapman *et al*, 1999) highly suggests that the interaction of β2GPI and anti-β2GPI with brain neurones may contribute to central nervous system pathologies.[10] Other still to be elucidated mechanisms can also disturb the functions of the other cell types to which β2GPI has been shown to bind.

In summary, it appears that β2GPI modulates thrombus formation and promotes the clearance of certain oxidation products. These potential functions of β2GPI could be altered by aPL/anti-β2GPI antibodies, which may partially explain the association between these antibodies and thrombosis and atherosclerosis. Moreover, the affinity of β2GPI for certain macromolecules may render them susceptible to aPL/anti-β2GPI immuno-reactivity, which can have considerable consequences depending on the involved cells or tissues. However, it is also clear from the above discussion that despite all the research into the potential functions of β2GPI, there is still no consensus regarding its precise physiological role or its role in thrombotic disorders, with the possible exception of the APS.

[10] In addition to other potential effects on cerebral endothelium such as those described above.

2 Aims of this study

2 Aims

The general purpose of this thesis was to enhance our understanding of the physiological role of β2GPI. Specific aims were to:

- establish a reliable assay to measure circulating level of β2GPI,
- examine the impact of inflammatory and thrombotic disorders on circulating level of β2GPI,
- examine the impact (if any) of polymorphism in the β2GPI (APOH) gene on the risk of developing common thrombotic disorders, and
- examine the expression of mRNA for β2GPI in a mouse model of sepsis.

3 Serum β2GPI concentration

3.1 Introduction

3.1.1 β2GPI level in normal individuals

Plasma β2GPI level has been shown to vary widely among individuals from a level that is immunologically undetectable (Bancsi *et al*, 1992; Takeuchi *et al*, 2000) to a level as high as 400 μg/mL (McNally *et al*, 1994b, 1995a&b). A mean at 200 μg/mL was observed in Caucasians, while individuals from African and Asian ancestry show a relatively lower plasma β2GPI concentration (Cleve, 1968; Koppe *et al*, 1970; Atkin *et al*, 1974; Propert, 1978; Walter *et al*, 1979; Sansom *et al*, 1991; McNally *et al*, 1993; Cassader *et al*, 1997). Several studies have found that age and gender do not seem to affect β2GPI level significantly (Cohnen, 1970; Sansom *et al*, 1991; Bancsi *et al*, 1992; Ichikawa *et al*, 1992; McNally *et al*, 1993; Mehdi *et al*, 1999), whereas other studies have observed a moderate effect of these two factors (Cleve, 1968; Propert, 1978; Crook *et al*, 1999). β2GPI concentration also shows a strong stability within individuals and does not seem to be dependent on their fasting/non-fasting or pre/post-prandial status (Cleve, 1968; McNally *et al*, 1993; Cardigan *et al*, 1998; Zahedi *et al*, 2004), even though conflicting reports are found in this matter (Polz *et al*, 1979c).

3.1.2 β2GPI level in disease

A substantial number of publications have dealt with β2GPI level in various pathological conditions, but none of them found a clear association between any of these conditions and a perturbed β2GPI level. Variations in plasma β2GPI concentration may however be associated with hypercoagulable states such as disseminated intravascular coagulation (Schousboe *et al*, 1980; Matsuda *et al*, 1993a). Interestingly, in these patients, the reduction in plasma β2GPI level is accompanied by an increased concentration of cleaved forms of β2GPI (Horbach *et al*, 1999). However, there is no conclusive report of patients with impaired blood coagulation (either thrombosis or a tendency to bleed) that is definitely related to low or high concentrations of (cleaved or not) serum β2GPI (Bancsi *et al*, 1992). The absence of detailed studies, particularly on common thrombotic disorders, is clearly a deficiency in our knowledge of β2GPI. In this context, it must also be emphasised again that several asymptomatic individuals with an undetectable level of β2GPI have been described in the literature (Bancsi *et al*, 1992; Takeuchi *et al*, 2000).

More specific observations have been made concerning β2GPI level in other disease states.

Inflammatory diseases: β2GPI seems to behave as a negative acute phase protein, its expression and plasma level being approximately halved under the influence of certain inflammatory mediators in *in vitro* and animal studies (Mehdi *et al*, 1991; Sellar *et al*, 1993). However, inflammatory diseases such as rheumatoid arthritis, chronic progressive polyarthritis, chronic glomerulonephritis, chronic pyelonephritis, ankylosing spondylitis, erythema nodosum, ulcerative colitis have also been investigated and none presented a β2GPI level that differed from normal values (Cleve, 1968; Cohnen, 1970). This is an important issue to be clarified, especially as inflammation may be associated with thrombosis (Cicala *et al*, 1998; Dhainaut *et al*, 2001) (see chapter 5).

Antiphospholipid syndrome and systemic lupus erythematosus: Patients with aPL were found to have a similar (De Benedetti *et al*, 1992; Ichikawa *et al*, 1992; Oosting *et al*, 1992) or increased (Galli *et al*, 1992a; Vlachoyiannopoulos *et al*, 1992; Kamboh *et al*, 1999a) β2GPI concentration compared to aPL-negative individuals. In patients with the APS or SLE, β2GPI level was found to be either diminished (Ichikawa *et al*, 1992; Kamboh *et al*, 1999a; Matsuda *et al*, 1993a), unchanged (Ichikawa *et al*, 1992), slightly increased (Cohnen, 1970) or significantly increased (McNally *et al*, 1995b; Nezlin, 2000) when compared to individuals without the APS or SLE. An increased level of β2GPI was demonstrated to be associated with a history of thromboembolism in patients with SLE (McNally *et al*, 1995b; George *et al*, 1999a). However, when β2GPI level was specifically tested against individual clinical features of the APS (previous thrombosis, spontaneous abortion or thrombocytopenia), no difference was found in β2GPI concentration (Ichikawa *et al*, 1992).

Hyperlipidaemia: The positive correlation between β2GPI concentration and the levels of plasma lipid constituents in hyperlipidaemic (hypercholesterolaemic and hypertriglyceridaemic) patients (McNally *et al*, 1994b; Crook *et al*, 1999; Mehdi *et al*, 1999), as well as the elevated level of β2GPI in these patients (Ichikawa *et al*, 1992; McNally *et al*, 1994b) suggest that a higher production of lipoproteins may be associated with increased β2GPI synthesis. SLE patients, which can present with a secondary hyperlipidaemic state - possibly caused by lupus nephritis (proteinuria) and/or prednisolone treatment (Ettinger *et al*, 1987), have a β2GPI level that positively correlates with total cholesterol and triglycerides (Ichikawa *et al*, 1992). In such patients, the equilibrium in the distribution of β2GPI between the lipoprotein fractions could be disturbed (McNally *et al*, 1994b), possibly providing immunogenic stimuli for aPL/anti-β2GPI antibody production (McNally *et al*, 1995a).

Liver dysfunction: As will be examined in chapter 5, the main location of β2GPI synthesis is thought to be the liver. Consequently, liver dysfunction could affect β2GPI level ([Cohnen, 1970; Quintarelli *et al*, 1994]). In patients with liver cirrhosis, plasma β2GPI level was found to be significantly decreased ([Cleve, 1968; Cohnen, 1970; Quintarelli *et al*, 1994]) and closely related to the degree of liver failure ([Quintarelli *et al*, 1994]). The latter observation supports the hypothesis that the hepatic parenchyma is the major source of serum β2GPI.

Diabetes: The little data that has been published as regards β2GPI level in diabetes show that plasma β2GPI level is significantly increased in diabetic patients (of both types I and II) as opposed to in non-diabetic subjects ([Cleve, 1968; Cassader *et al*, 1997; Ruiu *et al*, 1997]).

Cancers: A limited number of patients with cancer (lymphogranulomatosis, reticulosarcoma, multiple myeloma, macroglobulinaemia, stomach cancer) have also been examined, but only in stomach cancer did β2GPI level seem to be slightly decreased ([Cleve, 1968; Cohnen, 1970]).

Since no physiological function has been assigned to β2GPI, much of what has been observed about β2GPI variations cannot be ascribed to a specific cause. Numerous mechanisms could affect serum β2GPI concentration, such as change in synthetic rate, altered renal excretion, modified distribution within intra/extra-vascular compartments, haemodilution, variation in proteolysis rate or susceptibility, and/or fluctuation in the binding avidity to cells and/or other plasma constituents. Genetic and environmental factors may also play a significant role, and the former will be discussed in chapter 4. Clarification of the quantitative contribution made by these various factors is obviously difficult. Given the possible involvement of β2GPI in the regulation of coagulation, the aim of this chapter was to estimate the possible association between a variation in β2GPI concentration and thrombotic susceptibility.

3.2 Aims of chapter 3

Our aims in this part of the study were to:

- establish a sandwich β2GPI-specific enzyme-linked immunosorbent assay (ELISA),
- establish a reference range of serum β2GPI concentrations,
- measure circulating β2GPI concentration in inflammatory, thrombotic and pregnant states, and
- correlate serum β2GPI level with the presence of aPL (for some sub-groups of individuals only).

3.3 Materials and methods

3.3.1 Samples

3.3.1.I Subjects

Following informed consent from the tested individuals and approval from local ethical committees, blood samples were obtained from five different study groups.

- 334 **healthy individuals** without any apparent pathological condition at the time of blood sampling, including 269 individuals (202 females and 67 males), mostly laboratory and hospital employees (mean age at the time of blood sampling ± SD: 30.3 ± 11.7 years old) and 65 elderly individuals (50 females and 15 males) resident in nursing homes (mean age: 72.0 years old) who fulfilled the clinical criteria outlined in the SENIEUR protocol ([Ligthart *et al.* 1984]) and had no evidence of vascular disease or of prothrombotic risk factors.
- 121 patients (42 females and 79 males) who presented with an **acute coronary syndrome** (either cardiac infarction or unstable angina) (mean age ± SD: 65.3 ± 10.3 years old). Samples from these patients were kindly provided by Ross Murphy, Department of Cardiology, St James's Hospital, Dublin. All patients had received aspirin and heparin by the time the first blood sample was taken. Blood samples were taken at the time of presentation, and/or at 2, 4, 6 months after the cardiac event.
- 102 elderly patients (40 females and 62 males) with **non-haemorrhagic stroke** (mean age ± SD: 71.9 ± 12.2 years old). Samples from these patients were kindly provided by Wendy Livingstone, Trinity College Dublin - Smurfit Institute of Genetics. For 66 of these patients, blood samples were taken at the time of presentation, as well as 3 and 6 months after the stroke.
- 73 **women** (mean age ± SD: 30.5 ± 6.0 years old) with uncomplicated **pregnancy**. Samples from these women - who took part as controls in a genetic study - were kindly provided by Catriona Keenan, Trinity College Dublin - Smurfit Institute of Genetics. For 58 of these pregnant women, blood samples were taken at 8, 16, 26, and 36 weeks of pregnancy.
- 200 patients with an **elevated level of C-reactive protein (CRP)** (100 females and 100 males; mean age ± SD: 61.2 ± 19.4 years old) suffering from a variety of inflammatory disorders. CRP levels ranged between 10.2 and 390.0 mg/L as measured at the Department of Immunology, St James's Hospital. 64 additional patients (34 females and 30 males; mean age ± SD: 55.1 ± 21.0 years old) with a CRP level below 10.0 mg/L were also included for comparison.

3.3.1.II Blood collection

Venous blood samples from healthy donors and patients were collected into dry glass tubes without addition of anticoagulant and into plastic tubes with a one-tenth volume of 0.106 M trisodium citrate. Serum was prepared by allowing blood without anticoagulant to clot for 15 minutes at room temperature prior to centrifugation. Serum and plasma were separated by centrifugation at 2500 rpm for 10 minutes and divided into aliquots of 20 to 200 µl that were stored at -20°C or -70°C until use. Aliquots were used at most twice and then discarded.

3.3.2 Statistics

Depending on the number of sets of scores, comparisons between paired data were made using Wilcoxon or Friedman tests, and comparisons between non-paired data were made using Mann-Whitney or Kruskal-Wallis tests. Correlation analyses were performed using Spearman's rank correlation test. Statistical associations were determined using Fisher's exact probability test ([Wonnacott *et al*, 1998]). All analyses were performed using InStat GraphPad Software, Version 2.03, 1994 (PK Wallace, Wally Word). P values under 0.05 were considered statistically significant.

3.3.3 β2GPI detection techniques

Techniques to quantify β2GPI in serum have appreciably evolved in the last four decades. Originally performed using single ([Mancini *et al*, 1965]) and double (Ouchterlony) radial immunodiffusion techniques, Laurell rocket immuno-electrophoresis ([Laurell *et al*, 1966]) and crossed immuno-electrophoresis ([Laurell *et al*, 1965]), β2GPI quantification methods rapidly developed into more sensitive and more specific enzyme-linked immunosorbent-assays (ELISAs) using polyclonal and then monoclonal anti-β2GPI antibodies. It is noteworthy that strikingly comparable concentrations for β2GPI were found in healthy individuals with all detecting methods since 1968 (average ± SD of averages: 199.51 ± 19.7 µg/mL) (see references in chapter 3.1.1). One study in particular has shown a strong correlation between ELISA and Laurell rocket immuno-electrophoresis for the quantification of β2GPI ([McNally *et al*, 1993]). Today, the most commonly used techniques to measure β2GPI level are ELISAs, and these can be found in several (competitive and non-competitive) protocol designs.

3.3.4 β2GPI ELISA protocol[1]

β2GPI concentration was measured with a direct sandwich ELISA ([Lin *et al*, 2003]) (figure 3.1). γ-irradiated Nunc MaxiSorp™ ELISA plates (Nunc A/S, Roskilde, Denmark) certified by the manufacturer for consistency in adsorption of protein were incubated overnight at 4°C with 100 µL of a mouse monoclonal anti-human β2GPI antibody at a concentration of 3.2 µg/mL (Chemicon International Inc., Temecula, CA, USA) in coating buffer (15.0 mmol/L Na_2CO_3, 34.9 mmol/L $NaHCO_3$, pH 9.6). To remove any unbound antibody, the wells were then washed four times with phosphate-buffered saline with Tween®20 (PBST, 136.9 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na_2HPO_4, 1.5 mmol/L KH_2PO_4, pH 7.2, 0.05% v/v Tween®20).

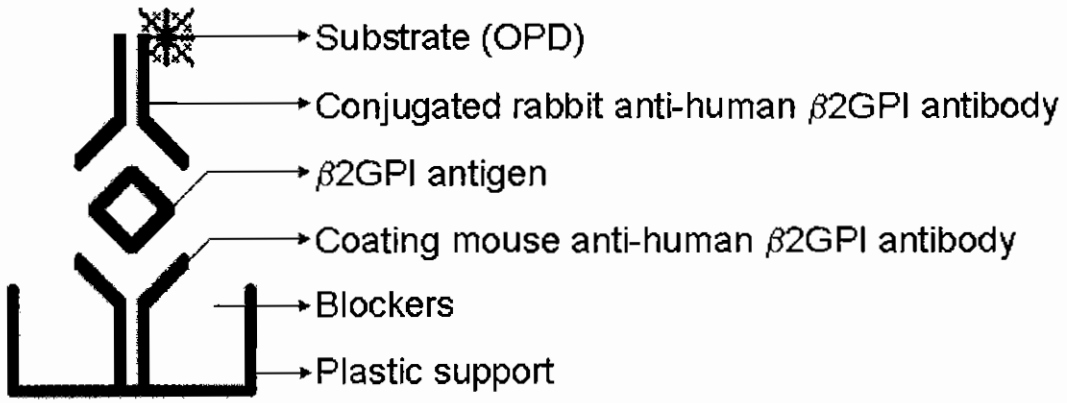

Figure 3.1: Diagram illustrating the succession of reagents in the β2GPI direct ELISA. Mouse monoclonal anti-β2GPI antibodies were attached to the solid phase by passive adsorption. After washing, serum samples or standards containing β2GPI antigen were added. After an incubation period of an hour and washing, detecting rabbit polyclonal enzyme-labelled anti-β2GPI antibodies were added. Following another incubation period of an hour and washing, the substrate was added. Colour was allowed to develop and then stopped with sulphuric acid and measured spectrophotometrically.

A pooled normal serum sample calibrated against purified β2GPI (Crystal Chem Inc., Chicago, USA) was used as standard. 100 µL of standards in a series of 2-fold dilutions (starting at 1 in 320 to 1 in 40,960 in PBST) and 100 µL of test samples (diluted at 1 in 3,000 in PBST) were added in duplicate. Two wells with PBST only were used as blanks. The plates were then incubated at 37°C for 1 hour. Following an additional wash step with PBST, 100 µL of a horseradish peroxidase-conjugated rabbit polyclonal anti-human β2GPI antibody (Dako A/S, Glostrup, Denmark) diluted at 1 in 1,000 in PBST were then added to each well and the plates were further incubated at 37°C for 1 hour. Colour was developed using 100 µL of orthophenylenediamine dihydrochloride (OPD; Dako A/S,

[1] Numerous problems were encountered in establishing the β2GPI assay. A full discussion of these difficulties and steps to resolve them is given in appendix G.

Glostrup, Denmark) with hydrogen peroxide (Merck) following a last wash with PBST. After an incubation of 5 minutes at room temperature and in the dark, 100 µL of 2.5 M sulphuric acid was added to stop colour development, and optical density (OD) was measured at 492 nm wavelength on a Multiskan EX Plate Reader Type 355 (Labsystems, Finland). OD values were processed through MultiCalc Advanced software version 1.5 (1985-1992; Wallac Oy, Finland). β2GPI concentrations in control and test samples were calculated from the standard curve.

3.3.5 Other tests

Albumin was measured using a bromocresol green protocol on Hitachi 747 in the Department of Biochemistry, St James's Hospital, Dublin.

C-reactive protein and **transferrin** were measured by nephelometry, using Behring Nephelometer Analyser II, in the Department of Immunology, St James's Hospital, Dublin.

Serum amyloid A, **anticardiolipin antibodies** (either IgG and IgM), and IgG **anti-β2GPI antibodies** were measured using ELISAs (respectively Tridelta Development Limited, Wicklow, Ireland; Orgentec Diagnostika GmbH, Mainz, Germany; and Pharmacia & Upjohn GmbH, Freiburg, Germany) following the manufacturers' protocols.

The acute phase proteins were measured in a subgroup of the 200 patients with inflammatory disorders and an elevated CRP level.

3.4 Results

3.4.1 Sample stability

To check the stability of β2GPI, samples stored for up to 3 years at -20°C and samples that were repeatedly frozen and thawed (up to seven times) at -20°C/room temperature (with aliquots removed after each cycle) were tested for the quantification of β2GPI antigen by the β2GPI-ELISA method.

Extended storage did not significantly affect β2GPI quantification. Indeed, serum β2GPI concentration remained mostly unchanged for up to 3 years when properly stored at -20°C, as can be seen in figure 3.2 which shows β2GPI level in three sera that were used as controls throughout this study (Friedman test P=0.2760).

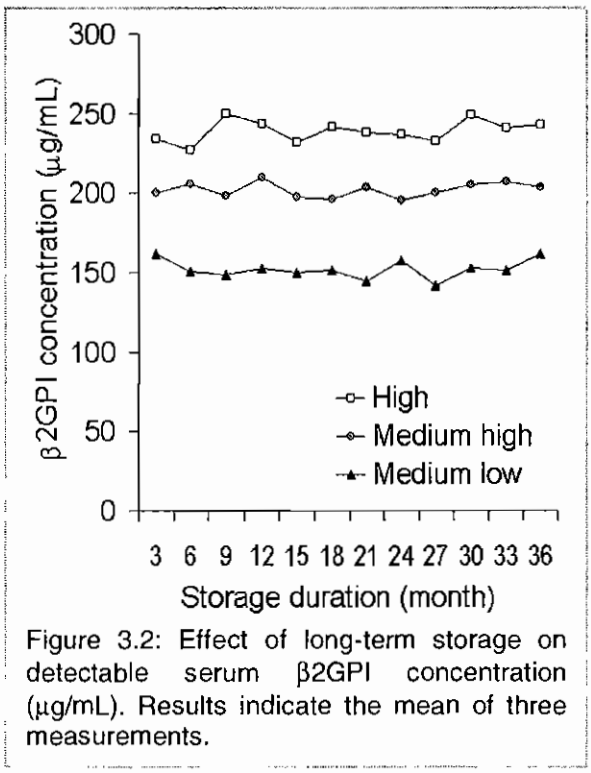

Figure 3.2: Effect of long-term storage on detectable serum β2GPI concentration (µg/mL). Results indicate the mean of three measurements.

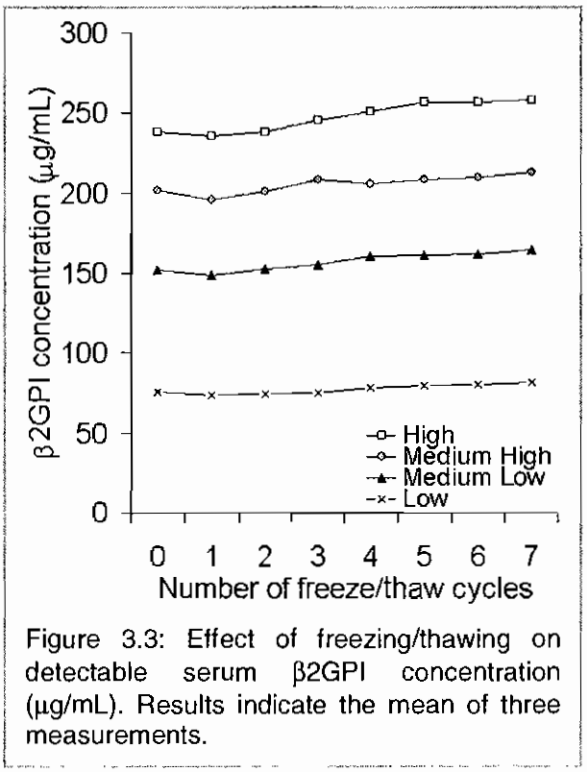

Figure 3.3: Effect of freezing/thawing on detectable serum β2GPI concentration (µg/mL). Results indicate the mean of three measurements.

However, as can be seen in figure 3.3, frequent freezing and thawing significantly affected β2GPI quantification, especially after two freezing/thawing cycles. On average, we found a 1.6% increase in β2GPI concentration per freezing/thawing cycle after two freezing/thawing cycles. These data show that handling of sera used for β2GPI quantification is an important potential source of assay variability. For this reason, we used in this study only properly stored samples that had undergone at most two freezing/thawing cycles.

3.4.2 Serum versus plasma

In order to test whether β2GPI quantification was affected by the activation of the clotting cascade, 50 paired serum/plasma samples were tested for β2GPI level. No difference was observed between serum and citrated plasma (after adjustment for dilution factor) (mean concentration ± SD: 158.4 ± 57.5 and 158.1 ± 61.5 µg/mL for serum and plasma respectively) (figure 3.4) (Mann-Whitney test P=0.937).

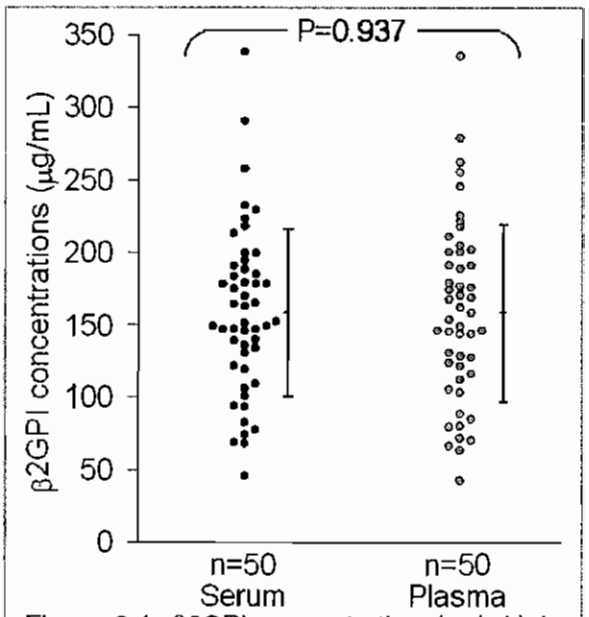

Figure 3.4: β2GPI concentration (µg/mL) in paired serum and plasma samples. Error bars represent mean ± SD.

3.4.3 β2GPI concentration in healthy individuals

Within the healthy group, there was a 14-fold width in the β2GPI distribution ranging from 20.8 to 296.8 µg/mL (average ± SD: 178.3 ± 46.2 µg/mL) with a histogram mildly skewed to the left. (figure 3.5). Although a slightly higher β2GPI level was found in males (183.0 ± 45.3 µg/mL) compared to females (176.8 ± 46.5 µg/mL), the difference was not statistically significant (Mann-Whitney P=0.4216) (figure 3.6).

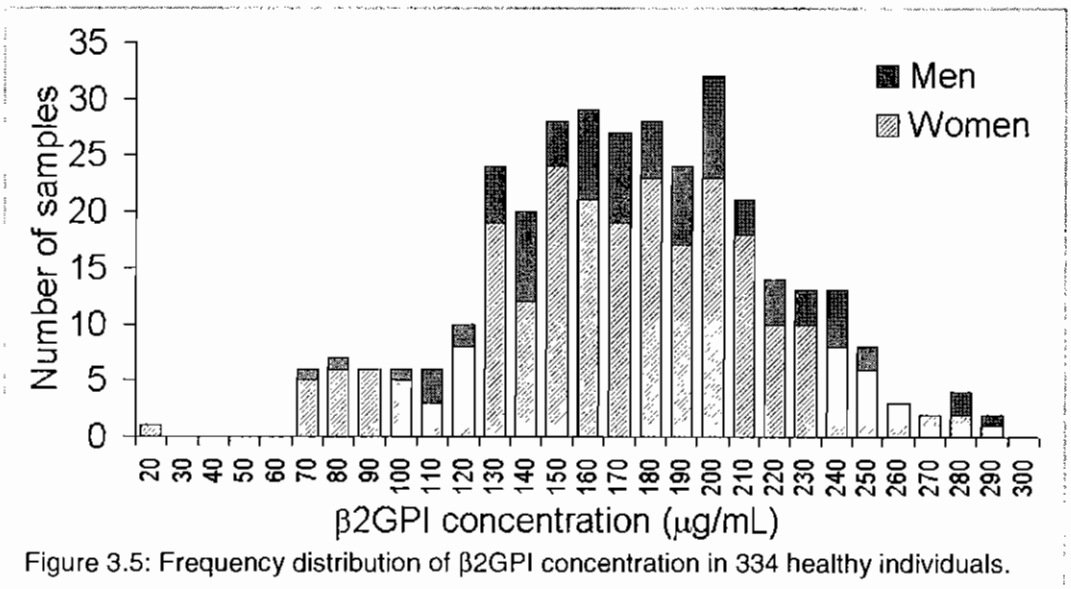

Figure 3.5: Frequency distribution of β2GPI concentration in 334 healthy individuals.

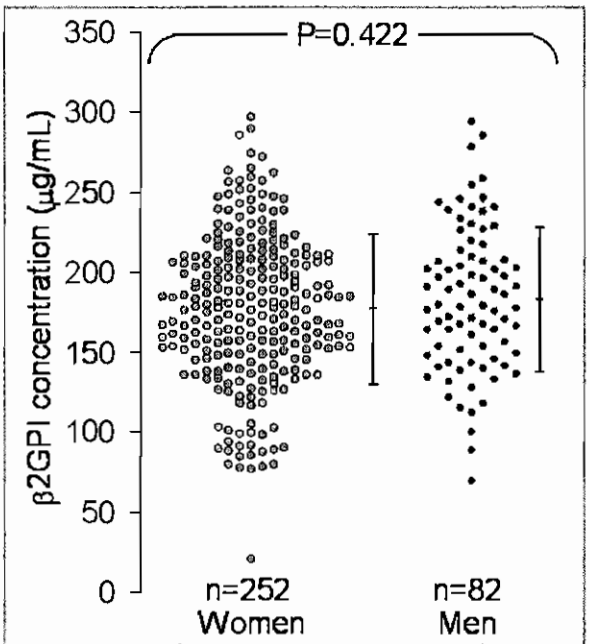

Figure 3.6: β2GPI concentration (μg/mL) in healthy women and men. Error bars represent mean ± SD.

Effect of age on β2GPI level: A Spearman rank correlation test revealed that there was a significant positive correlation between age and serum β2GPI level (Spearman rank test r=0.274; P<0.001) (figure 3.7).

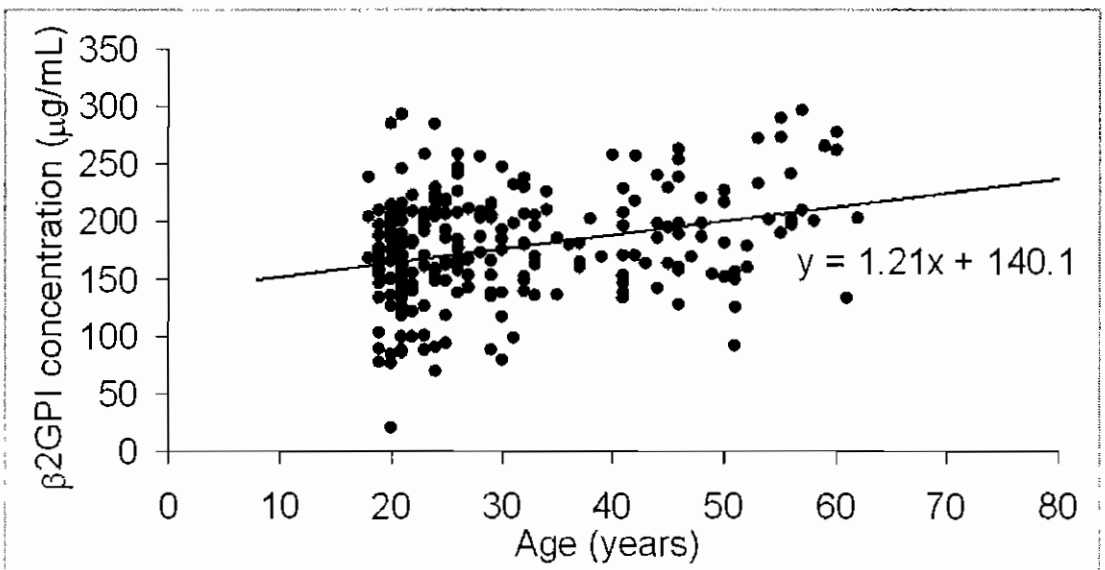

Figure 3.7: Scatter plot representing β2GPI concentration (μg/mL) versus the age of healthy individuals (n=269). The equation represents the regression equation for the trendline.

3.4.4 β2GPI, ACLA and anti-β2GPI antibody levels in patients with stroke

3.4.4.I β2GPI concentration in patients with stroke

In patients presenting with a stroke, β2GPI concentration at the time of presentation was significantly lower than in healthy age-matched individuals (mean ± SD: 170.2 ± 48.4 μg/mL and 187.5 ± 47.5 μg/mL respectively; Mann-Whitney P=0.0127) (figure 3.8). Over a 6-month follow-up period, β2GPI did not change significantly (Friedman test P=0.6565) (figures 3.9 & 3.10).

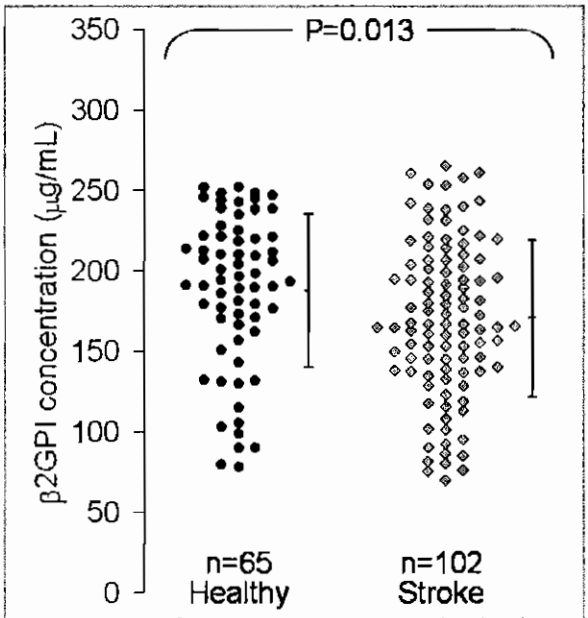

Figure 3.8: β2GPI concentration (μg/mL) in patients with stroke at the time of presentation to the hospital and in healthy elderly individuals. Error bars represent mean ± SD.

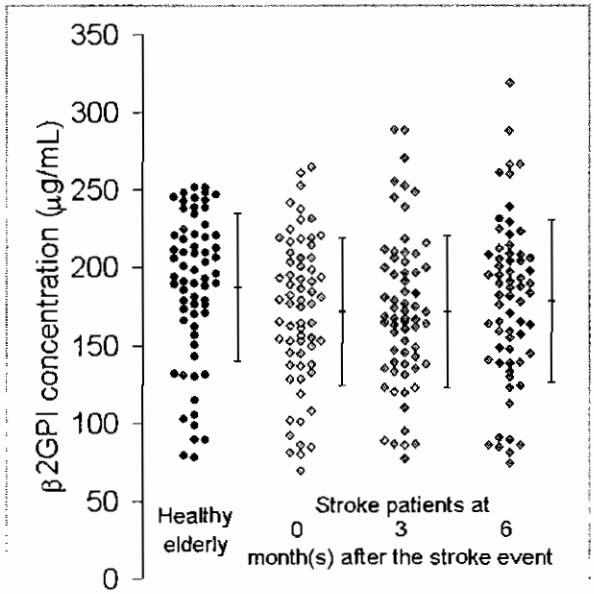

Figure 3.9: β2GPI concentration (μg/mL) in healthy elderly individuals (n=65) and patients with stroke at 0, 3 and 6 months after the stroke event (n=65). Error bars represent mean ± SD.

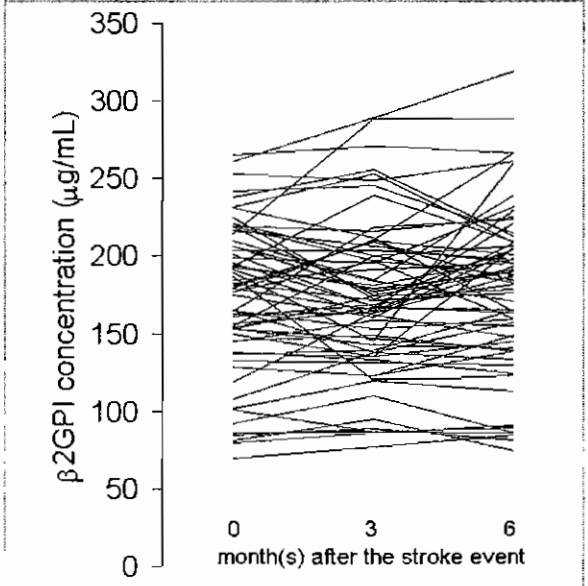

Figure 3.10: Trends in β2GPI concentration (μg/mL) in 65 patients with stroke over a 6-month period of time following the stroke event.

3.4.4.II ACLA concentration in patients with stroke

IgG ACLA levels in the stroke patients at the time of presentation to the hospital ranged from 0.78 to 55.18 GPL/mL with a mean value (± SD) of 6.01 (± 5.73) GPL U/mL. For seven individuals (6.9%), IgG ACLA level was considered positive according to the cut-off value established by the manufacturer (mean ± SD: 19.56 ± 16.00 GPL U/mL). A positive correlation was found between IgG ACLA level and the stroke patients' age (Spearman test r=0.227; P=0.0218), but no significant correlation was found between β2GPI and IgG ACLA levels (Spearman test r=-0.127; P=0.2027) (figure 3.11). β2GPI concentration was 10.7% higher in IgG ACLA-positive than in IgG ACLA-negative individuals (187.0 ± 52.8 versus 168.9 ± 48.2 μg/mL); however, this difference was not statistically significant (Student t-test P=0.3422) (figure 3.12). Over a 6-month follow-up period, IgG ACLA in 66 stroke patients did not change significantly (Friedman test P=0.4760) (figure 3.13).

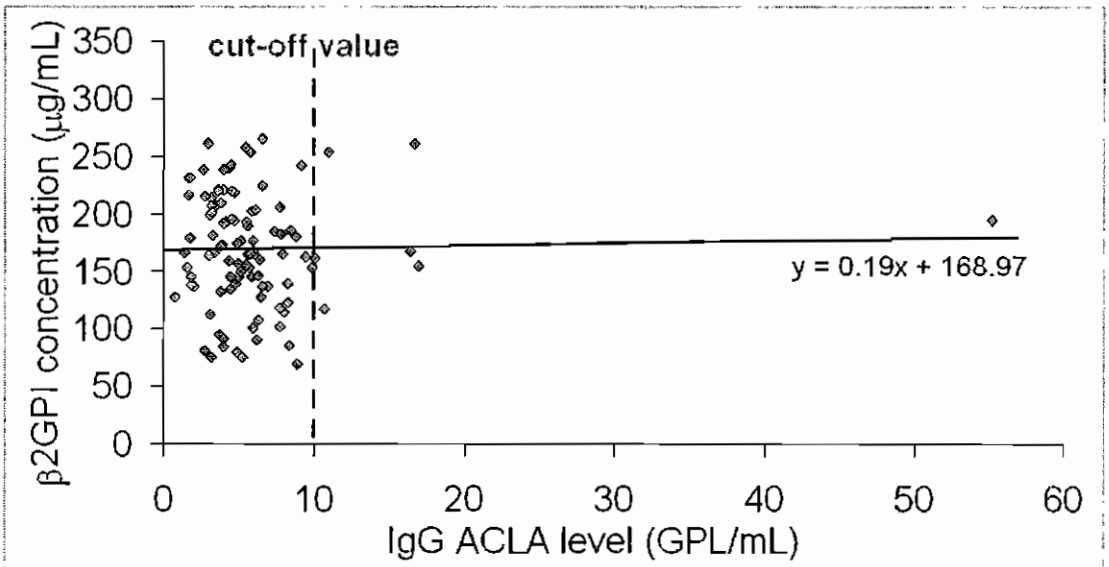

Figure 3.11: Scatter plot representing β2GPI (μg/mL) versus IgG ACLA levels (GPL U/mL) in 102 stroke patients (at the time of presentation to the hospital). The equation represents the regression equation for the trendline. The dotted line represents the manufacturer's cut-off value for IgG ACLA positivity.

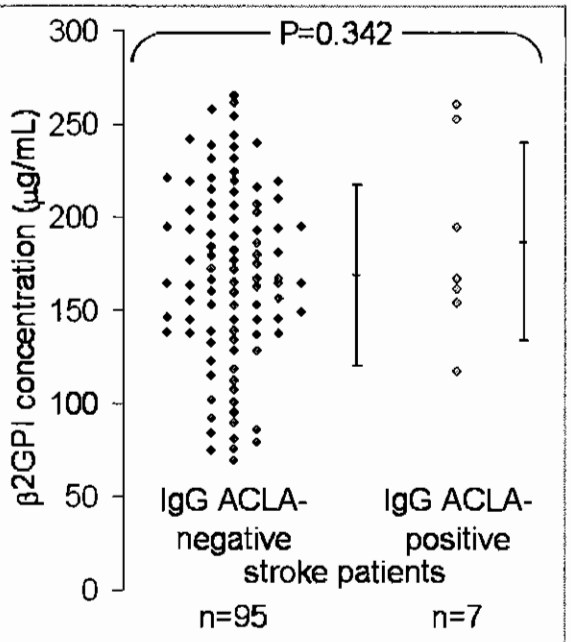

Figure 3.12: β2GPI concentration (μg/mL) in IgG ACLA-negative and positive patients with stroke. Error bars represent mean ± SD.

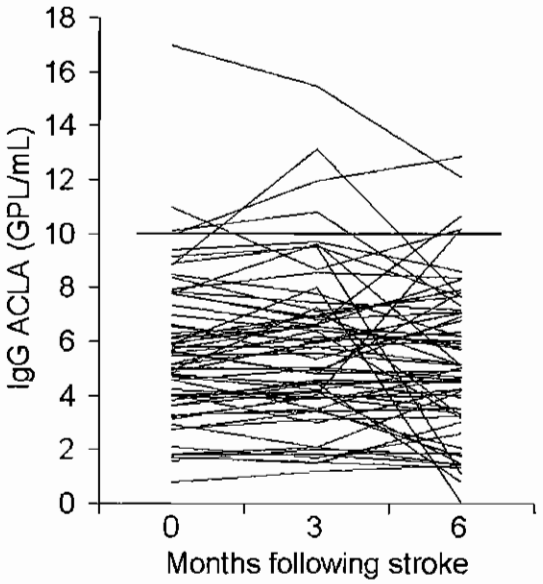

Figure 3.13: Trend in IgG ACLA level (GPL U/mL) in the 66 patients with stroke who were tested at the time of presentation to the hospital and at 3 and 6 months after the stroke. The thicker horizontal line represents the manufacturer's cut-off value for IgG ACLA positivity.

IgM ACLA levels ranged from an undetectable level to 36.19 MPL U/mL (mean ± SD: 3.26 ± 4.75 MPL U/mL). For nine individuals (9.2%), IgM ACLA level was considered positive according to the cut-off value established by the manufacturer (mean ± SD: 14.73 ± 8.81 MPL U/mL). In the seven IgG ACLA-positive stroke samples, IgM ACLA levels ranged from 0.89 to 12.10 MPL U/mL with a mean value (± SD) of 5.47 (± 4.01) MPL U/mL. For 3 of these patients, IgM ACLA level was considered positive according to the cut-off value established by the manufacturer. β2GPI concentration was about 8.4% lower in IgM ACLA-positive compared to IgM ACLA-negative individuals (158.2 ± 52.0 versus 172.8 ± 48.1 μg/mL); however, this difference was not statistically significant (Student t-test P=0.3928) (figure 3.14).

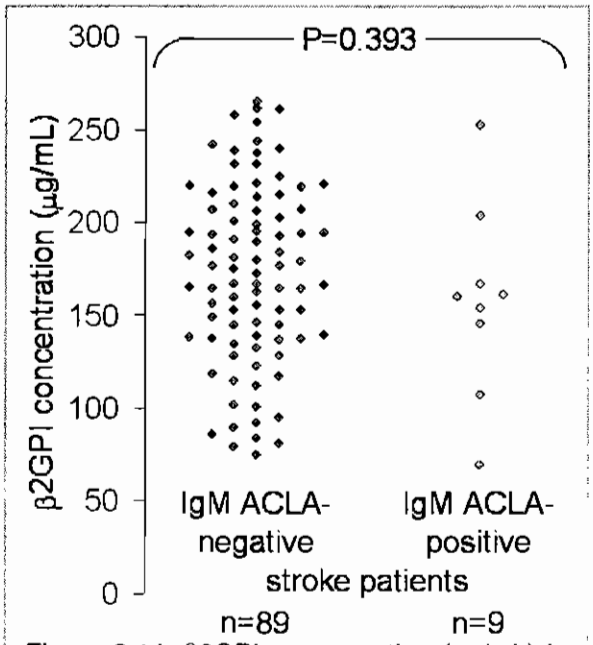

Figure 3.14: β2GPI concentration (μg/mL) in IgM ACLA-negative and positive patients with stroke. Error bars represent mean ± SD.

3.4.4.III Anti-β2GPI antibody concentration in patients with stroke

Anti-β2GPI antibody level was also tested in stroke patients who presented a positive IgG and/or IgM ACLA test. No correlation was found between anti-β2GPI antibodies and ACLA (Spearman test r=0.357; P=0.4316) or between anti-β2GPI antibodies and β2GPI levels (Spearman test r=-0.250; P=0.5887). Only one sample was positive for anti-β2GPI antibodies according to the cut-off value established by the manufacturer. This sample had a serum β2GPI concentration that was not statistically different from anti-β2GPI antibody-negative samples (154.6 versus 164.3 ± 55.2 µg/mL; P=0.8664) (figure 3.15).

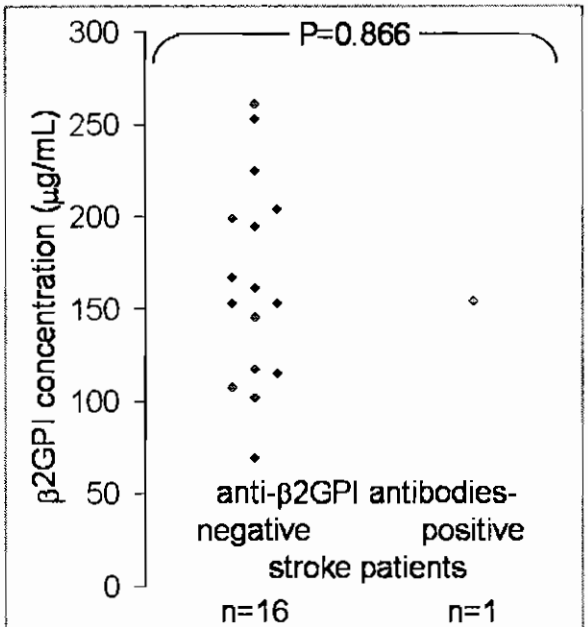

Figure 3.15: β2GPI concentration (µg/mL) in anti-β2GPI antibody-negative and positive patients with stroke. Error bars represent mean ± SD.

3.4.5 β2GPI concentration in patients with an acute coronary syndrome

β2GPI concentrations in patients presenting with an acute coronary syndrome ranged from 84.0 to 287.1 μg/mL (mean ± SD: 179.2 ± 50.3 μg/mL) and was not significantly different from the one in age-matched healthy individuals (Mann-Whitney P=0.109) (figure 3.16). However, when patients were subdivided into two age subgroups (≤ 65 or ≥ 66 years of age), younger patients had a higher β2GPI level than older patients (mean ± SD: 191.5 ± 47.6 and 167.9 ± 50.7 μg/mL respectively; Mann-Whitney P=0.043) (figure 3.17). Compared to healthy age-matched counterparts, β2GPI level was not significantly different in younger patients (Mann-Whitney P=0.682), but was significantly lower in older patients with acute coronary syndrome (Mann-Whitney P=0.046). Over a 6-month follow-up period, β2GPI level did not change significantly (Kruskal-Wallis test P=0.688) (figure 3.18).

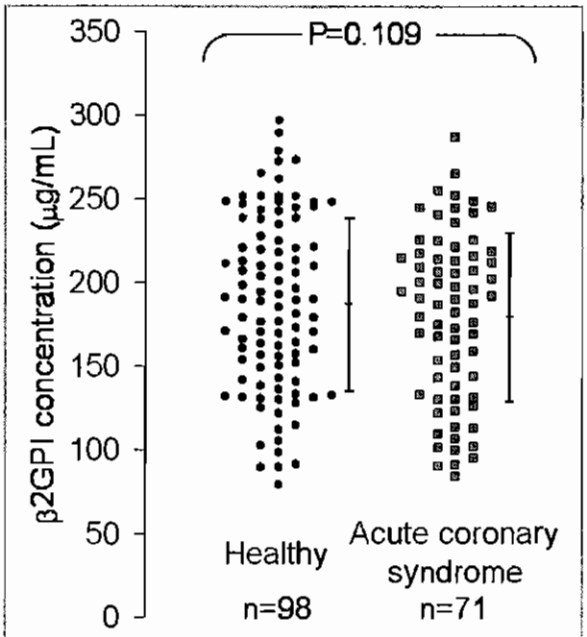

Figure 3.16: β2GPI concentration (μg/mL) in patients with an acute coronary syndrome at the time of presentation to the hospital and in healthy age-matched elderly individuals (H). Error bars represent mean ± SD.

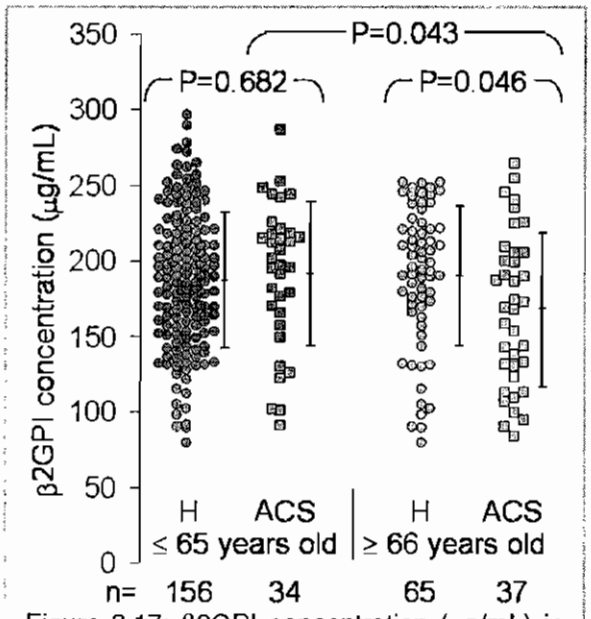

Figure 3.17: β2GPI concentration (μg/mL) in patients with an acute coronary syndrome (ACS) ≤ 65 and ≥ 66 years old at the time of presentation and in age-matched healthy controls (H). Error bars represent mean ± SD.

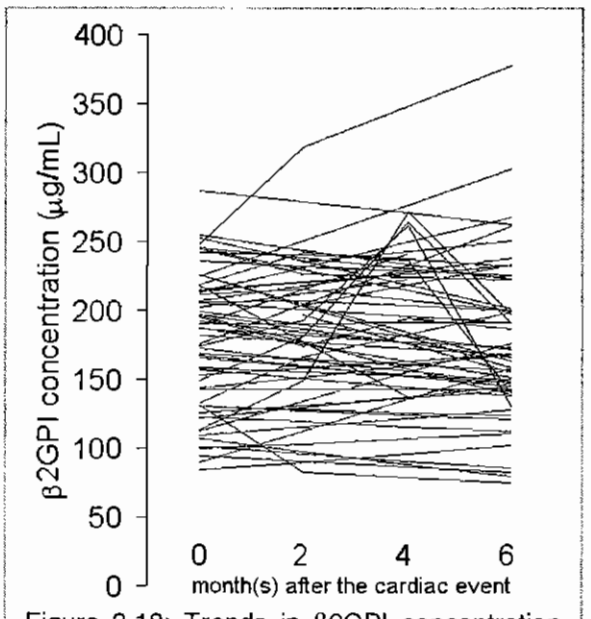

Figure 3.18: Trends in β2GPI concentration (μg/mL) in patients with an acute coronary syndrome over a 6-month period of time following the cardiac event.

3.4.6 β2GPI concentration in healthy pregnancy

In pregnant women, β2GPI concentration at 8 weeks of pregnancy was not statistically different from the one observed in non-pregnant women (mean ± SD: 174.1 ± 40.9 and 174.4 ± 45.4 μg/mL respectively; Mann-Whitney P=0.9695) (figure 3.19). However, β2GPI level showed a gradual decrease over the first 36 weeks of pregnancy (Friedman test P=0.0025), and at 36 weeks of pregnancy, β2GPI level (mean ± SD: 156.7 ± 49.2 μg/mL) was significantly lower than at 8 weeks of pregnancy (Wilcoxon test P<0.001) and than in the control non-pregnant group (Mann-Whitney P=0.0142). (figures 3.19 and 3.20).

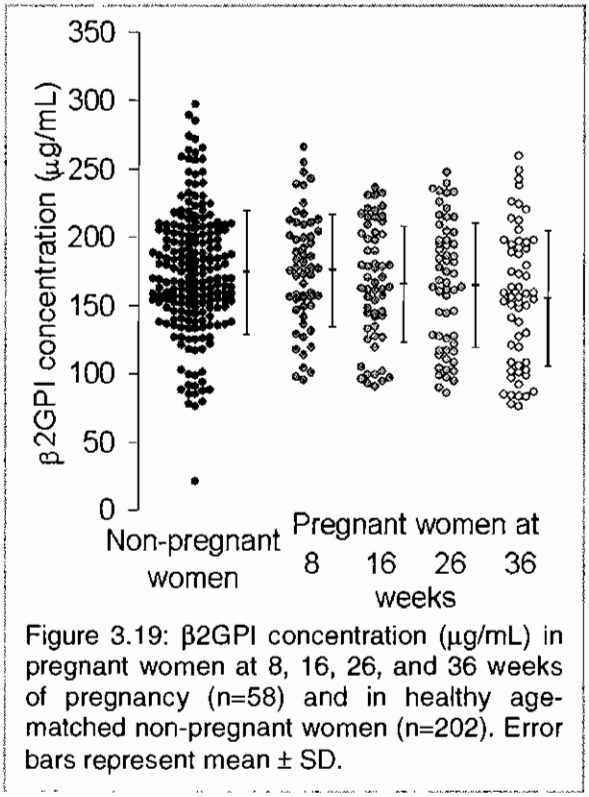

Figure 3.19: β2GPI concentration (μg/mL) in pregnant women at 8, 16, 26, and 36 weeks of pregnancy (n=58) and in healthy age-matched non-pregnant women (n=202). Error bars represent mean ± SD.

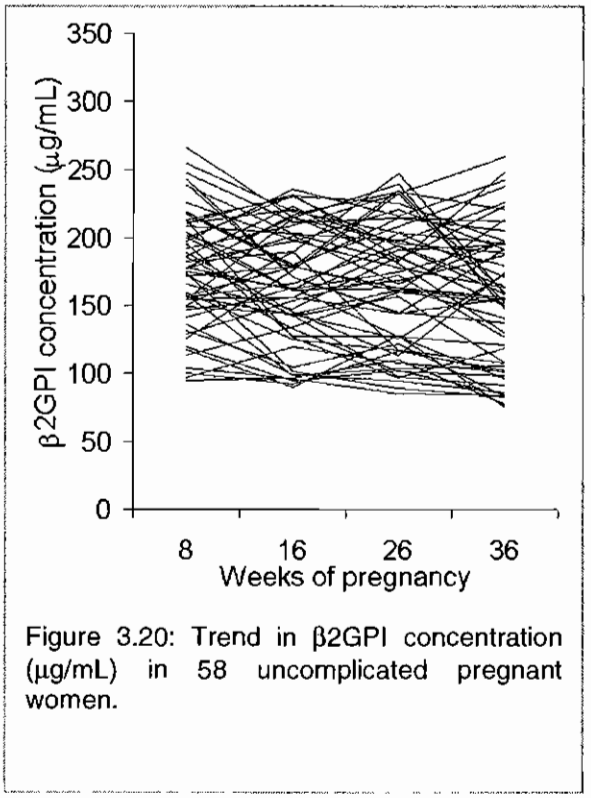

Figure 3.20: Trend in β2GPI concentration (μg/mL) in 58 uncomplicated pregnant women.

3.4.7 β2GPI concentration in patients with inflammatory disorders

Two groups of patients with inflammatory disorders were studied. In the first group of 120 patients with a raised erythrocyte sedimentation rate (ESR) (60 female and 60 male patients; mean age 53.0 ± 22.0 years), levels of β2GPI and several known acute phase reactants were measured and correlated. These patients included 64 patients with a normal CRP level (< 10.0 mg/L) and 56 patients with an elevated CRP level (> 10.1 mg/L). The latter group had a significantly lower β2GPI level than the former group (Mann-Whitney P=0.020). In addition, β2GPI level also showed a negative correlation with CRP concentration (Spearman rank correlation test r=-0.288 with P=0.0017) (figure 3.21), but a positive correlation with both albumin and transferrin (figures 3.22 & 3.23 respectively) (Spearman rank correlation test r=0.372 and 0.453 respectively with P<0.0001 for both).

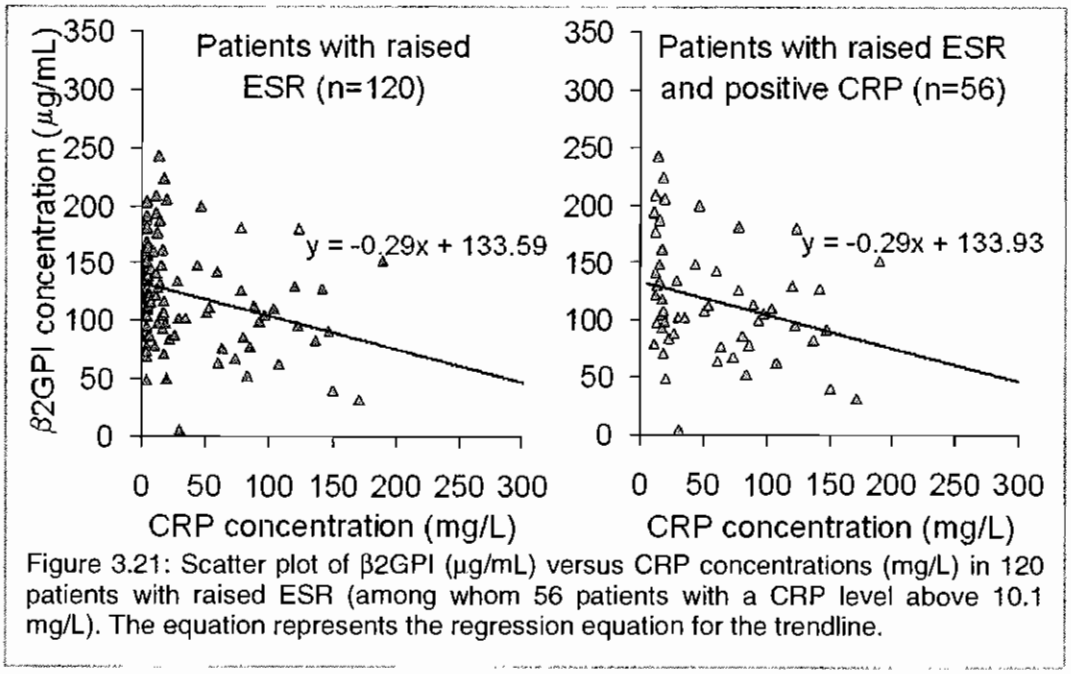

Figure 3.21: Scatter plot of β2GPI (μg/mL) versus CRP concentrations (mg/L) in 120 patients with raised ESR (among whom 56 patients with a CRP level above 10.1 mg/L). The equation represents the regression equation for the trendline.

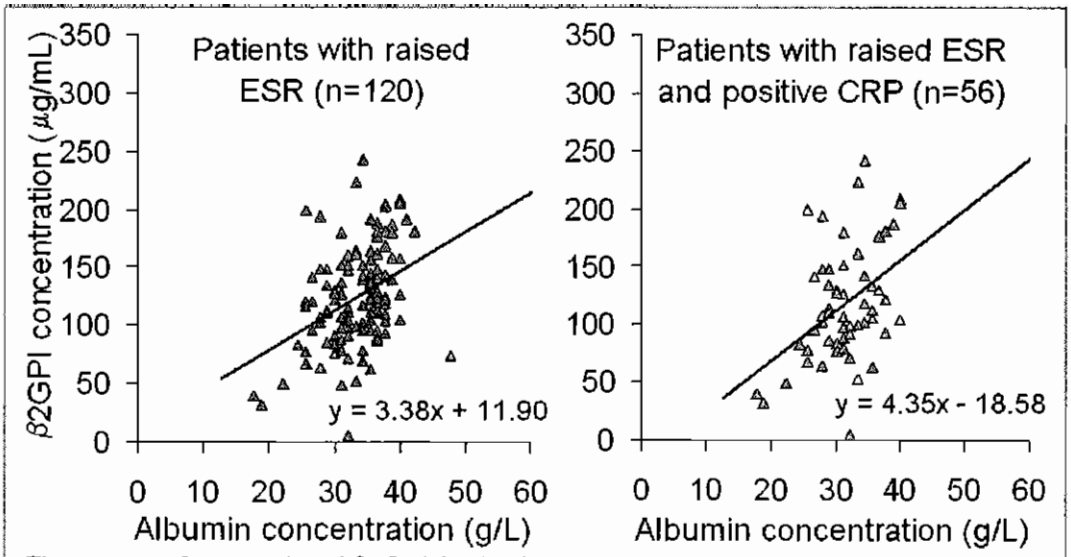

Figure 3.22: Scatter plot of β2GPI (μg/mL) versus albumin concentrations (g/L) in 120 patients with raised ESR (among whom 56 patients with a CRP level above 10.1 mg/L). The equation represents the regression equation for the trendline.

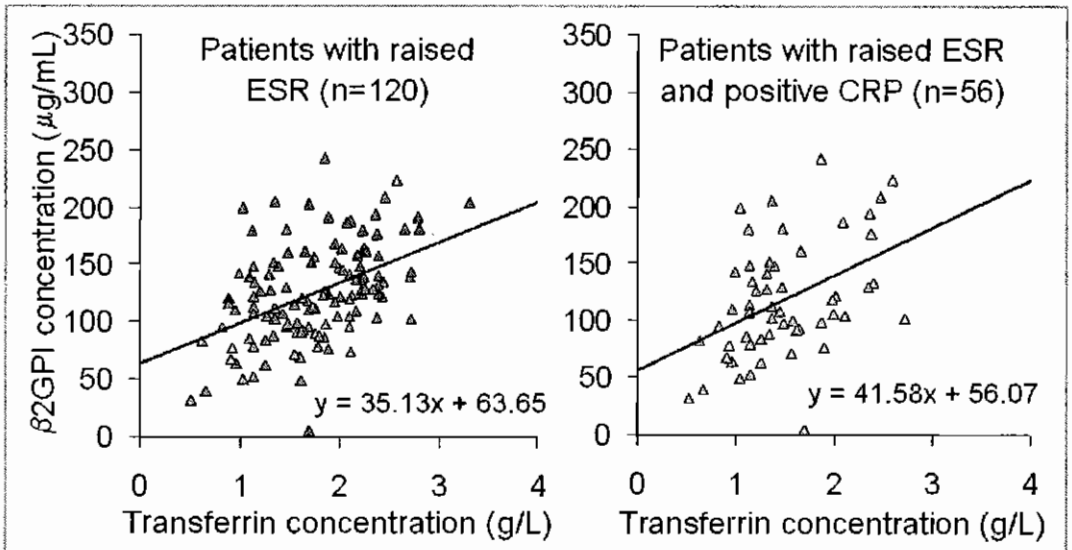

Figure 3.23: Scatter plot of β2GPI (μg/mL) versus transferrin concentrations (g/L) in 120 patients with raised ESR (among whom 56 patients with a CRP level above 10.1 mg/L). The equation represents the regression equation for the trendline.

Effect of increasing CRP level on β2GPI level: In order to study the effect of an increasing CRP value on β2GPI concentration, a larger group of 144 patients (74 female and 70 male patients; mean age 61.8 ± 18.6 years old) with a CRP level above 10.1 mg/L were tested for β2GPI concentration. In these individuals, β2GPI concentrations ranged between 57.9 to 316.1 µg/mL (mean ± SD: 163.4 ± 60.6 µg/mL) and again showed a strong negative correlation with CRP level (Spearman rank test r=-0.2838 with P=0.0006) (figure 3.24). For statistical purposes, the samples were subdivided into 4 quartiles with CRP level between 10.1 and 22.8 mg/L (Q1), 22.9 and 52.0 mg/L (Q2), 53.2 and 105.0 mg/L (Q3), and above 105.1 mg/L (Q4). As can be seen in figure 3.25, there were significant differences in β2GPI levels among the quartiles (Kruskal-Wallis test 0.0007) with an extremely significant decrease in β2GPI level in the quartile with the highest CRP values when compared to the healthy controls (mean ± SD: 186.6 ± 53.5 µg/mL (Q1), 166.9 ± 62.6 µg/mL (Q2), 167.2 ± 58.1 µg/mL (Q3), and 133.1 ± 57.7 µg/mL (Q4); Mann-Whitney tests comparing β2GPI levels in Q1 to Q4 versus healthy age-matched individuals: P=0.566; 0.010; 0.009; <0.001 respectively).

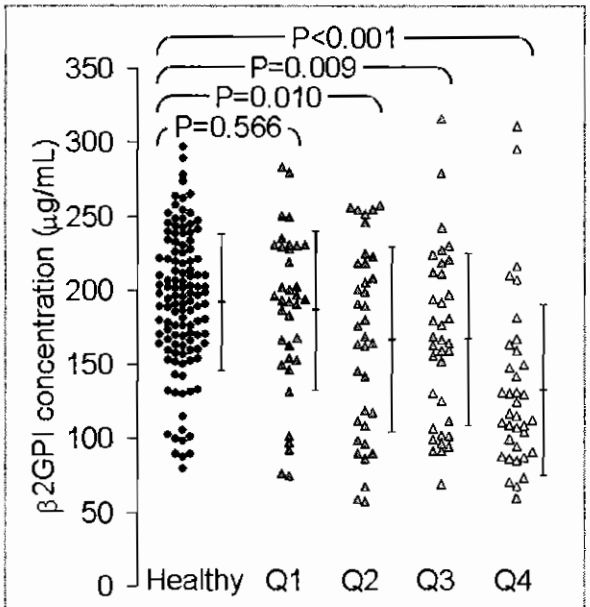

Figure 3.25: β2GPI concentration (µg/mL) in 144 patients with an elevated CRP level sorted by CRP level (quartiles 1 to 4, see text; n=36) and in healthy age-matched individuals (n=119). Error bars represent mean ± SD.

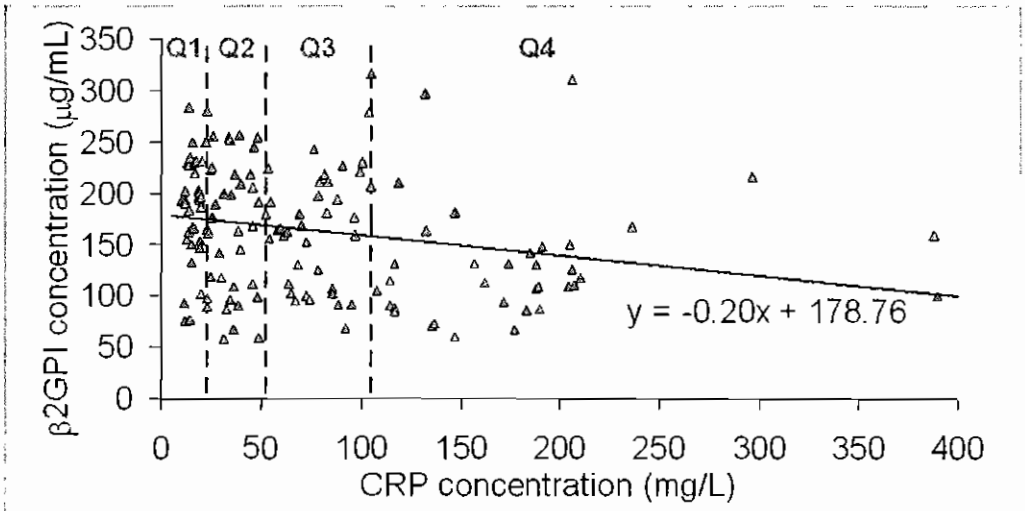

Figure 3.24: Scatter plot of β2GPI (µg/mL) versus CRP concentrations (mg/L) in 144 patients with an elevated CRP level. The equation represents the regression equation for the trendline. Dotted lines represent our arbitrary quartile limits.

3.5 Discussion

3.5.1 Sample stability

It is conceivable that the disruption caused by repeated freezing/thawing cycles of β2GPI-lipoprotein complexes - into which a significant proportion of the total plasma β2GPI content is thought to be located (Polz *et al*, 1979a,b,c & 1980; McNally *et al*, 1995a) - could increase the proportion of free β2GPI molecules, and thereby of β2GPI available for binding to the coated antibody. Indeed, we found a significant increase in β2GPI level following several freezing/thawing cycles, similarly to previous reports (McNally *et al*, 1995a). Although the β2GPI assay can be used to measure both free and bound β2GPI, this variability could have impaired the compatibility of our analyses. For this reason, we used only serum and plasma that were fresh or frozen/thawed at most once for β2GPI quantification in this study.

As observed in previous reports (Cleve, 1968; Norden *et al*, 1991), extended storage did not affect the measurement of β2GPI. This allowed the use of samples that had been stored at -20°C for up to three years.

3.5.2 Serum versus plasma

No significant difference in β2GPI concentration between serum and plasma samples was observed. These results are in agreement with previous reports that observed similar β2GPI level in serum samples and in sodium citrate, EDTA, and heparin plasmas (Ichikawa *et al*, 1992; McNally *et al*, 1993).

3.5.3 β2GPI concentration in healthy individuals

β2GPI concentrations in the group of healthy individuals had a mean (± SD) of 178.3 (± 46.2) μg/mL and ranged between 20.8 to 296.8 μg/mL (displaying a wide 14-fold range distribution with a bimodal distribution and a mildly skewed asymmetry to the left). These values obtained with our sandwich non-competitive ELISA protocol in sera collected from healthy volunteers were comparable to those obtained by others using radial immunodiffusion (Cleve, 1968; Cohnen, 1970; Propert, 1978; Walter *et al*, 1979; Hoeg *et al*, 1985; Ichikawa *et al*, 1992; Matsuda *et al*, 1993b; Quintarelli *et al*, 1994), immuno-electrophoresis (Bancsi *et al*, 1992; McNally *et al*, 1993), competitive (Brighton *et al*, 1996; Cassader *et al*, 1997; Ruiu *et al*, 1997) and non-competitive ELISA (Sansom *et al*, 1991; McNally *et al*, 1993, 1994b, 1995a&b; Crook *et al*, 1999; Mehdi *et al*, 1999; Yasuda *et al*, 2000b).

Individuals that had a β2GPI concentration below the lower 95% confidence limit of the normal distribution have been considered by some authors as carriers of the proposed Bg^D (deficient) codominant autosomal allele ([Cleve *et al*,, 1968 & 1969; Koppe *et al*, 1970; Bancsi *et al*, 1992]). 26 individuals (7.8%) showed a mean (± SD) β2GPI concentration of 87.2 (± 16.5) µg/mL. Among these individuals, one (0.3%) had a mean β2GPI concentration of 20.8 µg/mL, well below the lower 99% confidence limit of the (corrected) normal distribution. The mean β2GPI concentration (± SD) of the remaining 308 healthy individuals (92.2%) was 186.0 (± 39.2) µg/mL. If the individuals in these three subgroups were considered as being respectively Bg^N Bg^D heterozygotes, Bg^D Bg^D homozygous deficient, and Bg^N Bg^N homozygous normal, the frequency for the Bg^N gene would be 0.96 and the frequency for heterozygote Bg^N Bg^D would be 7.5%, close to the values previously reported for Caucasians ([Cleve, 1968; Bancsi *et al*, 1992]).

No significant difference was found between β2GPI level in female and male subjects (Mann-Whitney P=0.4216). However, there is inconsistency in the literature regarding this question. For some authors, gender does not seem to significantly affect β2GPI level ([Sansom *et al*, 1991; Bancsi *et al*, 1992; Ichikawa *et al*, 1992; McNally *et al*, 1993; Mehdi *et al*, 1999]), while other have shown significant differences between both genders ([Cleve, 1968; Propert, 1978; Crook *et al*, 1999]). We have shown that while males presented a slightly higher level of β2GPI than females, this difference was not statistically significant. The potential difference in levels of β2GPI in females and males is of interest as β2GPI is thought to be an inhibitor of the coagulation pathway and females have a higher incidence of thrombotic episodes during pregnancy and while receiving oestrogen therapy ([Gerstman *et al*, 1991; van Baal *et al*, 1999; Ridker *et al*, 2000]).

Interestingly, a significant positive correlation was observed between age and serum β2GPI level in healthy individuals (Spearman test r=-0.2744; P<0.0001), in line with previous reports ([Cleve, 1968; Mehdi *et al*, 1999]). The higher level associated with increasing age could indicate rising synthesis of β2GPI or reduced catabolism. In this regard, the age-related increase in the overall level of atherosclerosis ([Benditt *et al*, 1994]) may trigger increased synthesis of β2GPI as part of an attempt to inhibit atherosclerosis progression. β2GPI is abundantly present in atherosclerotic plaques in association with endothelial cells, macrophages, and CD4+ lymphocytes ([George *et al*, 1999b&c]) and has been shown to react directly with oxidized LDL ([Hasunuma *et al*, 1997; Kobayashi *et al*, 2003; Matsuura *et al*, 2003]). With its potential *in vivo* roles

as an activator of lipoprotein lipase ([Nakaya *et al*, 1980]) and a free-radical-scavenging antioxidant factor ([Lin *et al*, 2001]), β2GPI may function thus as a regulator of lipoprotein fractions in an unbalanced lipid environment and therefore be under increasing demand as atherosclerotic lesions progress.

3.5.4 β2GPI concentration during normal pregnancy

Pregnancy is thought to be a predisposing condition for thrombosis as it is accompanied by a marked increase in some of the plasma coagulation factors, especially in late pregnancy ([Pinto *et al*, 1988; Comeglio *et al*, 1997; Letsky *et al*, 1998 & 2000; Manten *et al*, 2004; Uchikova *et al*, 2005]).

Our results show that β2GPI level significantly decreases over 36 weeks of pregnancy (Friedman test P=0.002) and that β2GPI level is significantly reduced in pregnant women at 36 weeks of pregnancy when compared to those found in non-pregnant women or in 8-week pregnant women. This is in line with previous reports ([Cleve, 1968; Gleichmann *et al*, 1973]), although this reduction does not reach 50% as reported by Ganrot *et al.* ([1972]). It is highly probable that, as for many other blood components, the decrease of β2GPI level is at least partly explained by the dilution or redistribution of serum β2GPI molecules due to the increase in blood volume often exhibited by pregnant women ([Baylis *et al*, 1998; Letsky *et al*, 2000]).

There is, however, also reason to believe that a reduced β2GPI level may be caused by increasing consumption of the protein as pregnancy progresses. Since the placenta is a site where maternal blood is increasingly challenged with activators of the coagulation cascade, β2GPI could play an important role in local haemostasis particularly at the materno-foetal interface. The presence of β2GPI on the placenta - and more specifically on the syncytiotrophoblast and extravillous cytotrophoblast - ([Chamley *et al*, 1993b & 1997]) reinforces this possibility. Increased proteolysis could also be responsible for the decrease in β2GPI level. There is controversy as to whether the fibrinolytic activity rises during pregnancy concomitantly with an increased activation of coagulation ([Pinto *et al*, 1988; Comeglio *et al*, 1997; Letsky *et al*, 1998 & 2000; Choi *et al*, 2002; Uchikova *et al*, 2005]). If so, a marked increase in plasmin-mediated β2GPI proteolysis (to which β2GPI has been shown to be highly susceptible) could occur and significantly drive β2GPI level down ([Ohkura *et al*, 1998; Horbach *et al*, 1999; Matsuura *et al*, 2000]). Whether this physiological effect on β2GPI level has any significance remains to be determined.

3.5.5 β2GPI concentration in disease

3.5.5.I β2GPI concentration in patients with stroke or acute coronary syndrome

Patients presenting with stroke were found to have a reduced level of β2GPI in comparison to age-matched controls. It is possible that this lowered β2GPI level was present prior to the thrombotic event and significantly predisposed these individuals to stroke. Alternatively, the stroke itself could be the responsible factor for lowering serum β2GPI level. The observation that β2GPI level had not changed when these patients were followed up over six months after the stroke, however, makes it more likely that the stroke patients represent a group of individuals with lower β2GPI level *per se*.

When patients with acute coronary syndrome were taken as a whole, β2GPI level was not found to be significantly different than in age-matched controls. However, when divided into two subgroups according to their age, it was found that older cardiac patients (who were of the same age range as the stroke patients) did have a significantly lower serum β2GPI level than their age-matched counterparts. The same hypotheses can be drawn as for stroke patients: it is possible that a lower level of serum β2GPI predisposed elderly cardiac individuals to acute coronary syndrome or that the syndrome itself was responsible for lowering β2GPI level. Similarly to stroke patients, when the patients were followed up six months after the cardiac event, they showed no significant change in β2GPI level. It is therefore more likely that the elderly cardiac patients had a lowered β2GPI level before the cardiac event. The absence of difference in β2GPI level between younger healthy and cardiac individuals suggests that the role of β2GPI in acute coronary syndrome is probably more limited in younger people.

If the elderly patients who suffered from a stroke or an acute coronary syndrome had widespread atherosclerotic lesions, it is possible that β2GPI antigens had been "consumed" by these lesions. This may have happened either through physical binding of β2GPI to the atherosclerotic lesions or through ongoing immune-mediated response towards oxidized LDL that may further consume β2GPI through cross-reaction (Hörkkö *et al*, 1996 & 2000). From a clinical perspective, this variation in β2GPI level could significantly disturb β2GPI-mediated capacity to control platelet aggregation and thus clot formation, as well as LDL oxidative susceptibility (Lin *et al*, 2001).

3.5.5.II β2GPI concentration versus ACLA and anti-β2GPI antibody levels in patients with stroke

The majority (93.1%) of the stroke patients who were tested for ACLA did not have a significant level of either IgG or IgM ACLA. In the few individuals who were tested positive for IgG ACLA, the concentration of β2GPI did not significantly differ from the one found in those who were tested IgG ACLA negative (P=0.3422), which is in line with several other reports (Galli *et al*, 1992a; Oosting *et al*, 1992; Kamboh *et al*, 1999a).

Our results thus do not support a direct relationship between ACLA prevalence and concentration of β2GPI or anti-β2GPI antibodies. It is true that the association between stroke and aPL seems to be stronger in adults younger that 50 years old (Landi *et al*, 1983; Brey *et al*, 1990) and perhaps children (Triplett *et al*, 1998) compared to elderly patients (the cumulative odds ratio for ACLA has been estimated at 5.8 below age 50 and 2.5 at all ages), but these viewpoints are also controversial (Czlonkowska *et al*, 1992; Kushner *et al*, 1990). Although both ACLA and anti-β2GPI antibodies may share similarities in their activities, we suggest that those may arise from different and independent mechanisms that are not related to serum concentration of β2GPI. The functional activity of the latter may be compromised by aPL and/or anti-β2GPI antibodies, but as shown in the group of stroke patients, this could happen without any demonstrable change in the serum level of the protein.

3.5.5.III β2GPI concentration during acute inflammation

Some of our most interesting findings concerned the level of β2GPI in inflammation, and there has been much recent interest in the relationship between inflammation, atherosclerosis, and thrombosis (Hansson, 2005). Patients with acute inflammation are at increased risk of thrombosis mainly due to enhanced procoagulant activities and inhibited fibrinolysis (Cicala *et al*, 1998; Dhainaut *et al*, 2001). Patients with an elevated CRP level showed a strong negative correlation between β2GPI and CRP levels. More specifically, when these patients were sorted into four quartiles according to their CRP level, the individuals of the quartile with a low to moderate level of CRP (Q1, Q2, and Q3) had no or slightly (not statistically significant) decreased serum β2GPI concentration, while the individuals of the last quartile (Q4) with a CRP level above 105.1 mg/L (Q4) presented the most dramatic (and statistically highly significant) reduction in β2GPI level. Our findings also show that β2GPI level strongly correlates with albumin and transferrin levels, both known to be negative acute phase proteins. It is therefore likely that β2GPI is

a negative acute phase protein of which the synthesis is diminished in inflammation.

It is also reasonable to suggest from our results that β2GPI is gradually consumed as the inflammatory process progresses. Depending on the speed of onset and strength of potential compensatory mechanisms, this decrease might become significant. While a low to moderate degree of inflammation might not affect the capacity of β2GPI to face increasing demand, an elevated degree of inflammation characterised by a generalized prothrombotic environment might correspond to an exaggerated demand of β2GPI and subsequent excessive β2GPI consumption.

In addition, two other mechanisms could further deplete β2GPI level in these patients: increased apoptosis (due to microinfarcts resulting from excessive activation of coagulation) that is accompanied by substantial exposure of anionic phospholipids to which β2GPI avidly binds (Chonn *et al*, 1995; Balasubramanian 1997 & 1998; Levine *et al*, 1998; Manfredi *et al*, 1998a&b; Rovere *et al*, 1999), and fibrinolysis, although diminished in inflammation (Dhainaut *et al*, 2001), that may facilitate proteolysis of β2GPI, as previously mentioned (Ohkura *et al*, 1998; Horbach *et al*, 1999; Matsuura *et al*, 2000).

As β2GPI may have a role as an inhibitor of coagulation, it is interesting to note that the level of β2GPI is reduced during inflammation. The reduction in β2GPI could conceivably have an important influence on the pro-thrombotic tendency that is observed during inflammatory processes.

3.6 Conclusion

In this part of our study, we have developed a sensitive sandwich-direct ELISA for β2GPI quantification. This β2GPI ELISA has been shown to be a reliable assay for the quantification of β2GPI. We have shown a negative correlation between age, pregnancy, stroke, acute coronary syndrome (in older patients), inflammation and the level of β2GPI. This reduction in β2GPI level may have an important role to play in the pathogenesis of these conditions.

4 β2GPI polymorphism

4.1 Introduction

4.1.1 The β2GPI (APOH) gene

Although β2GPI is included in the apolipoprotein family for reasons mentioned in chapter 3, its nucleic acid sequence does not show any significant homology with any other apolipoprotein ([Day *et al*, 1992]). The β2GPI gene locus (referred to as APOH) has been located by *in-situ* hybridisation to the distal part of the long arm of chromosome 17 (more specifically 17q23-24) ([Steinkasserer *et al*, 1992b; Okkels *et al*, 1999]).[1]

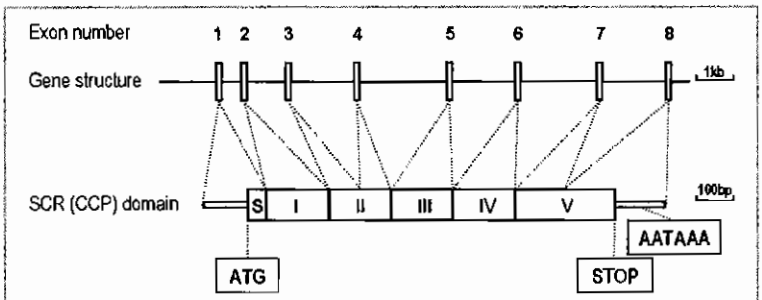

Figure 4.1: Schematic representation of the β2GPI (APOH) gene organisation. The positions of exons are shown as boxes, and the introns (not numbered) are shown as lines connecting the exons. The β2GPI domain (SCR) structure is shown below. ATG: translation initiation site; S: signal peptide; STOP: termination codon; AATAAA: polyadenylation site. From Sheng *et al.* ([1997]) and Okkels *et al.* ([1999]).

As described in the introductory chapter, β2GPI consists of five contiguous domains (or SCRs) that have been shown to be a common structural element in a large number of diverse proteins (see appendix E). These SCR gene units are generally inherited in a comparable fashion. Most of them are encoded by single individual exons, but some of them are encoded by two ("split") exons ([Fujisaku *et al*, 1989; Tosi *et al*, 1989; Rodriguez *et al*, 1991; Hillarp *et al*, 1993; Vik *et al*, 1993; Hobart *et al*, 1995]), and some other as two SCRs encoded together by one exon ([Vik *et al*, 1993; Fujisaku *et al*, 1989]). The human βGPI (APOH) gene consists of 8 exons spanning 18 kb ([Steinkasserer *et al*, 1991; Okkels *et al*, 1999]). It presents exon/intron splice junction sites that follow the GT/AG consensus sequence rule ([Sheng *et al*, 1997]) and the coded domains correspond to the exon/intron structure of the gene (with domains I, III, and IV encoded by single exons, and domains II and V encoded by two exons ([Sheng *et al*, 1997; Okkels *et al*, 1999])) (figure 4.1).

The coding sequence for the mature human β2GPI protein (326 amino acid residues) is shown in figure 4.2. It starts with a glycine and is preceded by a leader sequence of 19 amino acid residues.

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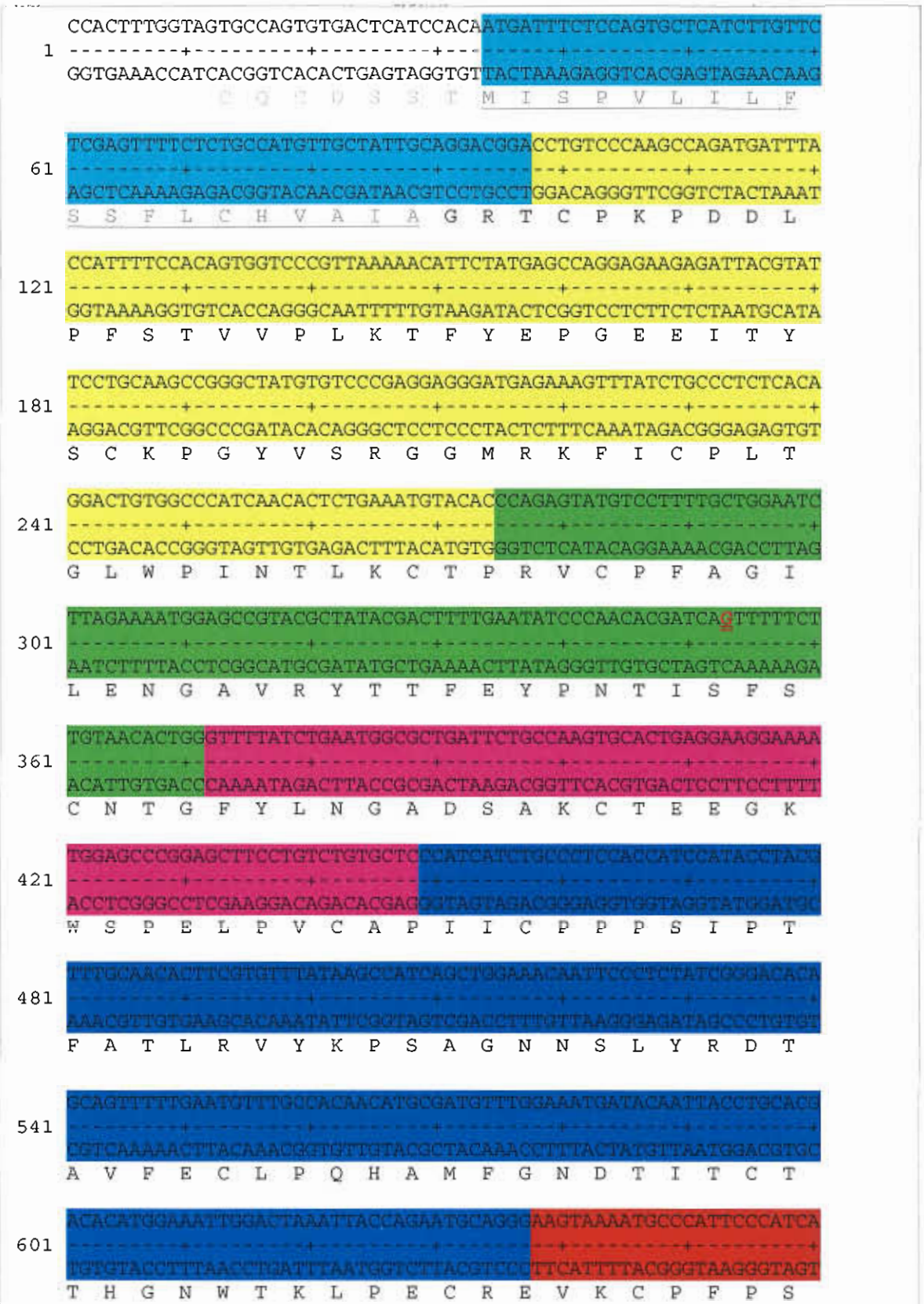
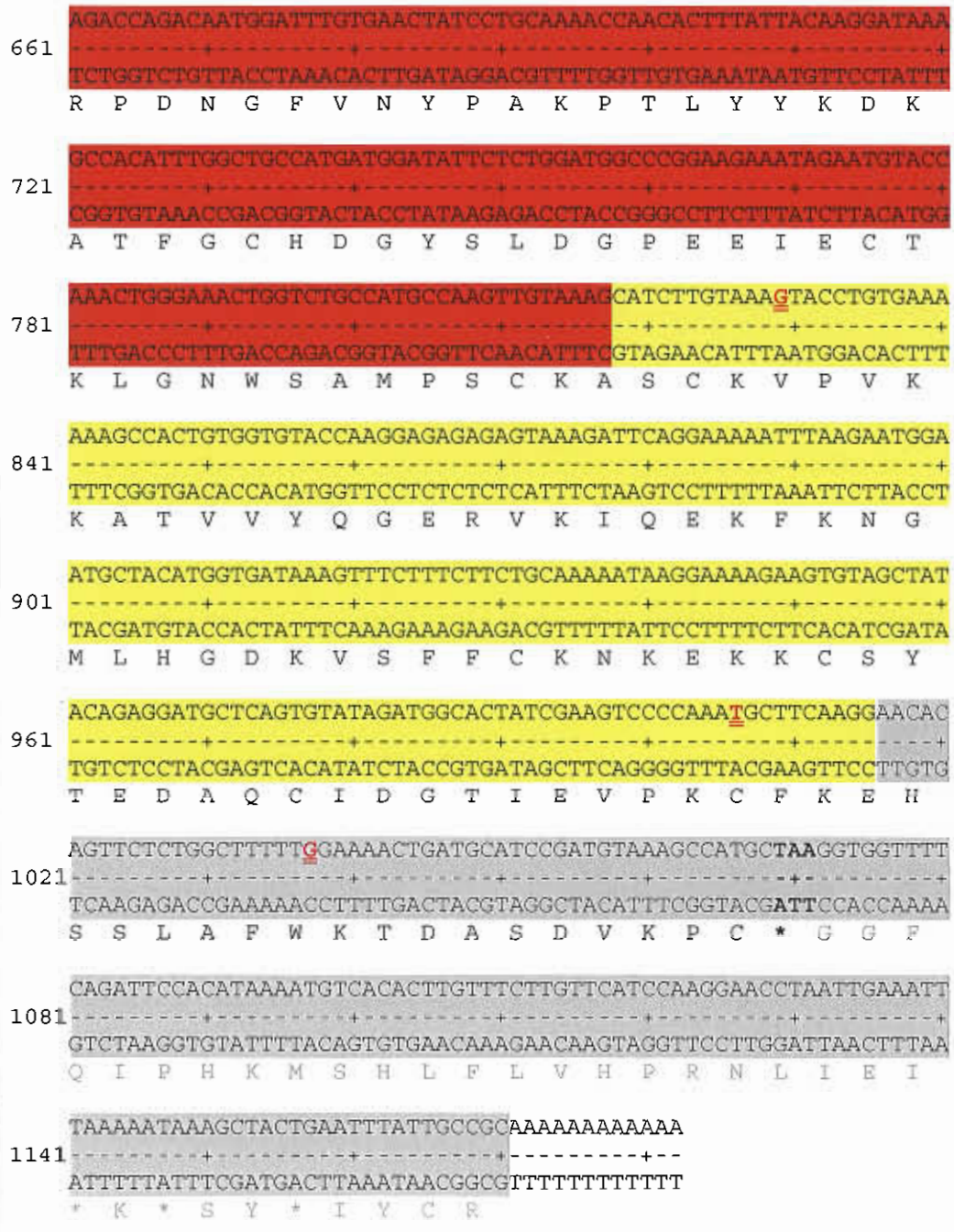
[1] To date, no other apolipoprotein gene is known to map to chromosome 17: the apo AII gene maps to chromosome 1; apo B to chromosome 2; apo D to chromosome 3; apo (a) to chromosome 6; apo AI, apo CIII, and apo AIV to chromosome 11; apo E, apo CI, and apo CII to chromosome 19 ([Karathanasis, 1985, Lusis, 1988; Utermann, 1989]). This suggests that the β2GPI (APOH) gene evolved from a duplication or dispersion of an original gene among structurally related but functionally divergent proteins.



Continued on next page

Figure 4.2: Nucleotide and deduced amino acid sequences of human β2GPI (see figure 1.3). Exons are colour differentiated. The point mutations studied in chapter 4 are double-underlined. Source: GenBank accession N° NM_000042.

Continued from previous page



4.1.2 β2GPI polymorphism[2]

The human β2GPI protein displays a genetically determined structural polymorphism that was initially detected by isoelectric focusing (IEF) in polyacrylamide gel followed by immunoblotting (Kamboh *et al*, 1988). This technique allowed the detection based on banding patterns of structurally different isoforms with different isoelectric points. These are thought to be due to an altered polypeptide chain owing to amino acid substitution in the given variant isoform and to variations in post-synthetic sialic acid contents in the different sub-fractions (with the degree of glycosylation rising as the isoelectric point falls) (Schousboe *et al*, 1983a; Gries *et al*, 1989). With this IEF-immunoblotting technique, four IEF banding patterns were first described and designated APOH*1, APOH*2, APOH*3, and APOH*4. Subsequently, the APOH*3 allele was subdivided into APOH*3^B and APOH*3^W based on specific monoclonal antibody reactivity (Kamboh *et al*, 1988; Sepehrnia *et al*, 1989).

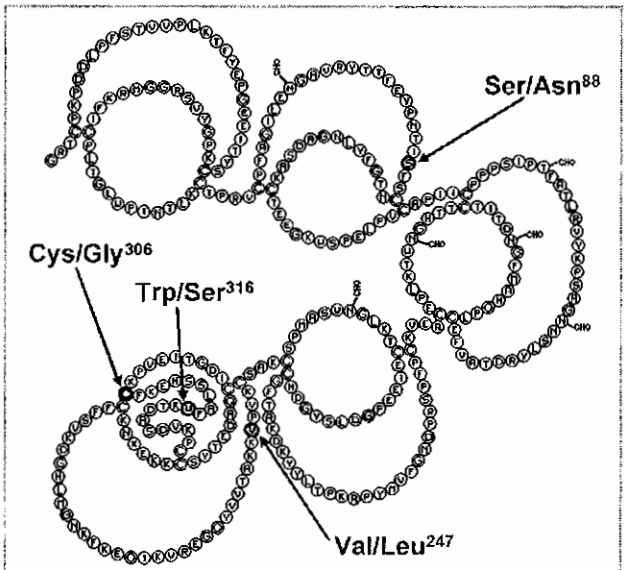


Figure 4.3: Schematic representation of β2GPI with the four point mutations that were studied in this chapter highlighted.

Both family and population studies have shown that APOH*2 is the most frequent, and most probably the parental, allele (Kamboh *et al*, 1988; Saha *et al*, 1992; Cassader *et al*, 1994), and that allele distribution shows ethnic variability (see below). The molecular basis of APOH*1 and APOH*3^W alleles has been established. The APOH*1 allele is differentiated from APOH*2 by a missense mutation (G→A) in exon 3 (in the second SCR) which alters the amino acid residue at codon 88 from a serine to an asparagine residue. The APOH*3^W allele is differentiated from APOH*2 by a missense mutation (G→C) in exon 8 (in the fifth SCR) which alters the amino acid residue at codon 316 from a hydrophobic tryptophan to a hydrophilic serine (Sanghera *et al*, 1997a&b). Recently, two missense mutations that alter the amino acid residues at codon 135 from an arginine to a histidine and at codon 141 form an alanine to an aspartic acid have been identified in the third domain of

[2] β2GPI polymorphism has been labelled according either to IEF-pattern denominations from Kamboh *et al.* (1988) or to the codon in which a base substitution has occurred (88, 247, 306 or 316). Point mutations in the β2GPI gene were also designated $Ser^{88}Asn$, $Val^{247}Leu$, $Cys^{306}Gly$, and $Trp^{316}Ser$ respectively according to the involved amino acid changes.

β2GPI. These two mutations correlated with the APOH*3^{B} and APOH*4 alleles respectively based upon protein typing. As little has been published on this matter in international papers since we published a poster at the 9th International Congress on Antiphospholipid Antibodies in Tours in 2000, these two mutations were not analysed in this study. Two other missense mutations (G→T) and (T→G), which do not account for any of the IEF-immunoblotting profiles, have been characterised in exon 7 (in the fifth SCR) substituting amino acid residues at codons 247 (from a valine to a leucine residue) and 306 (from a cysteine to a glycine) (Steinkasserer *et al*, 1993; Sanghera *et al*, 1997a&b; Gushiken *et al*, 1999; Hirose *et al*, 1999) (figure 4.3 and table 4.1 in chapter 4.3.3.II.b).

No thorough analysis of β2GPI polymorphism across ethnic groups has been performed to date. However, several studies have sporadically analysed them in groups of different ethnic backgrounds and shown that APOH*1 (i.e. Asn^{88}) and APOH*3^{W} (i.e. Ser^{316}) were much more frequent among non-Hispanic whites and Hispanics compared to blacks (Sanghera *et al*, 1997a), and that Leu^{247} was more frequent among Caucasians, less among African Americans, and least among Asians (Hirose *et al*, 1999). Fewer studies have been performed on the Gly^{306} mutation which appears to be unique to Caucasians, as it was not observed in populations of African ancestry (Sanghera *et al*, 1997a). The APOH*4 allele seemed to be confined to the latter population.

The exact effect of these mutations on the level, binding properties, functions, or antigenicity of β2GPI, or indirectly on other plasma proteins, has not been fully clarified.

4.1.3 Potential effect of β2GPI polymorphism

Given that β2GPI is thought to be involved *in vivo* in the clearance of triglycerides from plasma (Wurm, 1984), various studies have attempted to relate IEF patterns with plasma lipids without being able to draw any definite conclusions. Several studies carried out in US whites (Eichner *et al*, 1989b; Kaprio *et al*, 1991), Chinese (Saha *et al*, 1993) and Siberian populations (Kamboh *et al*, 1996) found no relationship between β2GPI IEF patterns and any particular lipid trait, while two other studies reported that the APOH*3 allele was associated with a higher plasma triglyceride level in Italians (Cassader *et al*, 1994) and Nigerians (Sepehrnia *et al*, 1989). APOH*1 was also found to have a lowering effect, and APOH*4 an elevating effect, on HDL_{3c} (Sepehrnia *et al*, 1989).

Other studies attempted to correlate the presence of antiphospholipid antibodies (aPL) and/or anti-β2GPI antibodies with β2GPI polymorphism, and particularly with the Leu^{247} mutation. This mutation substitutes a valine to a leucine residue in a location between the phospholipid-binding site of the fourth and fifth domains of β2GPI, the former domain being thought to bear cryptic epitopes that are recognized by anti-β2GPI antibodies from patients with the APS (Igarashi *et al*, 1996; Matsuura *et al*, 1995; Koike *et al*, 1998). The expression of a valine at position 247 has been proposed to induce a conformational change in the protein that may be associated with auto-reactivity (Hirose *et al*, 1999). In patients with the primary APS with anti-β2GPI antibodies, there was an increased frequency of valine at position 247 when compared to control subjects or patients with the primary APS without anti-β2GPI antibodies (Atsumi *et al*, 1999; Hirose *et al*, 1999; Prieto *et al*, 2003; Yasuda *et al*, 2005). In addition, βGPI bearing a valine at position 247 has shown higher binding by anti-β2GPI antibodies than β2GPI bearing a leucine at the same position (Yasuda *et al*, 2000a). However, because Val^{247} is commonly present in healthy populations, because up to 21 % of anti-β2GPI antibody-positive patients have been found to be homozygous for the $Leu^{247}Leu$ genotype (Atsumi *et al*, 1999; Prieto *et al*, 2003), and because some patients with the primary APS have been found to have serum anti-β2GPI antibodies regardless of the amino acid present at position 247 (Hirose *et al*, 1999, Atsumi *et al*, 1999), other mechanisms must be involved in the induction of an anti-β2GPI autoantibody response.

As the fifth domain of β2GPI is thought to mediate the binding of the protein to phospholipids (Hunt *et al*, 1993 & 1994, Lauer *et al*, 1993; Sheng *et al*, 1996), any mutation in this domain has the potential to alter the physiological properties of β2GPI. The two

structural mutations at positions 306 and 316 are located in the C-terminal tail of this domain. Both mutations are thought to render β2GPI unable to bind negatively charged phospholipids (Sanghera *et al*, 1997a&b; Horbach *et al*, 1998; Kamboh *et al*, 1998; Mehdi *et al*, 2000a&b; Nash *et al*, 2003). The Ser^{316} mutation disrupts the integrity of the highly conserved hydrophobic region at positions 313 to 316 that is thought to closely interact with anionic phospholipids (Sanghera *et al*, 1997b), while the Gly^{306} mutation causes a disruption of the disulfide bond between Cys^{281} and Cys^{306} that is thought to be essential for the clustering of positively charged amino acids and the conserved hydrophobic region, and thereby for the binding to anionic phospholipids (Steinkasserer *et al*, 1991; Hunt *et al*, 1993 & 1994). Studies have shown that both mutations result in a markedly reduced binding of β2GPI to phospholipid surfaces, especially in homozygous and compound heterozygous states (Sanghera *et al*, 1997b).

Since they reduce β2GPI's capacity to bind to phospholipids, it has been hypothesised that both mutations may preclude the production of phospholipid/β2GPI-dependent aPL (Kamboh *et al*, 1995; Sanghera *et al*, 1997a&b). Although some authors have found no clear association of either mutation with aPL (Gushiken *et al*, 1999; Camilleri *et al*, 2003), others have found that the Ser^{316} mutation seems to confer some protection against aPL production (Kamboh *et al*, 1999a). On the other hand, the Ser^{316} mutation was not found to protect against the production of anti-β2GPI antibodies (Horbach *et al*, 1998; Gushiken *et al*, 1999; Kamboh *et al*, 1999a). This supports the hypothesis that anti-β2GPI antibodies are directed against at least two epitopes of β2GPI (Cabral *et al*, 1995), one involving a phospholipid-induced neo-epitope and another a phospholipid-free epitope. However, due to the low frequency of both mutations at codons 306 and 316, larger-scaled studies are required to prove such statements.

Alternatively, the reduced binding capacity of native β2GPI to anionic phospholipids could rather be a reflexion of a lowered concentration of plasma β2GPI - that have been associated with both mutations at codons 306 and 316 in a dose-dependent fashion (Ruiu *et al*, 1997; Sanghera *et al*, 1997b; Kamboh *et al*, 1999a; Mehdi *et al*, 1999). However, this association was not absolute, and more recent data from *in vitro* mutagenesis and expression studies show that neither mutation has an effect on the level of expression or secretion of recombinant β2GPI in transfected COS-1 cells (Mehdi *et al*, 2000a & 2003). Thus, it has been proposed that the two mutations are rather in linkage disequilibrium with one or several other functional mutation(s) that are yet to be identified.

On the basis of plasma β2GPI variation in family and population data, β2GPI plasma concentration has been proposed to be controlled by two putative codominant autosomal alleles, named Bg^N (normal) and Bg^D (deficient). The frequency of this deficiency-coding allele is thought to be around 6 % in Caucasian populations and much higher in African and Asian populations (around 20 %) (Cleve *et al*, 1968 & 1969; Koppe *et al*, 1970; Kamboh *et al*, 1988; Sepehrnia *et al*, 1989; Bancsi *et al*, 1992). Depending on the authors, β2GPI plasma concentration is thought to be below 60 µg/mL in homozygous Bg^DBg^D individuals, to exceed 150 µg/mL in homozygous Bg^NBg^N individuals, and to range between these two values in heterozygous Bg^NBg^D individuals (Cleve, 1968; Koppe *et al*, 1970; Atkin *et al*, 1974; Propert, 1978; Walter *et al*, 1979; Kamboh *et al*, 1988; Singh *et al*, 2002a&b). However, the molecular basis of the Bg^D allele has yet to be established, and in several instances the quantitative variation failed to conform to a Mendelian inheritance pattern. Neither the Gly^{306} nor the Ser^{316} mutations show a direct correlation with the Bg^D allele. Recently, a functional mutation in the promoter region of β2GPI (-1C→A at the β2GPI transcriptional initiation site) was proposed to directly affect the synthesis of mRNA and consequently β2GPI level (Mehdi *et al*, 2003). This mutation was also found to be in strong linkage disequilibrium with the Ser^{316} mutation and to confer a significant protective effect against the occurrence of aPL (Mehdi *et al*, 2003). However, this mutation was also observed in individuals with relatively high β2GPI plasma level (Mehdi *et al*, 2003). Thus, this newly discovered mutation alone in the promoter region does not explain all plasma reductions of β2GPI concentration. The molecular basis of plasma β2GPI deficiency thus seems to be heterogeneous, and might be influenced by several other genetic and/or non-genetic factors (Cleve, 1968, Walter *et al*, 1979).

In summary, mutations within the β2GPI (APOH) gene that might affect β2GPI stability, antigenicity, concentration or binding properties could have a significant influence on the clinical susceptibility of carriers of such mutations to thrombotic pathologies. However, population variability at the β2GPI (APOH) locus is not well documented, and even less so for patients with thrombotic diseases.

4.2 Aims of chapter 4

Our aims in this part of the study were to:

- examine the distribution of four well-characterised β2GPI mutations in a representative cohort of healthy Irish citizens,
- evaluate the association of these genetic variations with the risk for thrombosis, by analysing them in case-control cohorts of patients suffering from thrombotic disorders,
- estimate the impact of these mutations on serum β2GPI level in healthy individuals and stroke patients, and
- assess whether these mutations were associated with the presence of anticardiolipin antibodies (ACLA) and anti-β2GPI antibodies.

Before analysing these mutations, we also performed some IEF-immunoblotting testing on the serum samples from a selection of healthy individuals and stroke patients in order to identify β2GPI IEF patterns. As will be discussed below, due to the complexity and controversial character of IEF-immunoblotting analyses, we quickly abandoned this technique and moved to DNA-based genetic engineering techniques.

4.3 Materials and methods

4.3.1 Samples and statistics

4.3.1.I Subjects

Following informed consent from blood donors and approval from local ethical committees, blood samples were obtained from four different study groups.

- 323 **healthy Irish individuals** (male:female ratio 1:3.2, mean age ± SD: 33.5 ± 14.0 years) predominantly young students, nurses, laboratory workers (52 of these individuals were analysed by IEF-immunoblotting);
- 113 patients with **non-haemorrhagic stroke** (male:female ratio 1.6:1, mean age ± SD: 72.0 ± 12.3 years old) (81 of these patients were analysed by IEF-immunoblotting);*
- 360 patients with **early onset acute coronary syndrome** (ACS) defined as either cardiac infarction or unstable angina presenting in males under 55 years old (285 subjects with mean age ± SD: 51.7 ± 6.7 years old) or females under 60 (75 subjects with mean age ± SD: 56.3 ± 6.8 years old);**
- 47 females with **recurrent foetal loss** (RFL) (mean age ± SD: 33.2 ± 6.5 years old; 9 (19.1 %) with ≥ 3 foetal miscarriages);***
- 39 individuals with diagnosed **peripheral thrombotic disorders** (including pulmonary embolism and deep vein thrombosis) (male:female ratio 1:1.6, mean age ± SD: 40.2 ± 15.2 years old).***

4.3.1.II Blood collection

Venous blood samples were collected into dipotassium EDTA (1.5 mg/mL blood) and promptly treated for DNA extraction or stored at -70°C until DNA extraction.

4.3.2 Statistics

Allele frequencies were determined by means of the gene count method. Hardy-Weinberg equilibrium (HWE) was checked by means of the χ^2 test. Differences in allele or genotype frequency between groups of individuals were evaluated by means of the χ^2 test or Fisher's exact probability test. β2GPI concentrations in different cohorts were compared using the ANOVA, Mann-Whitney and student t tests. All analyses were performed with InStat GraphPad Software, Version 2.03, 1994 (PK Wallace, Wally Word) or SPSS Inc., version 11.0 for Windows, 2001. P values under 0.05 were considered statistically significant. Haplotype

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Samples were kindly donated by * W. Livingstone, Trinity College Dublin - Smurfit Institute of Genetics, Dublin; ** D.C. Shields, Department of Clinical Pharmacology, Royal College of Surgeons in Ireland, Dublin; *** J. Vaughan, Dublin Institute of Technology, Dublin.

frequencies were initially investigated using the STATA hapipf function (Mander *et al*, 2001).

4.3.3 Analyses of β2GPI polymorphism

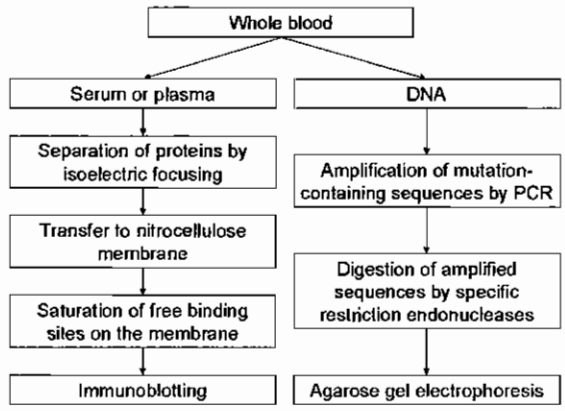


Figure 4.4: Schematic outline of the procedures used for β2GPI polymorphism analysis.

Genetically determined β2GPI structural polymorphism was detected with isoelectric protein focusing (IEF) followed by protein immunoblotting with specific antibodies, and with restriction fragment length polymorphism analysis (PCR-RFLP) (figure 4.4).

4.3.3.I IEF-immunoblotting for β2GPI polymorphism

The analysis of β2GPI structural polymorphism by isoelectric focusing (IEF)-immunoblotting consisted in a sequence of steps (appendix H). Proteins were first separated by electrophoresis, then transferred to a sheet of nitrocellulose membrane, and eventually detected immunologically (figure 4.5). The principle of immunoblotting was first described by Towbin *et al.* (1979a&b) and the technique has been reviewed extensively since (Gershoni *et al*, 1982; Towbin *et al*, 1984; Huisman, 1986; Stott, 1989). The combination of the two techniques (electrophoresis on polyacrylamide gels followed by immunoblotting) presents several non-negligible advantages: it is very sensitive and cost-effective, it does not require specific or expensive equipment, and it requires very diluted antiserum solutions.

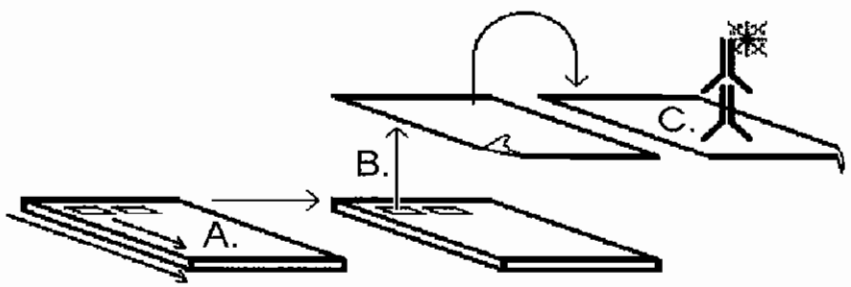


Figure 4.5: Schematic outline of the IEF-immunoblotting protocol: A: separation of proteins by IEF; B: protein transfer on a nitrocellulose membrane followed by saturation of free binding sites; C: successive incubations with the primary antibody, washing, incubation with the enzyme-linked secondary antibody, washing, and then visualization by histochemical staining.

4.3.3.I.a IEF-immunoblotting modus operandi for β2GPI polymorphism

The protocol used in this study was based on that described by Kamboh *et al.* ([1988]). IEF was carried out in thin-layer polyacrylamide gels (0.43 mm Clean-IEF polyacrylamide gel T=5%, C=3%, Pharmacia Biotech, Uppsala, Sweden) using a Multiphor® II platform and a LKB Biochrom 2103 Power Supply. Gels were first re-hydrated for two hours in a GelPool™ flat tray with a mixture of amphoteric and additive compounds (2.2 % Pharmalyte 4-6.5 and 4.4 % Pharmalyte 5-8, Pharmacia Biotech; 3.1 M Urea, BDH). The gel was then placed on the electrophoretic platform damped in kerosene and covered with two wick paper strips, one saturated with sodium hydroxide on the cathode side and one saturated with sulphuric acid on the anode side. A prefocusing step was performed at 700 constant volts for 20 minutes, and then 1 to 2 µl of serum samples were applied onto the gel near the cathode. The power supply settings for the electrophoresis were 500 volts, 8 mA, and 8 W for 20 minutes for the sample entrance step, 1600 volts, 14 mA, and 14 W for 3 hours for the focusing step, and 2000 volts, 14 mA, 18 W for 10 minutes for the band sharpening step (figure 4.5-A).

Electrophoretically separated proteins were then transferred onto a sheet of 0.45 µm pore size nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) pre-soaked with Tris-buffered saline (TBS) (2 mM Trisma Baze, Sigma Chemical Co, St Louis, MO, USA; 15 mM NaCl, Merck) by simple contact diffusion for 2 hours at room temperature (figure 4.5-B). After the protein transfer, the membrane was washed three times 5 minutes with TBS and then incubated in a blocking 5 % w/v non-fat dry milk solution (powder skimmed milk, Marvel, Merseyside, UK) for 15 minutes. After blocking, the membrane was probed overnight with rabbit polyclonal anti-human β2GPI antibodies (Dako) (1:500, v/v) in TBS (figure 4.5-C). Horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako) (also 1:500, v/v) in TBS were then added for two hours. Lastly, the membrane was stained with a solution of 0.85 mM 3-amino-9-ethylcarbazole (Sigma Chemical Co) dissolved in a reaction mixture (dimethyl sulphoxide, BDH; 3 mM CH_3COOH, BDH; 7 mM CH_3COONa, Merck; pH 5.1) to which H_2O_2 was added just before staining. The reaction of colour development was allowed to proceed for 5 to 10 minutes at room temperature and stopped with tap water.

4.3.3.I.b Optimization of the IEF-immunoblotting method for the detection of β2GPI polymorphism

Several parameters of the IEF-immunoblotting assay were tested prior to analysing serum samples from healthy individuals and patients. These minor modifications in the protocol from Kamboh *et al.* ([1988]) included the ampholyte pH range, the concentration of urea in the polyacrylamide gel re-hydration solution, the time and the power intensity used for the electrophoretic step, the concentration and the duration of the blotting steps with both antibodies, and the duration of the incubation step with the substrate.

4.3.3.II PCR-RFLP for β2GPI polymorphism

Since the early 1980s, researchers have increasingly favoured DNA polymorphism to analyse genetic traits. This was a direct consequence to the progress in molecular biology, especially the discovery and implementation of the polymerase chain reaction (PCR) methodology and its derivatives, such as microsatellite marker analyses and restriction fragment length polymorphism (RFLP) analyses. In principle, DNA variations can be divided into three categories: those that have a functional - and potentially pathogenic - role (quantitatively altering gene expression or qualitatively affecting the encoded protein's function), those that have no effect on the gene's expression and function but can be used as genetic markers, as a consequence of gene linkage disequilibrium, and those that have neither a functional nor a marker role.

Single nucleotide polymorphisms (SNPs) are the most common type of human DNA genetic variations ([Thomson *et al*, 1999; Wang *et al*, 1998b]). They are stable, heritable, bi-allelic single base pair differences that typically occur on average at a frequency (density) of 1 to 10 per 1,000 nucleotides in the human genome ([Lodish *et al*, 2003]). Numerous techniques have been used to detect these mutations, the simplest of which being a polymerase chain reaction (PCR) followed by allele-specific restriction enzyme digestion (RFLP). This genotyping test is based on the ability of DNA segments to be cut by specific (restriction) endonuclease enzymes depending on the presence (or absence) of an alteration at the recognition site generated by a bi-allelic polymorphism.

4.3.3.II.a Genomic DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes using standard methods (either a salting out or a phenol/chloroform-based procedure) ([Dracopoli *et al*,

[2003]) (appendices I, J & K). Some of the DNA samples that were donated to us had been stored for a few months to several years (without significant DNA degradation).

4.3.3.II.b PCR-RFLP modus operandi for β2GPI polymorphism

The protocols for β2GPI (APOH) gene polymorphism analyses performed by PCR followed by RFLP (figure 4.4) (appendix L) using *Bst*B I, *Nsi* I, *Rsa* I, *Tsp*509 I (New England Biolabs Inc., Berverly, MA, USA) and *Cvi*J I * (ChimerX, Milwaukee, WI, USA) for restriction endonucleases were partially based on those from Steinkasserer *et al.* ([1993]), Sanghera *et al.* ([1997a]) and Gushiken *et al.* ([1999]).

The four mutations that were analysed in this part of the study were located in exons 3, 7, and 8 (table 4.1). The first mutation consists in a missense mutation (G→A) at codon 88 in exon 3 that alters the amino acid residue from Ser to Asn creating a restriction site for *Tsp*509 I (figure 4.6). The second mutation consists in a missense mutation (G→T) at codon 247 in exon 7 that alters the amino acid residue from Val to Leu annihilating a restriction site for *Rsa* I (figure 4.7). The third mutation consists in another missense mutation in exon 7 (T→G) at codon 306 that alters the amino acid residue from Cys to Gly annihilating a restriction site for *Nsi* I (figure 4.8), and creating a restriction site for *Cvi*J I * (figure 4.9). The fourth mutation consists in a missense mutation (G→C) at codon 316 in exon 8 that alters the amino acid residue from Trp to Ser creating a restriction site for *Bst* B I (figure 4.10).

Table 4.1: Nature and location of the base (in nucleotide sequence) and amino acid (in peptide sequence) substitutions corresponding to the four tested mutations at codons 88, 247, 306 and 316 with the restriction endonuclease used for their detection.

Codon position	Base substitution	Amino acid substitution	SCR (1)	Exon	Restriction endonuclease	Recognition site	IEF (2)
88	G > A	Ser > Asn	2	3	*Tsp*509 I	5'...|AATT ...3' 3'... TTAA|...5'	1
247	G > T	Val > Leu	5	7	*Rsa* I	5'...GT|AC...3' 3'...CA|TG...5'	Nil
306	T > G	Cys > Gly	5	7	*Cvi*J I *	5'...Pu-G|C-Py...3' 3'...Py-C|G-Pu...5'	Nil
306	T > G	Cys > Gly	5	7	*Nsi* I	5'...A TGCA|T...3' 3'...T|ACGT A...5'	Nil
316	G > C	Trp > Ser	5	8	*Bst* B I	5'...TT|CG AA...3' 3'...AA GC|TT...5'	3^{w}

(1): SCR: short consensus repeat.
(2): corresponding isoelectric-immunoblotting pattern as described by Kamboh MI *et al.* ([1988]).
*: *Cvi*J I is the first restriction endonuclease that was used in this study to detect the mutation at codon 306 (with the primer set-1 in table 4.2). This enzyme worked perfectly well but was rather expensive and its supply was very inconsistent. We therefore designed a second set of primers (set 2 in table 4.2) into which diagnostic restriction sites were introduced in order to allow the use of *Nsi* I.

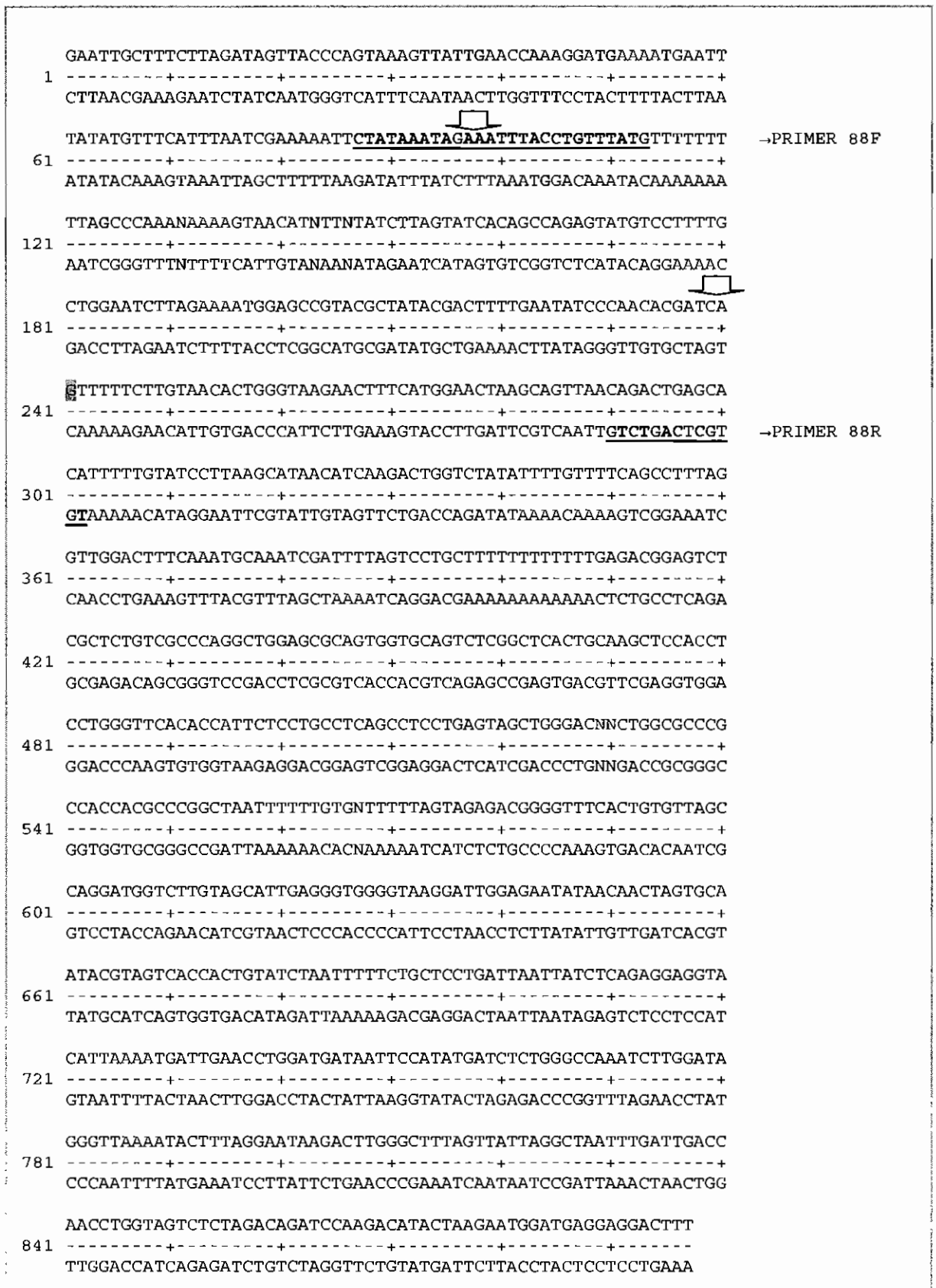


Figure 4.6: Nucleotide sequence of human β2GPI gene, exon 3.
The missense mutation at codon 88 in the 3rd exon of the β2GPI gene (G → A) (location highlighted in grey) created a restriction site for *Tsp*509 I. Primer sequences are underlined and restriction sites for *Tsp*509 I are marked with arrows.
GenBank accession N° Y11494 - HSAPOHEX3.

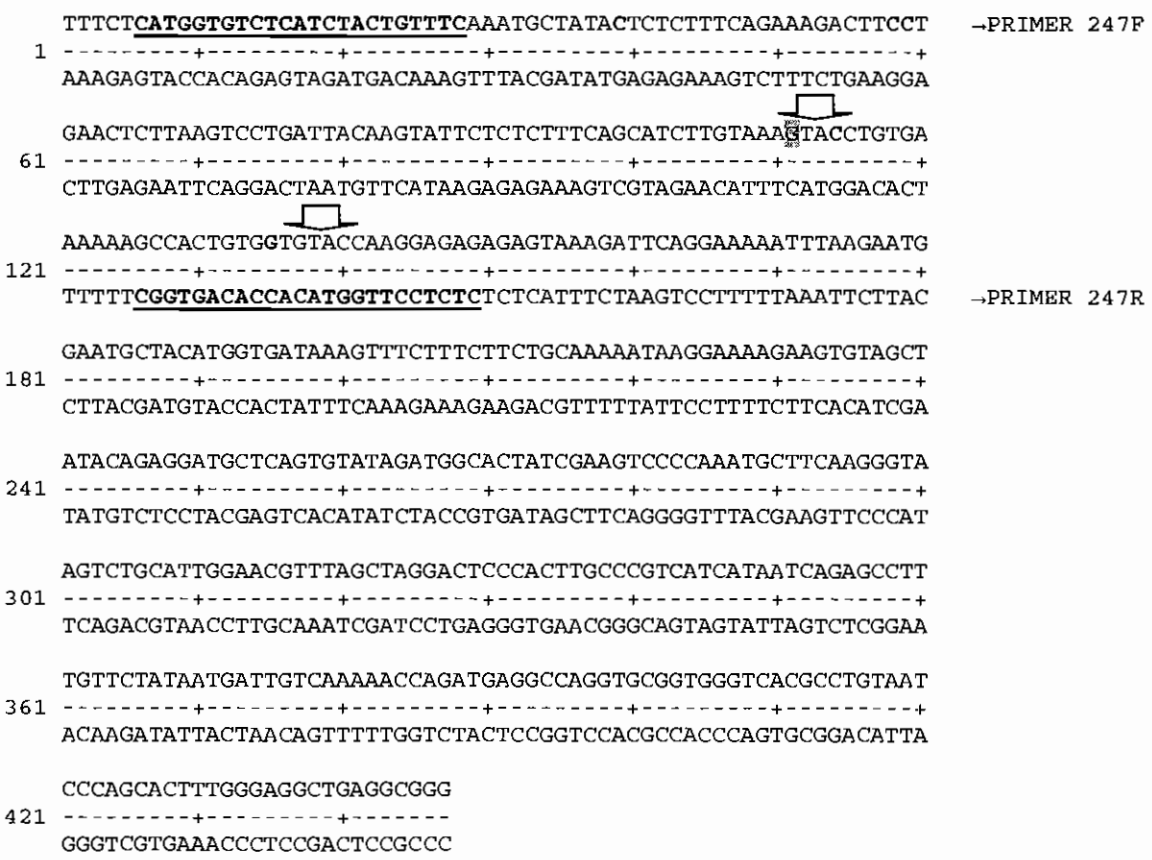


Figure 4.7: Nucleotide sequence of human β2GPI gene, exon 7.
The missense mutation at codon 247 in the 7th exon of the β2GPI gene (G → T) (location highlighted in grey) annihilated a restriction site for *Rsa* I. Primer sequences are underlined and restriction sites for *Rsa* I are marked with arrows.
GenBank accession N° Y11497 - HSAPOHEX7.

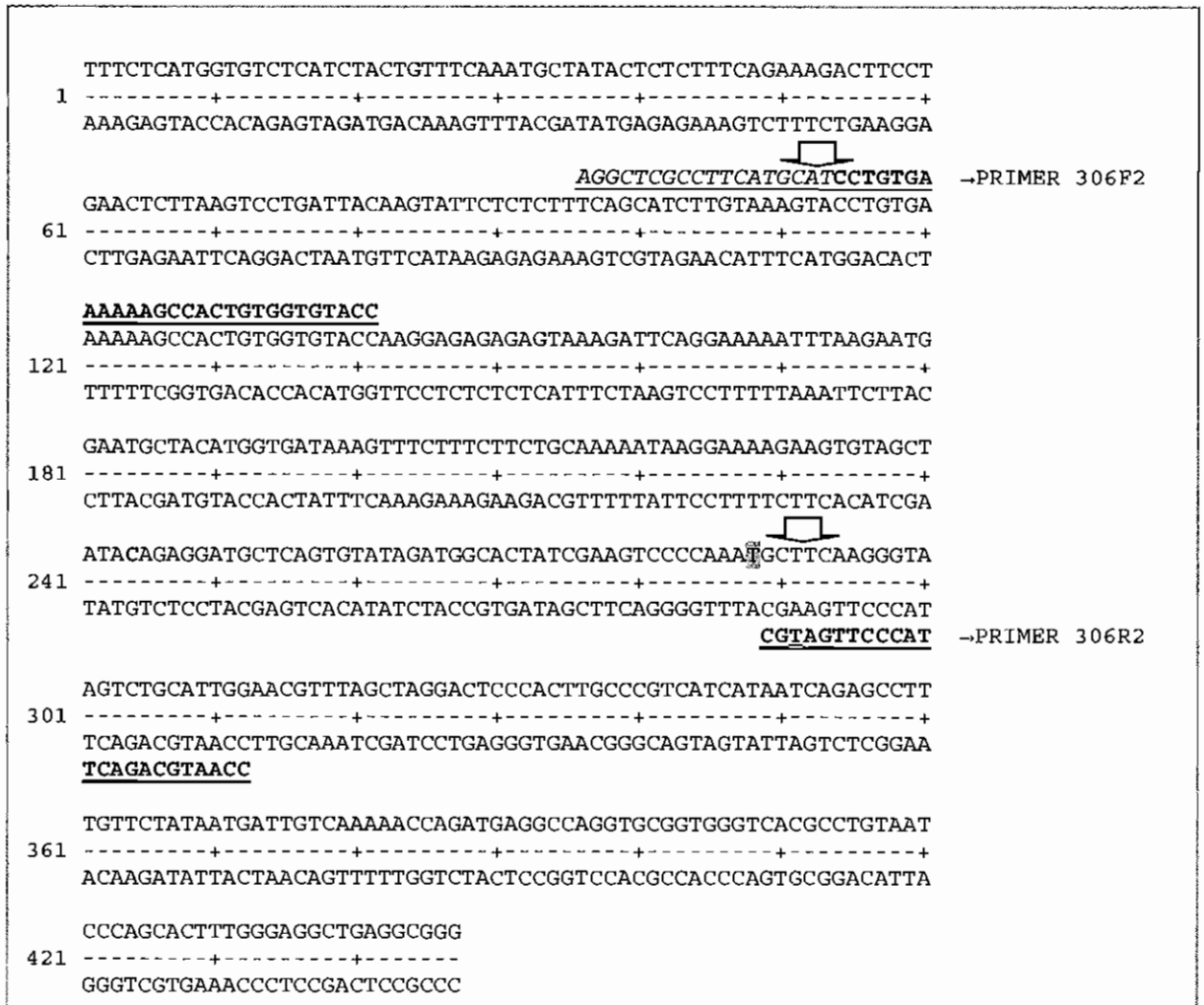


Figure 4.8: Nucleotide sequence of human β2GPI gene, exon 7.
The missense mutation at codon 306 in the 7th exon of the β2GPI gene (T → G) (location highlighted in grey) annihilated a restriction site for *Nsi* I. Primer sequences are underlined: primer 306F2 contained an "engineered" segment (in italics) and primer 306R2 contained a single base pair alteration (A → T). Restriction sites for *Nsi* I are marked with arrows.
GenBank accession N° Y11497 - HSAPOHEX7.

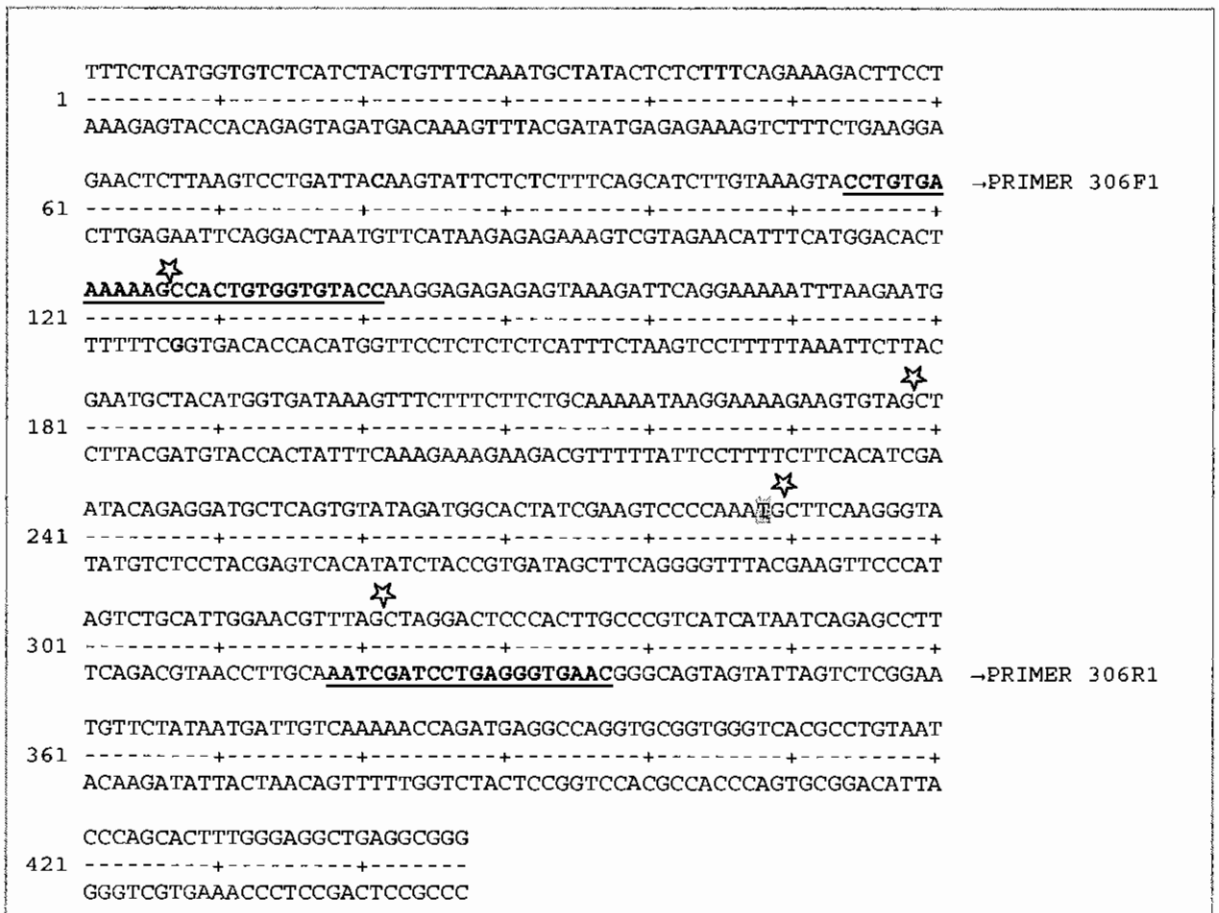


Figure 4.9: Nucleotide sequence of human β2GPI gene, exon 7.
The missense mutation at codon 306 in the 7th exon of the β2GPI gene (T → G) (location highlighted in grey) created a restriction site for *CviJ* I. Primer sequences are underlined and restriction sites for *CviJ* I are marked with stars.
GenBank accession N° Y11497 - HSAPOHEX7.

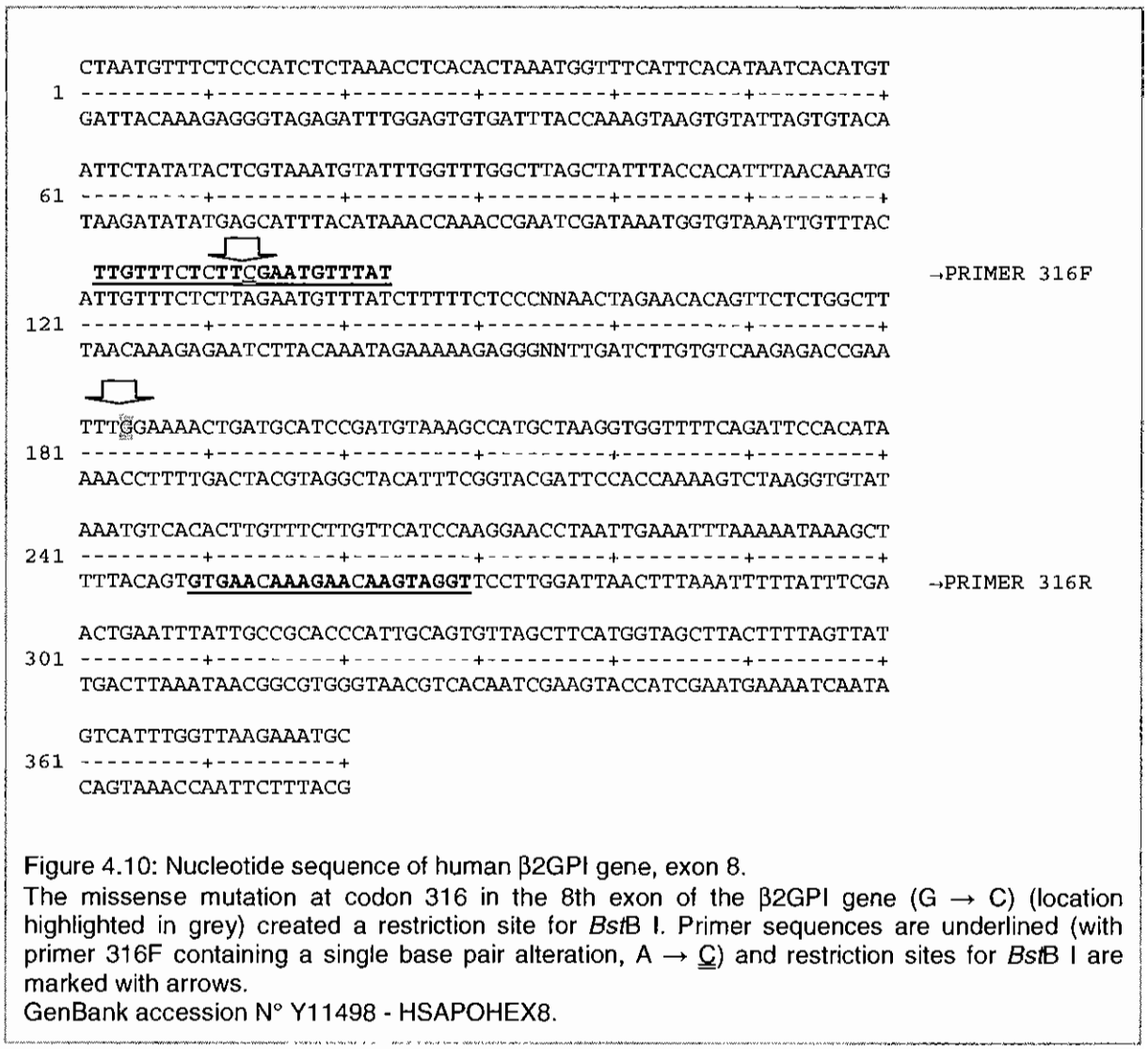


Figure 4.10: Nucleotide sequence of human β2GPI gene, exon 8.
The missense mutation at codon 316 in the 8th exon of the β2GPI gene (G → C) (location highlighted in grey) created a restriction site for *Bst*B I. Primer sequences are underlined (with primer 316F containing a single base pair alteration, A → C) and restriction sites for *Bst*B I are marked with arrows.
GenBank accession N° Y11498 - HSAPOHEX8.

The design of optimal primer pairs and selection of restriction endonucleases were performed with Primer Premier software version 5.0 (Premier Biosoft International, Palo Alto, CA, USA) following the generally accepted rules (size, distance of binding, G/C content, T_m values ([Breslauer *et al*, 1986]), complementary sequences, secondary structures, etc) as reviewed by Hildebrandt *et al.* ([1999]). A requirement in our design was that the amplified sequences should contain at least two restriction sites, one created/annihilated by the missense mutation and one elsewhere (figures 4.6 to 10). This allowed the visual confirmation by agarose gel electrophoresis that the restriction endonucleases presented proper endonuclease activity independently of the mutation. In particular, primer sets for the mutations at codons 306 and 316 required some engineering in order to contain a restriction site for, respectively, *Nsi* I and *Bst* BI for control purposes (figures 4.6 to 10).

Portions of exons 3, 7 and 8 were thus amplified by PCR using specific primers (Sigma-Genosys Ltd, Cambridgeshire, UK) (table 4.2). Approximately 0.5 µg of each DNA sample was PCR-amplified in a final volume of 25 µL with 100 ng of each sense (forward) and antisense (reverse) primer, one unit of recombinant *Taq* or Platinum® *Taq* DNA polymerase (Gibco Life Technologies, Paisley, Scotland, UK), 200 µM of dNTP, 20 mM Tris-HCl pH 8.4, 50 mM KCl, and $MgCl_2$ at a concentration varying from 1.5 to 3.2 mM. A premix of all the components of the reaction mixture other than DNA was made and added to tubes prior to adding 1 µL of test DNA. After an initial denaturation for 5 minutes at 95°C, the mixture was subjected to 35 to 45 30-second incubation cycles at 95°C (for denaturation), 55 to 59°C (for annealing), and 72°C (for extension), and a final round was extended for 5 minutes at 72°C. Negative controls included "no *Taq*", "no Primer", and "no target sequence" controls. The $MgCl_2$ concentration, number of cycles and annealing temperature required optimization and differed from one mutation to another (table 4.3). Optimization and sample testing were first performed in 0.5 mL Eppendorf tubes and then on 96-well thin-wall polycarbonate microtitre plates (ThermoHybaid, Ashford, Middlesex, UK; and USA Scientific Plastics, Milton Keynes, UK) covered by TD sealing tape and a silicone foam compression pad (ThermoHybaid). PCR-thermocycler blocks were from Hybaid (Omne-E model; Ashford, Middlesex, UK).

Table 4.2: Primer sets used to PCR amplify the DNA sequences containing the restriction site related to the four analysed β2GPI missense mutations.

Codon position	Orientation	Label	Sequence (5'→3')
88	Forward	88F	CTA TAA ATA gAA ATT TAC CTg TTT ATg
	Reverse	88R	TgT gCT CAg TCT gTT AAC Tg
247	Forward	247F	CAT ggT gTC TCA TCT ACT gTT TC
	Reverse	247R	CTC TCC TTg gTA CAC CAC AgT ggC
306	Forward set-1	306F1	CCT gTg AAA AAA gCC ACT gTg gTg TAC C
	Reverse set-1	306R1	CAA gTg ggA gTC CTA gCT AA
	Forward set-2	306F2	Agg CTC gCC TTC ATg CAT CCT gTg AAA AAA gCC ACT gTg gTg TAC C
	Reverse set-2	306R2	CCA ATg CAg ACT TAC CCT TgA TgC
316	Forward	316F	TTg TTT CTC TTC gAA TgT TTA T
	Reverse	316R	Tgg ATg AAC AAg AAA CAA gTg

Table 4.3: Laboratory conditions for PCR cycles.

Codon position	$MgCl_2$ concentration (mM)	DNA polymerase	PCR temperature (°C)*	Number of cycles
88	3.2	Taq, recombinant	95/58/72	45
247	1.5	Platinum® Taq	95/60/72	35
306	1.5	Platinum® Taq	95/60/72	35
316	1.5	Platinum® Taq	95/52,50/72	40

* Temperatures correspond to cycling denaturation, annealing, and extension temperatures.

The amplified sequences for each mutation at codons 88, 247, 306 and 316 were 216, 144, 216/224 (with *Nsi* I / *CviJ* I) and 148 bp long respectively. Digestion of these PCR-amplified products was performed with the corresponding restriction endonuclease enzyme (1 U per µL of PCR products) for 2 hours using the buffers supplied and at the temperature prescribed by the manufacturer (table 4.4). Following restriction, the fragments were separated by electrophoresis on 2 % agarose gels (Pronadisa or Gibco) containing ethidium bromide. The fragments' sizes were related to a 100 bp DNA (molecular marker) ladder (Gibco) and to undigested fragments.

Table 4.4: Restriction endonuclease digestion laboratory conditions and size of undigested and digested fragments.

Codon position	Optimal restriction endonuclease temperature (°C)	Size of undigested fragments (bp)	Size of digested fragments when the mutation is absent (bp)	Size of digested fragments when the mutation is present (bp)
88	65	216	203+13	143+60+13
247	37	144	107+26+11	133+11
306	37 (*CviJ* I)	224	112+83+16+13	112+51+32+16+13
306	37 (*Nsi* I)	216	178+21+17	199+17
316	65	148	136+12	85+51+12

4.3.3.II.c Optimization of the PCR-RFLP method for β2GPI polymorphism

As a start, we used the primer sequences and PCR protocols referred by Sanghera *et al.* ([1997a]) for the mutations at positions 88 and 316, by Steinkasserer *et al.* ([1993]) for the mutation at position 247, and by Gushiken *et al.* ([1999]) for the mutation at position 306. Some of these primer sets gave very poor quality amplification products. To remedy this, we designed several other sets of primers, including one for the mutation at codon 306 that allowed the use of the (much cheaper) restriction endonuclease *Nsi* I. As mentioned earlier, the foremost conditional feature in the design of our primer sets was that the amplified sequences had to contain at least two restriction sites, one dependent on and one independent of the presence of the tested mutation. Amplification and restriction efficiency was confirmed by gel electrophoresis.

In order to find the optimal PCR conditions for each amplicon, a selection of DNA samples from healthy individuals were tested with the four above-mentioned primer sets at various magnesium concentrations (range: 0.25 mM to 4.5 mM) and annealing temperatures (range: 45°C to 65°C) ([Saiki *et al*, 1988; Eckert *et al*, 1990]). Platinum® *Taq* DNA polymerase ([Westfall *et al*, 1998]) was also tested in place of recombinant *Taq* DNA polymerase, and the number of amplification cycles was also sometimes modified (up to 45 cycles). These variations significantly improved amplification specificity and were subsequently used for patient samples (optimal conditions for each mutation are summarised in table 4.3). The use of additional compounds such as DMSO (up to 5 %) that can affect DNA melting temperature ([Pomp *et al*, 1991, Varadaraj *et al*, 1994, Baskaran *et al*, 1996]), as well as modifications of cycling (in particular extension) time, and of concentrations of dNTPs and primers were also tested, but did not improve specificity, while increasing experiment duration and contamination risk.

4.3.3.III ELISA for β2GPI, anticardiolipin and anti-β2GPI antibodies

Serum β2GPI concentration was determined by a capture enzyme-linked immunosorbent assay (ELISA) ([Lin *et al*, 2003]) adapted from the protocol of McNally *et al.* ([1993]) as described in chapter 3. IgG and IgM anticardiolipin antibodies and anti-β2GPI antibodies were measured using commercialised ELISA kits (respectively Orgentec Diagnostika GmbH, Mainz, Germany and Pharmacia & Upjohn GmbH, Freiburg, Germany) following the manufacturers' protocols.

4.4 Results

4.4.1 IEF-immunoblotting assay

4.4.1.I IEF-immunoblotting assay optimization

Some modifications of the tested parameters significantly affected the efficiency of the method, especially the urea concentration in the gel re-hydration solution. A concentration of around 3M was an absolute requirement in order to obtain proper IEF bands (otherwise bands disappeared or shifted). No other ampholyte pH range worked as well as the one described by Kamboh *et al.* ([1988]). Prolongation and/or shortening of the different stages of the electrophoretic step, as well as modification of the power settings, negatively affected the resolution of the assay, while some adjustments in the immunoblotting step, particularly higher antibody concentration, slightly improved the sensitivity of the assay but were counterbalanced by the appearance of undesirable background. Somewhat higher specificity could be obtained with a lower antibody concentration but this was counterbalanced by a longer operational time.

4.4.1.II IEF-immunoblotting patterns

β2GPI IEF-immunoblotting patterns have been categorised according to their isoelectric-point differences (i.e. the differences in migration distance from the cathode). Three major patterns, designated APOH 1-1, 2-2 and 3-3, composed of five major flanking isoforms, have been described (figure 4.11). Their differentiation is not only made according to their respective banding pattern relative to each other but also by the additional presence of minor (i.e. less intense) flanking isoforms. For instance, the most acidic major pattern has two of these minor flanking isoforms, while the intermediate major pattern presents one minor band (cathodally localized) and the third major pattern none. Combination of the major patterns results in band doublets composed of the exact combination of the above major bands associated with the corresponding minor bands.

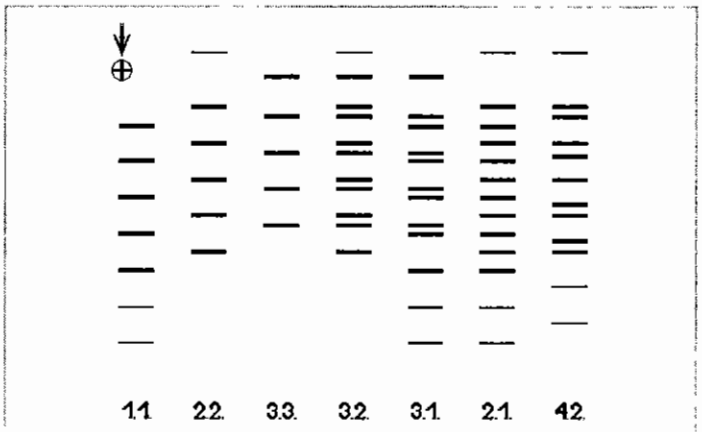


Figure 4.11: Schematic representation of β2GPI phenotypes detected on IEF 5 % polyacrylamide gel containing 3M urea in pH 4-7 (pH 5-8 and 4-6.5). If the rare "African" APOH*4 allele is excluded, there are six phenotypes remaining, called APOH 1-1, 2-2, 3-3, 2-1, 3-1, and 3-2. The anode is at the bottom of the figure and the cathode at the top. From Kamboh *et al.* ([1988]).

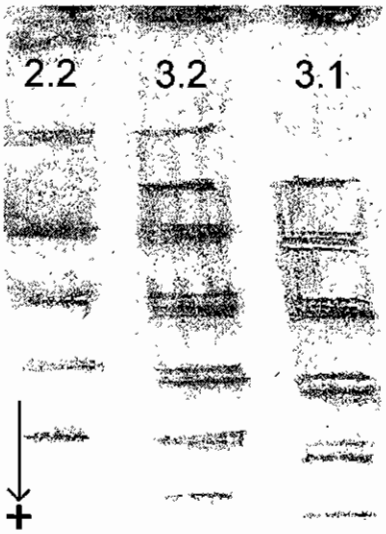


Figure 4.12: Example of an IEF-immunoblot with monospecific β2GPI antiserum. The anode is at the bottom of the figure and the cathode at the top. Three clearly distinguishable β2GPI IEF-patterns are visible on this nitrocellulose membrane.

52 healthy individuals and 81 stroke patients were tested for β2GPI polymorphism using IEF-immunoblotting. A typical example of a gel picture resulting from IEF-immunoblotting is shown in figure 4.12. To facilitate the identification of the β2GPI IEF-patterns, controls with known β2GPI isoforms kindly donated by Mr. Kamboh MI were used and the distinction of the IEF-patterns was made by comparison of the IEF-immunoblots with the schematic representation of IEF-patterns from Kamboh *et al.* ([1988]) (figure 4.11).

4.4.1.III IEF-immunoblotting genotype and allele frequencies

The β2GPI IEF-patterns (genotypes), estimated allele frequencies (estimated by allele counting) and heterozygosities of the two tested populations (namely healthy individuals and stroke patients) are presented in tables 4.5 and 4.6.

Table 4.5: β2GPI genotypes detected by IEF-immunoblotting in healthy individuals and patients with stroke (n: number of samples; expected Hardy-Weinberg equilibrium percentages).

β2GPI genotypes	Healthy individuals		Stroke patients	
	n	% (eHW)	n	% (eHW)
APOH 1:1	0	0.0 % (0.0 %)	0	0.0 % (0.0 %)
APOH 2:1	0	0.0 % (1.7 %)	0	0.0 % (2.3 %)
APOH 3:1	1	1.9 % (0.2 %)	2	2.5 % (0.1 %)
APOH 2:2	42	80.7 % (80.0 %)	72	88.9 % (86.9 %)
APOH 3:2	9	17.3 % (17.2 %)	7	8.6 % (10.4 %)
APOH 3:3	0	0.0 % (0.9 %)	0	0.0 % (0.3 %)

Table 4.6: β2GPI allele frequency and heterozygosity in healthy individuals (n=52) and patients with stroke (n=81) estimated through IEF-immunoblotting (n: number of samples).

Allele frequency	Healthy individuals	Stroke patients
APOH*1	0.0096	0.0123
APOH*2	0.8942	0.9321
APOH*3	0.0961	0.0556
Heterozygosity	0.191	0.128

In some cases, the electrophoretic position of some isoform patterns was unfortunately not clear enough to allow absolutely correct assignment of a phenotype in full agreement with the schematic representation of Kamboh *et al.*

([1988]), and therefore these tables contain summarised data that should be considered as provisional. Only three IEF-patterns were observed in both healthy subjects and stroke patients (figure 4.12), with the APOH 2:2 and 3:2 IEF-patterns being by far the most common genotypes (with a frequency above 80 % for the former) in both populations, which is similar to previous reports. The remaining phenotype (3:1) was much less frequent, with a frequency below 3.0 %. No APOH*4 pattern was found in any of the samples. Both the healthy and the stroke groups were in agreement with Hardy-Weinberg equilibrium (P=0.8313 and 0.5551 respectively). Although the APOH*3 pattern can be subdivided based on differential monoclonal antibody reactivity ([Kamboh *et al*, 1988]), such segregation was not performed in this study.

The relative frequency for each allele in our group of healthy individuals obtained by IEF-immunoblotting was found to be very similar to the values found in other Caucasian populations (table 4.7). The most frequent allele was by far APOH*2 with a frequency around 90 %. Although two groups found significantly different numbers from our results (with P values of 0.0415 and 0.0276), most did not (with P values ranging from 0.0540 to 0.3829). When considered as genotypes, our data in the healthy group was remarkably similar to the previously reported values, with the exception of two (out of seven) groups (P=0.0490 and 0.0016) (table 4.7). When compared to the healthy cohort, the stroke patients presented neither a significantly different β2GPI genotype distribution (P=0.3227), nor a significantly different β2GPI allele distribution (P=0.4488).

Table 4.7: β2GPI allele and genotype distributions among various populations of healthy Caucasian individuals estimated through IEF-immunoblotting (with P value from χ^2 test for comparison with distribution from this study).

Reference		Kamboh	Richter	Richter	Kaprio	Cassader	Ruiu	Mehdi	average	sum	This
Year		1988	1988	1988	1991	1994	1997	1999			study
n		196	187	238	453	217	278	449	288	2018	52
Origin		USA	Austria	Germany	USA	Italy	Italy	USA			Ireland
Genotype	1:1	2	3	1	0	1	0	0	1	7	0
	2:1	19	17	23	34	0	1	48	20	142	0
	3:1	0	2	3	6	0	0	0	2	11	1
	2:2	153	144	190	365	190	243	351	234	1636	42
	3:2	21	21	21	45	25	32	44	30	209	9
	3:3	1	0	0	3	1	2	6	2	13	0
	P value	0.0490	0.1323	0.0794	0.1503	0.2001	0.1201	0.0016	0.2100	0.1476	
Allele	APOH*1	0.059	0.067	0.059	0.044	0.005	0.002	0.053	0.041	0.041	0.010
	APOH*2	0.883	0.872	0.891	0.893	0.933	0.933	0.884	0.898	0.898	0.894
	APOH*3	0.059	0.061	0.050	0.063	0.062	0.065	0.062	0.061	0.061	0.096
	APOH*4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	P value	0.0540	0.0415	0.0276	0.1168	0.3829	0.2068	0.0710	0.1300	0.1026	

4.4.2 Extracted DNA concentration, purity and integrity

The used protocols provided high yield and purity of un-degraded DNA preparations. The DNA extraction yield was estimated by absorption spectroscopy, considering that 50 μg/mL of dsDNA give an OD value of 1.0 at a wavelength of 260 nm. Diluted samples (at 1 in 100) that presented an OD value below 0.1 (i.e. a dilution-corrected concentration of 0.5 μg/μL) were discarded, and new samples of the corresponding blood were obtained for DNA re-extraction.

Extracted DNA was considered as relatively "pure" only when the ratio of the absorbance at wavelengths of 260 nm and 280 nm (A_{260nm}/A_{280nm}) was above 1.7. DNA that did not satisfy this criterion was rejected and new samples of the corresponding blood were obtained for re-extraction. The mean ratio for all (accepted) extracted DNA was 1.871 (range: 1.705 - 2.151).

Small aliquots (~1 μg) of each DNA extract were run by electrophoresis on 1.0 % agarose gels and stained with ethidium bromide to estimate integrity (and concentration). Extracted genomic DNA that did not present a single sharp band (i.e. with no smearing) were discarded.

Only two of the DNA samples were found to be inadequate for PCR works as revealed by the absence of any transcript amplification.

4.4.3 PCR-RFLP assay[3]

4.4.3.I PCR-RFLP assay optimization

The PCR-RFLP assay required some optimization at each step of the reaction, namely the primer design, the PCR reaction, and the endonuclease digestion. Each primer set required a specific magnesium concentration and annealing temperature. For that purpose, different concentrations of magnesium were tested at different temperatures for each amplicon. Figure 4.13 represents an example of the resulting optimization checkerboard.

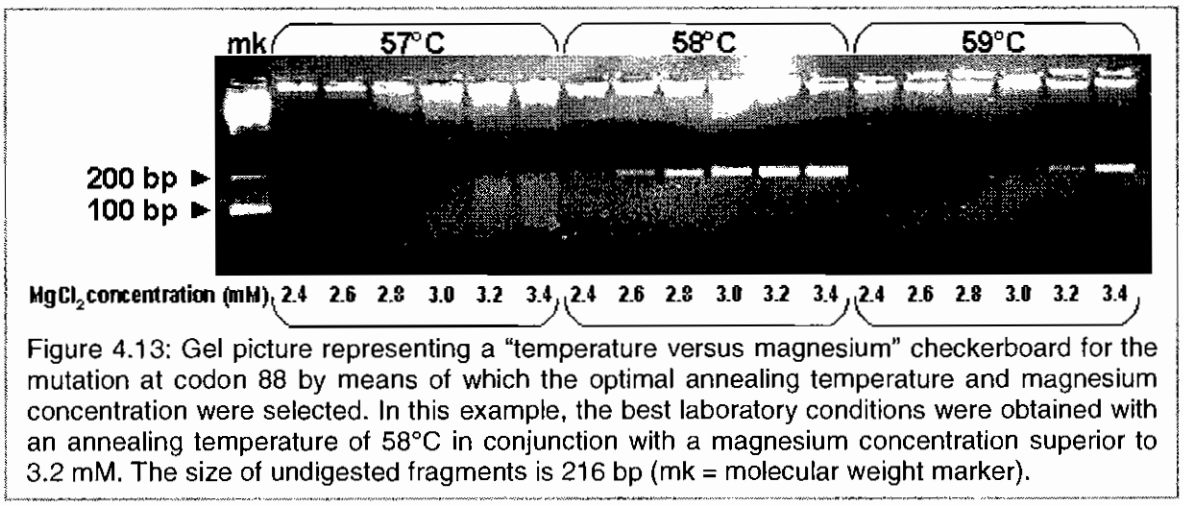


Figure 4.13: Gel picture representing a "temperature versus magnesium" checkerboard for the mutation at codon 88 by means of which the optimal annealing temperature and magnesium concentration were selected. In this example, the best laboratory conditions were obtained with an annealing temperature of 58°C in conjunction with a magnesium concentration superior to 3.2 mM. The size of undigested fragments is 216 bp (mk = molecular weight marker).

4.4.3.II PCR-RFLP agarose gels

All healthy individuals and patients with thrombotic disorders were tested for the four mutations at codons 88, 247, 306 and 316 using RT-PCR. Figures 4.14 to 4.17 show examples of agarose gel pictures that were obtained for the four mutations following restricted digestion of the respective PCR-amplified sequences (the fragments' sizes are summarized in table 4.4 in Materials & Methods).

[3] Alleles were labelled with the codon position at which the point mutation occurs and with the corresponding amino acid (i.e. Ser^{88} and Asn^{88}, Val^{247} and Leu^{247}, Cys^{306} and Gly^{306}, and Trp^{316} and Ser^{316} for codons 88, 247, 306, and 316 respectively). Genotypes were labelled using the same nomenclature (i.e. $Ser^{88}Ser$, $Ser^{88}Asn$ and $Asn^{88}Asn$; $Val^{247}Val$, $Val^{247}Leu$ and $Leu^{247}Leu$; $Cys^{306}Cys$, $Cys^{306}Gly$ and $Gly^{306}Gly$, and $Trp^{316}Trp$, $Trp^{316}Ser$ and $Ser^{316}Ser$ for codons 88, 247, 306, and 316 respectively).

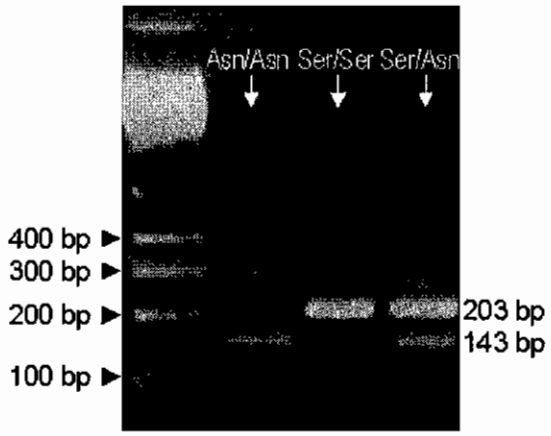


Figure 4.14: Example of a gel picture for the mutation at position 88. The amplified sequence of a length of 216 bp was cut by *Tsp*509 I into two fragments of 13 and 203 bp. The latter fragment was further cut into two fragments of 60 and 143 bp when the Asn[88] mutation was present.

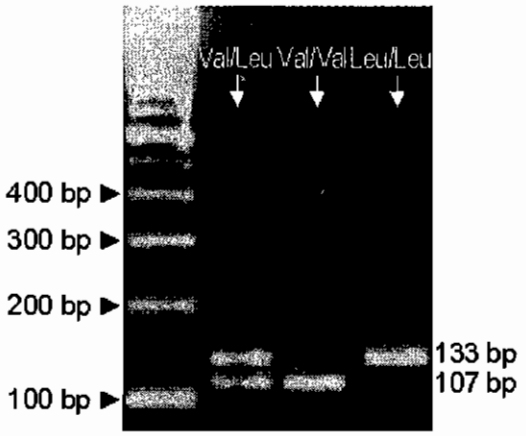


Figure 4.15: Example of a gel picture for the mutation at position 247. The amplified sequence of a length of 144 bp was cut by *Rsa* I into two fragments of 11 and 133 bp. The latter fragment was further cut into two fragments of 26 and 107 bp when the Leu[247] mutation was absent.

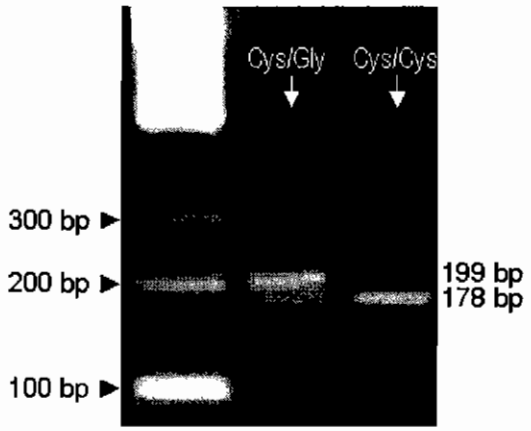


Figure 4.16: Example of a gel picture for the mutation at position 306. The amplified sequence of a length of 216 bp was cut by *Nsi* I into two fragments of 17 and 199 bp. The latter fragment was further cut into two fragments of 21 and 178 bp when the Gly[306] mutation was absent.

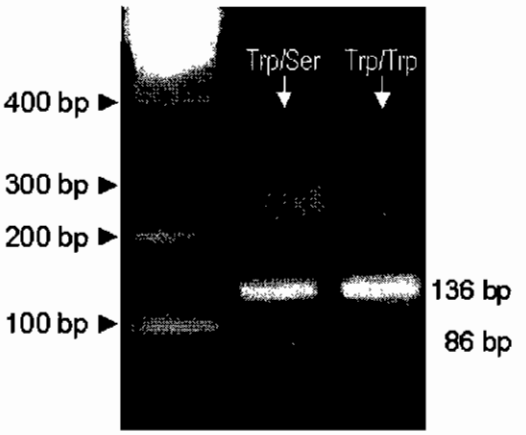


Figure 4.17: Example of a gel picture for the mutation at position 316. The amplified sequence of a length of 148 bp was cut by *Bst* B I into two fragments of 12 and 136 bp. The latter fragment was further cut into two fragments of 51 and 85 bp when the Ser[316] mutation was present.

4.4.3.III PCR-RFLP genotype and allele frequencies

Observed genotype frequencies for each group of individuals showed no significant deviation from those expected under Hardy-Weinberg equilibrium (HWE) tested at each locus (table 4.8 and appendix O).

Table 4.8: β2GPI genotype frequencies in healthy individuals, and in patients with stroke, early onset acute coronary syndrome (ACS), recurrent foetal loss (RFL) or another thrombotic event (from a mixed bag of thrombotic disorders) (Other T).

Codon		88			247			306			316		
		SerSer	SerAsn	AsnAsn	ValVal	ValLeu	LeuLeu	CysCys	CysGly	GlyGly	TrpTrp	TrpSer	SerSer
Healthy	#	286	33	2	156	134	29	304	16	0	288	26	0
	%	89.1	10.3	0.6	48.9	42.0	9.1	95.0	5.0	0.0	91.7	8.3	0.0
Stroke	#	105	7	0	48	60	4	103	10	0	104	9	0
	%	93.8	6.3	0.0	42.9	53.6	3.6	91.2	8.8	0.0	92.0	8.0	0.0
ACS	#	308	30	0	200	111	38	327	32	0	338	19	0
	%	91.1	8.9	0.0	57.3	31.8	10.9	91.1	8.9	0.0	94.7	5.3	0.0
RFL	#	46	1	0	27	16	4	42	5	0	44	3	0
	%	97.9	2.1	0.0	57.4	34.0	8.5	89.4	10.6	0.0	93.6	6.4	0.0
Other T	#	38	1	0	19	19	1	37	2	0	37	2	0
	%	97.4	2.6	0.0	48.7	48.7	2.6	94.9	5.1	0.0	94.9	5.1	0.0

#: number of samples. As not all DNA samples were amplified for a given genetic marker, the total numbers of healthy individuals and patients vary slightly between the mutations examined.

Carrier frequencies of the mutation in the five tested populations ranged between 2.1 and 10.9 % for Asn^{88}, between 42.6 and 57.1 % for Leu^{247}, between 5.0 and 10.6 % for Gly^{306}, and between 5.1 and 8.3 % for Ser^{316} (table 4.9).

Table 4.9: β2GPI allele and carrier frequencies in healthy individuals, and in patients with stroke, early onset acute coronary syndrome (ACS), recurrent foetal loss (RFL) or another thrombotic event (from a mixed bag of thrombotic disorders) (Other T).

Codon		88		247		306		316	
		Ser	Asn	Val	Leu	Cys	Gly	Trp	Ser
Healthy	AF	0.942	0.058	0.699	0.301	0.975	0.025	0.959	0.041
	CF	99.4%	10.9%	90.9%	51.1%	100.0%	5.0%	100.0%	8.3%
Stroke	AF	0.969	0.031	0.696	0.304	0.956	0.044	0.960	0.040
	CF	100.0%	6.3%	96.4%	57.1%	100.0%	8.8%	100.0%	8.0%
ACS	AF	0.956	0.044	0.732	0.268	0.955	0.045	0.973	0.027
	CF	100.0%	8.9%	89.1%	42.7%	100.0%	8.9%	100.0%	5.3%
RFL	AF	0.989	0.011	0.745	0.255	0.947	0.053	0.968	0.032
	CF	100.0%	2.1%	91.5%	42.6%	100.0%	10.6%	100.0%	6.4%
Other T	AF	0.987	0.013	0.731	0.269	0.974	0.026	0.974	0.026
	CF	100.0%	2.6%	97.4%	51.3%	100.0%	5.1%	100.0%	5.1%

AF: allele frequency; CF: carrier frequency.

4.4.3.IV Comparisons with published genotype and allele frequencies in other healthy Caucasian individuals

The following tables (4.10 & 4.11) present the genotype and allele frequencies for the four β2GPI mutations in Western European and North American Caucasians reported in the literature. Compared to these values, the healthy Irish cohort did not present significantly different allele or genotype frequencies for any of the four tested point mutations (as evaluated by χ^2-test) except for values found one American (Eichner *et al*, 1989b) and two Italian studies (Cassader *et al*, 1994; Ruiu *et al*, 1997).

Tables 4.10 & 4.11: Genotype and allele frequencies for the four β2GPI mutations in healthy Western European and North American Caucasians as reported in the literature.

	Year	1988	1988	1993	1994	1997	1998	1999
	Author	Richter	Richter	Steinkasserer	Cassader	Ruiu	Horbach	Atsumi
	#	187	239	34	217	278	65	39
	Origin	Austria	Germany	UK	Italy	Italy	Netherlands	UK
88	Ser/Ser	165 (88.2%)	212 (88.7%)		216 (99.5%)	277 (99.6%)		
	Ser/Asn	19 (10.2%)	26 (10.9%)		0 (0.0%)	1 (0.4%)		n.a.
	Asn/Asn	3 (1.6%)	1 (0.4%)		1 (0.5%)	0 (0.0%)		
	P value	0.5578	0.9250	n.a.	0.0000	0.0000	n.a.	
88	SER	349 (93.3%)	450 (94.1%)		432 (99.5%)	555 (99.8%)		75 (96.2%)
	ASN	25 (6.7%)	28 (5.9%)		2 (0.5%)	1 (0.2%)		3 (3.8%)
	P value	0.5541	0.9467		0.0000	0.0000		0.4852
247	Val/Val			20 (58.8%)				
	Val/Leu			12 (35.3%)				n.a.
	Leu/Leu			2 (5.9%)				
	P value	n.a.	n.a.	0.5217	n.a.	n.a.	n.a.	
247	VAL			52 (7.6%)				55 (70.5%)
	LEU			16 (23.5%)				23 (29.5%)
	P value			0.2590				0.9121
316	Trp/Trp						62 (95.4%)	
	Trp/Ser						3 (4.6%)	n.a.
	Ser/Ser						0 (0.0%)	
	P value	n.a.	n.a.	n.a.	n.a.	n.a.	0.3117	
316	TRP						127 (97.7%)	73 (93.6%)
	SER						3 (2.3%)	5 (6.4%)
	P value						0.3215	0.3561

	Year	1988	1989b	1991	1997a	1999	1999a	1999
	Author	Kamboh	Eichner	Kaprio	Sanghera	Hirose	Kamboh	Mehdi
	#	196	443	453	661	118	175	449
	Origin	USA	USA	USA	USA	USA	USA	USA
88	Ser/Ser	175 (89.3%)		413 (91.2%)	585 (88.5%)			401 (89.3%)
	Ser/Asn	19 (9.7%)	n.a.	40 (8.8%)	74 (11.2%)			48 (10.7%)
	Asn/Asn	2 (1.0%)		0 (0.0%)	2 (0.3%)			0 (0.0%)
	P value	0.8648		0.1891	0.6979	n.a.	n.a.	0.2431
88	SER	369 (94.1%)	862 (97.3%)	866 (95.6%)	1244 (94.1%)			850 (94.7%)
	ASN	23 (5.9%)	24 (2.7%)	40 (4.4%)	78 (5.9%)			48 (5.3%)
	P value	0.9446	0.0025	0.2293	0.9035			0.7232
247	Val/Val					51 (43.2%)	88 (53.0%)	
	Val/Leu					58 (49.2%)	66 (39.8%)	
	Leu/Leu					9 (7.6%)	12 (7.2%)	
	P value	n.a.	n.a.	n.a.	n.a.	0.4064	0.6225	n.a.
247	VAL					160 (67.8%)	242 (72.9%)	
	LEU					76 (32.2%)	90 (27.1%)	
	P value					0.5482	0.3313	
306	Cys/Cys						162 (82.6%)	
	Gly/Gly						13 (7.4%)	
	Gly/Gly						0 (0.0%)	
	P value	n.a.	n.a.	n.a.	n.a.	n.a.	0.2714	n.a.
306	CYS						337 (96.3%)	
	GLY						13 (3.7%)	
	P value						0.2787	
316	Trp/Trp				592 (89.6%)		158 (91.9%)	410 (91.3%)
	Trp/Ser				60 (9.1%)		14 (8.1%)	38 (8.5%)
	Ser/Ser				9 (1.4%)		0 (0.0%)	1 (0.2%)
	P value	n.a.	n.a.	n.a.	0.1034	n.a.	0.9570	0.7012
316	TRP				1244 (94.1%)		330 (95.9%)	858 (95.5%)
	SER				78 (5.9%)		14 (4.1%)	40 (4.5%)
	P value				0.1060		0.9579	0.7665

#: number of samples; P values from χ^2-test comparing reported values with those from this study. n.a.: not available.

4.4.3.V PCR-RFLP haplotype analysis

In order to determine the most appropriate analytic approach, we estimated haplotype frequencies. Each minor variant occurred independently against a background of the commonest haplotype. Given the absence of haplotypes comprising combinations of more than one minor variant, the most appropriate analysis was to test the effect of each variant allele independently (i.e. genotypic analysis of each variant position).

4.4.3.VI PCR-RFLP in thrombotic diseases

Contingency χ^2-tests were used to assess the association between genotype and the risk of diseases. Odds ratios (OR) were calculated at all loci for the heterozygous state relative to the homozygous non-carrier state and for allele carriage. Given the scarcity of the homozygous genotype state with mutation, it was not possible to calculate OR for that genotype except at the 247 locus (detailed results in appendix O).

The relative risk for ACS was found to be a little less than twice as low among heterozygotes at position 247 than among homozygous non-carrier subjects (P=0.0088; OR=0.646; 95%CI: 0.466-0.896), but such significance was not observed in homozygous carriers of the mutation (P=0.9353; OR=1.022; 95%CI: 0.604-1.731). Inversely, at position 306, the relative risk for ACS was found to be a little less than twice as high among heterozygotes as among homozygous non-carrier subjects (P=0.0470; OR=1.859; 95%CI: 1.000-3.457). No genotypic association with the thrombotic disease states was observed at the other two positions. When considered across allele frequencies, results showed no significant association between any of the tested alleles and any particular disease (see appendix O).

4.4.3.VII PCR-RFLP versus β2GPI concentration

In 166 healthy individuals and 113 stroke patients, mutations in the β2GPI (APOH) gene and β2GPI serum concentration were determined. The mutations at codons 306 (Gly^{306}) and 316 (Ser^{316}) had a significant lowering effect on serum β2GPI concentration ($P<0.001$ for both in healthy subjects, and $P<0.0001$ and 0.0061 respectively in stroke patients). In contrast, the mutations at codons 88 (Asn^{88}) and 247 (Leu^{247}) had no significant impact on serum β2GPI concentration ($P=0.9391$ and 0.1499 respectively in healthy subjects, and $P=0.5717$ and 0.6798 respectively in stroke patients) (table 4.12 and figures 4.18 and 4.19). When looked at from an allele perspective in healthy subjects, Asn^{88} had no significant impact on serum β2GPI concentration (P=0.9837), Leu^{247} showed a very modest increasing effect (of about 7%) on serum β2GPI concentration (P=0.0358), and Gly^{306} and Ser^{316} were associated with a much stronger decrease in serum β2GPI concentration (of about 54% and 36% respectively) (P<0.0001 for both). Similar differences were found in patients with stroke (table 4.13), although with a smaller amplitude.

Table 4.12: Mean serum β2GPI concentration and standard deviation (SD) (µg/mL) in healthy individuals and stroke patients according to β2GPI genotype.

Codon		88			247			306			316		
		SerSer	SerAsn	AsnAsn	ValVal	ValLeu	LeuLeu	CysCys	CysGly	GlyGly	TrpTrp	TrpSer	SerSer
Healthy	#	145	19	2	86	58	20	157	8	0	147	14	0
	average	177.3	174.8	187.1	170.8	181.2	192.7	182.1	81.9		183.2	112.0	
	SD	49.8	47.4	6.9	49.8	48.2	48.5	44.9	31.8	n.a.	46.1	32.1	n.a.
	P value	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		<0.0001			<0.0001	
Stroke	#	100	5	0	43	58	4	96	10	0	97	9	0
	average	170.2	158.1		164.9	171.7	156.6	175.0	109.5		172.6	128.4	
	SD	47.1	24.1	n.a.	49.5	46.3	7.0	41.6	54.3	n.a.	46.4	30.4	n.a.
	P value		n.s.		n.s.	n.s.	n.s.		<0.0001			0.0061	

#: number of samples; P values from student t-tests for comparisons versus concentration in homozygous non-carriers; n.s.: not significant; n.a.: not applicable. As not all DNA samples were amplified for a given genetic marker, the total number of controls and cases varies slightly between the mutations examined.

Table 4.13: Mean serum β2GPI concentration and standard deviation (SD) (µg/mL) in healthy individuals and stroke patients according to β2GPI allele carriage.

Codon		88		247		306		316	
		Ser	Asn	Val	Leu	Cys	Gly	Trp	Ser
Healthy	#	309	23	230	98	322	8	308	14
	average	177.2	177.0	173.4	185.9	179.6	81.9	180.0	112.0
	SD	49.5	43.2	49.4	48.1	47.1	31.8	47.8	32.1
	P value		n.s.		0.0358		<0.0001		<0.0001
Stroke	#	205	5	144	66	202	10	203	9
	average	169.9	158.1	167.6	169.8	171.7	109.5	170.6	128.4
	SD	46.6	24.1	48.0	43.7	44.4	54.3	46.5	30.4
	P value		n.s.		n.s.		<0.0001		0.0076

#: number of alleles; P values from student t-test; n.s.: not significant. As not all DNA samples were amplified for a given genetic marker, the total number of controls and cases varies slightly between the mutations

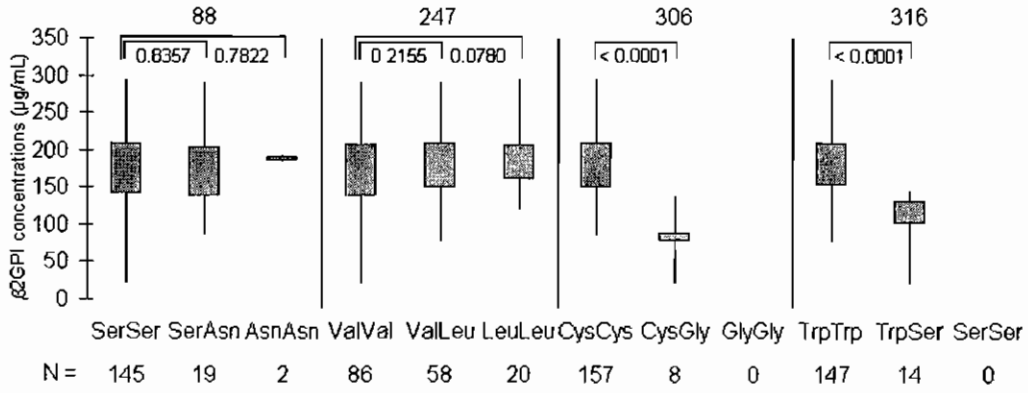


Figure 4.18: Boxplots showing the interquartile ranges of β2GPI concentration in healthy individuals according to their β2GPI genotype. N indicates the number of individuals in each genotype. Extended bars represent concentration range.

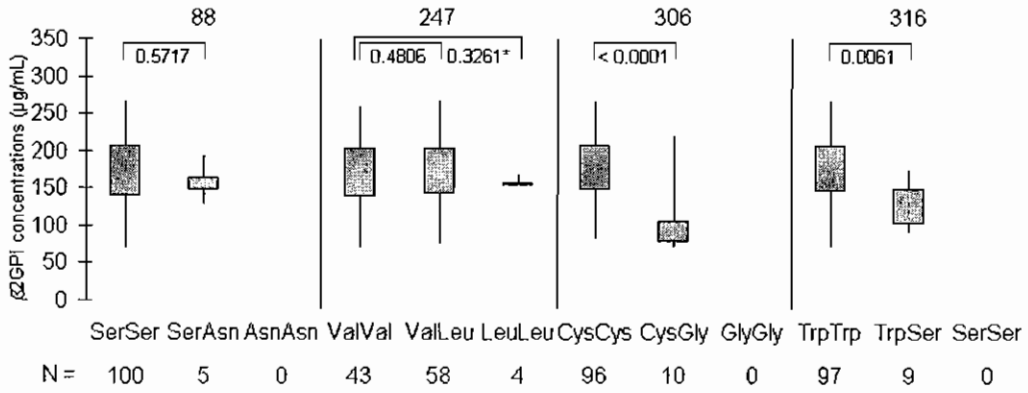


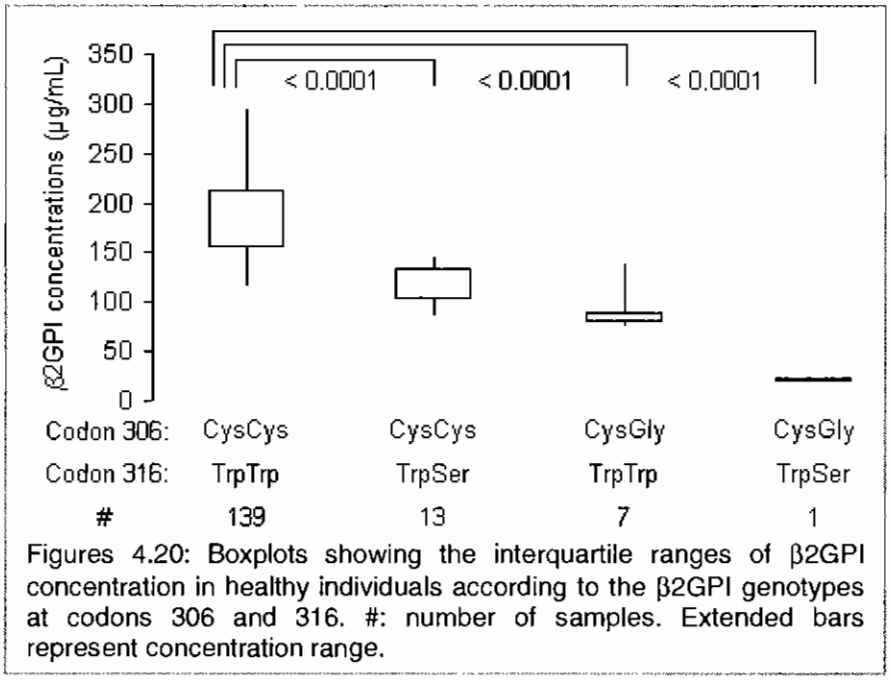
Figure 4.19: Boxplots showing the interquartile ranges of β2GPI concentration in patients with stroke according to their β2GPI genotype. N indicates the number of individuals in each genotype. Extended bars represent concentration range.

The effects of Gly^{306} and Ser^{316} were apparently additive as the mean serum β2GPI concentration in healthy individuals was 188.0 µg/mL in homozygotes without any of the mutations (Cys^{306}Cys-Trp^{316}Trp), 119.1 and 90.6 µg/mL in heterozygotes for the mutation at, respectively, codon 306 (Cys^{306}Gly-Trp^{316}Trp) and 316 (Cys^{306}Cys-Trp^{316}Ser), and 20.8 µg/mL in the only individual who was heterozygous for both mutations (Cys^{306}Gly-Trp^{316}Ser). Similar results were found in stroke patients: 179.8 µg/mL in homozygotes without any of the mutations, and 128.4 and 109.5 µg/mL in heterozygotes for the mutations at codons 306 and 316. No compound heterozygote was found in this group of patients (table 4.14 and figures 4.20 & 4.21). Not all patients with mutations at codons 306 and 316 had a low circulating level of β2GPI, indicating that these mutations were probably not interfering with antibody capture in the ELISA assay.

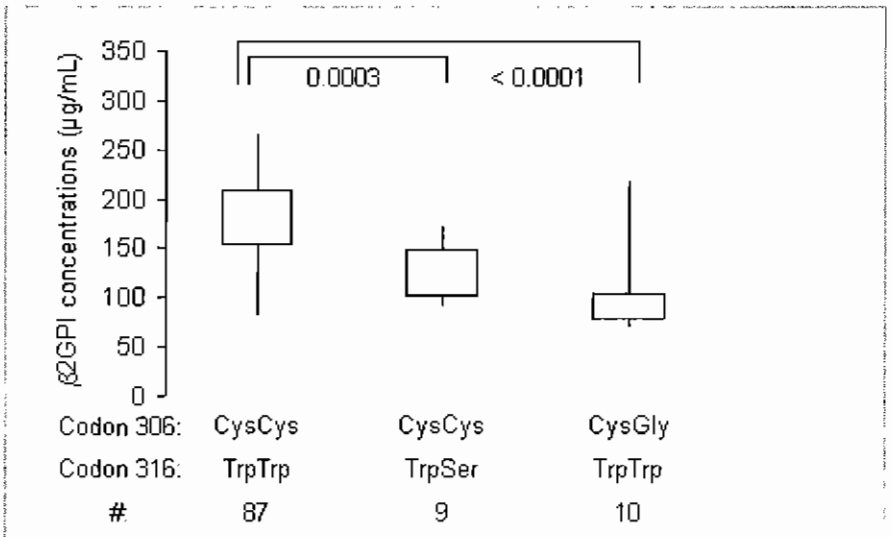
Table 4.14: Mean serum β2GPI concentration and standard deviation (SD) (μg/mL) in healthy individuals and stroke patients according to β2GPI genotype at both codons 306 and 316.

	306 316	CysCys TrpTrp	CysCys TrpSer	CysCys SerSer	CysGly TrpTrp	CysGly TrpSer	CysGly SerSer	GlyGly TrpTrp	GlyGly TrpSer	GlyGly SerSer
Healthy	#	139	13	0	7	1	0	0	0	0
	average	188.0	119.0		90.6	20.8				
	SD	42.0	19.2	n.a.	21.6	n.a.	n.a.	n.a.	n.a.	n.a.
	P value		<0.0001		<0.0001	0.0001				
Stroke	#	87	9	0	10	0	0	0	0	0
	average	179.8	128.4		109.5					
	SD	39.7	30.4	n.a.	54.3	n.a.	n.a.	n.a.	n.a.	n.a.
	P value		0.0003		<0.0001					

#: number of samples; P values from student t-tests comparing β2GPI level versus homozygous non-carriers at both codons; n.a.: not applicable. As not all DNA samples were amplified for a given genetic marker, the total number of controls and cases varies slightly between the mutations examined.



Figures 4.20: Boxplots showing the interquartile ranges of β2GPI concentration in healthy individuals according to the β2GPI genotypes at codons 306 and 316. #: number of samples. Extended bars represent concentration range.



Figures 4.21: Boxplots showing the interquartile ranges of β2GPI concentration in patients with stroke according to the β2GPI genotypes at codons 306 and 316. #: number of samples. Extended bars represent concentration range.

4.4.3.VIII PCR-RFLP versus ACLA and anti-β2GPI antibodies in patients with stroke

In addition to being tested for the four mutations in the β2GPI (APOH) gene and serum β2GPI concentration, most of the stroke patients were also tested for IgG and IgM ACLA. When any of these tests was positive, anti-β2GPI antibody level was also measured. The majority of the stroke patients did not have a significant level of either IgG or IgM ACLA, and the few individuals who were tested positive (according to cut-off values established by the manufacturer) did not present major differences in terms of genotype or allele frequencies at any of the four tested codons when compared to those who were tested negative (detailed results in appendix O). Out of the IgG or IgM ACLA-positive individuals, only one individual had a significant level of anti-β2GPI antibodies, and this individual did not carry any of the tested mutations except the one at codon 247. No significant difference was observed in the overall serum concentration of IgG and IgM ACLA and anti-β2GPI antibodies between either heterozygotes or homozygous carriers and homozygous non-carriers.

IgG ACLA-positive patients presented a very slightly increased serum β2GPI concentration compared to IgG ACLA-negative patients (respectively 187.0 ± 52.8 and 168.6 ± 47.3), while IgM ACLA-positive patients presented a slightly lower serum β2GPI concentration than IgM ACLA-negative patients (respectively 158.2 ± 52.0 and 172.3 ± 47.4) (see appendix O). However, these differences were not statistically significant (respectively P=0.3244 and 0.4004).

4.5 Discussion

In view of the proposed role for β2GPI in the regulation of coagulation and thrombosis, it is possible that polymorphic variations in its encoding gene could affect its function and be associated with thrombotic disease. In this study, we examined the incidence of four point mutations in the β2GPI (APOH) gene in a Western European population and studied the effect of these mutations on circulating level of the protein and the risk of selected common thrombotic disorders.

4.5.1 β2GPI polymorphism detected by IEF-immunoblotting

Our initial aim was to characterize β2GPI polymorphism using IEF-immunoblotting, as this technique was available in our laboratory. Numerous population studies have shown that β2GPI IEF polymorphism displays a significant ethnic variability.[4] When comparing our cohort of healthy individuals from Ireland with other Caucasian populations (Americans and Western Europeans), it was found that there was no significant difference between them in either the allele or the genotype distributions. Identical patterns between these populations included the higher frequency of the APOH 2:2 genotype (80.8 %), the higher frequency of the APOH*2 allele (89.4 %), and the absence of the APOH*4 allele. However, contrarily to other studies which reported identical frequencies for APOH*1 and APOH*3, we found the APOH*3 allele to be about 10 times more frequent than the APOH*1 allele. When compared to individuals of other ethnic origins (such as Africans and Asians), the Irish population presented higher frequency of the APOH*3 allele (respectively 0.0825 and 0.0588 versus 0.0961), and lower frequency of the APOH*1 allele (respectively 0.0103 and 0.0511 versus 0.0096). Since the Irish population is relatively homogenous compared to other Caucasian populations (principally due to the geographical situation of the island), it is highly probable that the genetic pool of the Irish population is more limited. Therefore, the differences in the frequencies of the β2GPI isoforms between our data and some of the previous reports could represent a real finding. As our study involved a relatively small number of samples (52 healthy individuals and 81 patients with stroke), a larger number of normal controls would be required to confidently estimate this difference.

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4 Kamboh *et al*, 1988, 1991, 1996, 1999a & 2004; Richter *et al*, 1988; Eichner *et al*, 1989a&b; Sepehrnia *et al*, 1989; Crews *et al*, 1991, 1993 & 2004; Cleve *et al*, 1992; Saha *et al*, 1992; Sanghera *et al*, 1997a; Kim *et al*, 1998; Singh *et al*, 2002a&b; Tsunoda *et al*, 2002; Vitale *et al*, 2002

In order to examine the possible association between β2GPI IEF polymorphism and susceptibility to thrombus, 81 stroke patients were tested by IEF-immunoblotting and compared to the healthy subjects. As no significant difference was found in the allele and genotype distributions between cases and controls (respectively P=0.4488 and 0.3227), our data indicate that the genetic variation in the β2GPI (APOH) gene identified by IEF does not seem to be a significant risk factor for stroke. We are aware that the numbers of healthy individuals and stroke patients that we tested with IEF-immunoblotting are relatively small. Since polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) methods have been shown to be fundamentally much more sensitive and specific than the IEF-immunoblotting technique, and owing to the complexity and controversial specificity of the latter, we abandoned IEF-immunoblotting and used genetic engineering techniques instead.

4.5.2 β2GPI polymorphism detected by PCR-RFLP

Four point mutations in the β2GPI (APOH) gene have been well characterised at (codon) positions 88, 247, 306, and 316 (Steinkasserer *et al*, 1993; Sanghera *et al*, 1997a&b). Two of these mutations (coding for codons 88 and 316) correspond to two APOH patterns (respectively APOH*1 and APOH*3^{W}), while the other two mutations (coding for codons 247 and 306) do not correspond to any IEF pattern. Observed genotype frequencies for each tested group of individuals showed no significant deviation from those expected under Hardy-Weinberg equilibrium (HWE). This is an indication of the quality and homogeneity of our sample, as calculation of HWE has been suggested to serve as a crude quality check on the data and gross deviations from HWE often indicate genotyping errors or population admixture.

4.5.2.I Comparison with previously published frequencies in other healthy Caucasian individuals

In our healthy study population, the incidence of each tested point mutation was in line with previously reported observations made in other Western European and North American Caucasians, except in one American and two Italian studie (tables 4.10 and 4.11). It is noticeable that allele frequency ranges for the mutations at codons 88 and 316 from North Americans are within those from Western Europeans (table 4.15). This could be the result of a relatively larger genetic pool in Europe from which most US Caucasians are originated. As regards the mutation at codon 247, the ranges differed slightly between the two continents with a slightly higher frequency of the Val^{247} in European Caucasians. Interestingly, the

Irish data for this mutation were within the American range but not within the European range, although no statistical difference was observed (as P=0.7342 and 0.5217 respectively).

Table 4.15: Allele frequency range for the four mutations in healthy Western European and North American Caucasians (excluding the Italian reports ([Cassader *et al*, 1994; Ruiu *et al*, 1997]) and Eichner *et al.*'s study ([1989b]).

Codon	Allele	Western Europe Caucasians	US Caucasians	This study
88	Ser	0.9332 - 0.9615	0.9410 - 0.9558	0.9424
	Asn	0.0668 - 0.0385	0.0590 - 0.0442	0.0576
247	Val	0.7051 - 0.7647	0.6780 - 0.7289	0.6991
	Leu	0.2949 - 0.2353	0.3220 - 0.2711	0.3009
306	Cys	n.a.	0.9629	0.9750
	Gly		0.0371	0.0250
316	Trp	0.9359 - 0.9769	0.9410 - 0.9593	0.9586
	Ser	0.0641 - 0.0231	0.0590 - 0.0407	0.0414

4.5.2.II Haplotype analysis

Because there was no *a priori* reason to focus on particular genotypes, they were first analysed in terms of haplotypes. Haplotype analysis ignores subjects and analyses the chromosome data under the assumption of HWE. Departures from HWE might however not necessarily lead to reduced accuracy because a violation of HWE causes an excess of homozygotes and hence reduces the need for phase information. To infer haplotypes, one relies on algorithms, such as the expectation maximisation algorithm. Whatever the number of loci taken into consideration, when analysing the healthy individuals with no missing genotype for the four tested mutations (n=310), it appeared that if rare haplotypes (defined as haplotypes with an imputed frequency of less than 5 %) were merged, all of possible haplotypes were merged with the exception of those differentiated by the 247 locus. This reflects the very small number of carriers of the mutation at codons 88, 306 and 316 positions. Each minor variant thus occurred independently against a background of the commonest haplotype, and given the absence of haplotypes comprising combinations of more than one minor variant, haplotype analysis could simply be reduced to genotypic analysis of each variant position.

4.5.2.III PCR-RFLP in thrombotic diseases

At position 88, no significant association was found between the Asn^{88} allele and any of the thrombotic states examined, in line with a previously published study that found no association between the mutation at codon 88 and cerebral infarction (Xia *et al*, 2003b). This suggests that the serine to asparagine missense mutation at codon 88 neither affects the molecule's function nor represents a significant risk factor for thrombosis.

At position 247, the heterozygous state was associated with a lower occurrence of ACS (*P*=0.0088; OR: 0.646; 95%CI: 0.466-0.896) suggesting that the Leu^{247} allele confers some protection against ACS. This was partially in line with the results of a previous study performed in patients with the APS (Atsumi *et al*, 1999).[5] However, this association was confirmed neither by the homozygous state with the mutation (when compared to homozygous non-carriers; P=0.9353; OR: 1.022; 95%CI: 0.604-1.731), nor by statistical analyses based on allele frequencies (P=0.1810; OR: 0.850; 95%CI: 0.670-1.079). Moreover, no similar association was observed in the other thrombotic states examined.

Individuals who were heterozygous at position 306 had a higher occurrence of ACS, even though this association was of marginal significance (*P*=0.0470; OR: 1.859; 95%CI: 1.000-3.457) and allele analysis showed that the relative risk of a cardiac event was also of marginal significance, with carriers of the Gly^{306} allele having a little less than twice a higher risk of ACS than non-carriers (*P*=0.0512; OR: 1.819; 95%CI: 0.989-3.348). With regard to the other specific thrombotic disease examined, there was no significant association as assessed by allele analysis, despite the fact that the occurrence of the Gly^{306} allele in patients with stroke and RFL was about twice as frequent as in healthy individuals (respectively 8.8 and 10.6 % versus 5.0 %).

By contrast, heterozygotes at position 316 seemed marginally less likely to suffer from thrombotic disorders but our results did not reach statistical significance, which concurs with a recent report (Xia *et al*, 2003a). On the other hand, our results do not support a previous study that reported that the Ser^{316} mutation alone might predispose to thrombosis (Gushiken *et al*, 1999). It is noteworthy that the frequency of the

[5] Several other studies found similar results but in non-Caucasian individuals, namely Mexicans (Prieto *et al*, 2003) and Asians (Hirose *et al*, 1999; Yasuda *et al*, 2000a & 2005).

Ser^{316} allele was lower in all the tested groups (from 5.1% to 8.0%) compared to the healthy individuals (8.3 %), particularly in the cardiac patients (5.3%).

β2GPI from homozygotes for the Gly^{306} or the Ser^{316} mutations or for both mutations has been shown to present markedly reduced or no binding to anionic phospholipids, while β2GPI from homozygotes without any of the mutations or from individuals presenting heterozygosity for only one of the two mutations (Gly^{306} or Ser^{316}) has been shown to present normal phospholipid binding (Sanghera *et al*, 1997a&b; Horbach *et al*, 1998; Mehdi *et al*, 2000a&b; Nash *et al*, 2003). Interestingly, the slight increase in occurrence of thrombotic diseases among carriers of the mutation at codon 306 was only observed in individuals without the mutation at codon 316 (P=0.0328; OR: 1.901; 95%CI: 1.045-3.485; results not shown). However, this does not suggest that the latter mutation has any protective function against thrombotic diseases. Regrettably, our sample contained very few heterozygotes for either of the codons and not a single homozygous carrier of any of these two mutations. This did not allow us to determine whether the cumulated presence of both mutations can impede regulation of the coagulation cascade by β2GPI molecules and whether such a state actually differs from homozygous carrier states. As both mutations seem to be present at very low frequencies, larger-scale studies are required to prove any influential role of these mutations.

4.5.2.IV PCR-RFLP versus β2GPI concentration

β2GPI concentration varies widely among individuals, particularly when of different ethnic backgrounds. Although family data indicate that this variation is under genetic control ([Cleve, 1968; Koppe *et al*, 1970; Propert, 1978]), its molecular basis is still unclear. In this study, we conducted association studies between the four mutations described above and serum β2GPI concentration. In the healthy individuals, no significant variation in β2GPI concentration was observed in relation to the genotypes at codons 88 and 247 (individuals with the latter presented a marginally increased β2GPI level) (ANOVA test P=0.9391 and 0.1499 respectively). On the other hand, significant and independent associations were observed between the genotypes at codons 306 and 316 and extremely reduced β2GPI serum concentrations (P<0.0001 for both mutations). These findings are similar to earlier results ([Ruiu *et al*, 1997; Horbach *et al*, 1998; Kamboh *et al*, 1999a; Mehdi *et al*, 1999 & 2003]). Analyses performed in patients with stroke yielded very similar findings.

One may question the ability of the antibodies (particularly the capture monoclonal antibody) used in the β2GPI ELISA to equally recognize the different β2GPI variants. In view of our results, however, we think it unlikely that the studied β2GPI mutations caused any interference with the ELISA. Firstly, there was very little difference in serum β2GPI concentration between carriers and non-carriers of the mutations at codons 88 and 247. Secondly, not all of the patients with the mutations at codons 306 and 316 had a reduced level of the protein and in some instances the level of β2GPI was increased. This indicates that the lower levels of β2GPI detected in carriers of the mutations at positions 306 and 316 do not reflect poor binding of the detecting ELISA antibodies.

In line with a previous report ([Kamboh *et al*, 1999a]), the effects of the mutations at codons 306 and 316 were additive: β2GPI concentrations of 188.0, 119.0, 90.6, and 20.8 μg/mL were found in, respectively, homozygotes without any of the mutations ($Cys^{306}Cys$-$Trp^{316}Trp$), heterozygotes at position 316 with homozygosity at codon 306 ($Cys^{306}Cys$-$Trp^{316}Ser$), heterozygotes at position 306 with homozygosity at codon 316 ($Cys^{306}Gly$-$Trp^{316}Trp$), and in heterozygotes at both positions ($Cys^{306}Gly$-$Trp^{316}Ser$). A similar picture was observed in patients with stroke: 179.8, 128.4, and 109.5 μg/mL of β2GPI were observed in homozygotes without any of the mutations and heterozygotes with homozygosity at codon 306 and at codon 316 respectively (this group contained no heterozygote for both

mutations).

Interestingly, the impact of both mutations at codons 306 and 316 seemed to be related to the proposed Bg^D allele (see Chapter 3.5.3). The only individual who could be classified as homozygote for the Bg^D allele (with a β2GPI concentration of 20.8 μg/mL) was indeed the only compound heterozygote detected among the 313 healthy individuals who were genotyped for both mutations at codons 306 and 316. However, the association between the two mutations with reduced β2GPI concentration cannot be considered as exclusive. This is because more than half of the individuals in the quartile with the lowest β2GPI concentrations in both the healthy and the stroke groups carried a mutation neither at codon 306 nor at codon 316, and because several heterozygotes for the mutations at codons 306 or 316 were found in the other quartiles. Our results thus do not seem to link the two mutations to an altered expression of β2GPI, in line with previous *in vitro* mutagenesis and expression studies (Mehdi *et al*, 2000a&b). This absence of strict association between a low serum β2GPI concentration and the Gly^{306} and Ser^{316} mutations[6] suggests that they do not represent absolute regulators of serum β2GPI concentration. This does not exclude however that they may be in linkage disequilibrium with one or several other, still to be identified, functional mutation(s) that is (are) the actual independent lowering factor(s) for serum β2GPI concentration. Alternatively, other mechanisms, including non-genetic factors, could be involved in variations of serum β2GPI concentration (Cleve, 1968; Walter *et al*, 1979). In this regard, our study suggests that a variety of inflammatory stimuli may affect β2GPI concentration (see chapter 3).

One should note that the reduction in serum β2GPI concentration associated with the presence of the two mutations was slightly less marked in patients with stroke than in healthy individuals. When subjects of the latter healthy group were sorted according to age in two subgroups of equal size, aged ≤ 22 and ≥ 23 years, it appeared that not only was the average serum β2GPI concentration (± SD) significantly higher in the older subgroup compared to the younger one (199.5 ± 46.5 versus 159.7 ± 45.6 μg/mL; $P<0.0001$),[7] but fewer heterozygotes at both codons 306 and 316 were also observed in the older subgroup (5.4 % in the older

[6] As well as with the recently reported mutation in the promoter region of the β2GPI (APOH) gene with which the Ser^{316} mutation seems to be in linkage disequilibrium (Mehdi *et al*, 2000a&b).
[7] In line with what was described in chapter 3.

subgroup versus 18.7 % in the younger subgroup). When tested against the older healthy subgroup, the stroke group presented a significantly lower serum β2GPI level (mean ± SD: 169.1 ± 47.6 versus 199.5 ± 46.5 μg/mL; P<0.0001) and significantly more heterozygotes at both codons 306 and 316 (18.4 versus 5.4 %). Interestingly, the stroke and the younger healthy subgroups presented similar percentages of heterozygotes at both codons 306 and 316 (respectively 18.4 % and 18.7 %). Stroke patients thus represent a population that presents a higher occurrence of both mutations at codons 306 and 316, particularly when tested against older healthy controls. The absence of clear differences in allele and genotype frequencies at both codons 306 and 316 between the stroke group and our entire healthy group could be explained by the composition of the latter group. Most of the healthy subjects tested in this part of our study were relatively young compared to the stroke patients. It is theoretically possible that this healthy group may have included individuals that were predisposed to thrombosis - and stroke in particular - due to genetic defects in the β2GPI (APOH) gene, but whose phenotype was exempt of thrombotic symptoms due to their young age. It can thus not be excluded that the relative differences observed in allele and genotype frequencies at both codons 306 and 316 would actually be substantially more solid if we had genuinely healthy elderly subjects for comparison against the different diseased groups, particularly the stroke one.

As a conclusion, our findings provide some insight into the possible involvement of these mutations in the pathogenesis of thrombosis without highlighting a clear causative role for the tested mutations in the reduction of serum β2GPI concentration. The association between the mutation at codon 306 and the thrombotic disorders as a group and cardiac disease separately, is likely to be very weak. Thrombosis is a multifactorial process and it is certain that there are many different genetic and environmental risk factors involved. In addition, it is difficult to assess whether the observed increased incidence of thrombosis in those individuals heterozygous at position 306 is independent of the effect of this mutation on circulating level of the protein. Furthermore, the individuals heterozygous at position 316 also had a reduced level of β2GPI but no increase in the incidence of thrombosis.

4.5.2.V PCR-RFLP versus ACLA and anti-β2GPI antibodies

The presence of autoantibodies that react with anionic phospholipids in patients with certain autoimmune diseases, such as SLE and the primary APS, has been associated with an increased risk of thromboembolic events. Some of these antibodies have been shown to react with β2GPI either on its own or as part of a complex epitope that includes β2GPI and the bound anionic phospholipids (see introductory chapters and appendix B). Since genetically determined structural variation in β2GPI may modify the antigenicity of the molecule, the presence of any of the four tested mutations can potentially affect the presence of β2GPI-dependent aPL and/or anti-β2GPI antibodies, and thus influence the clinical course of patients with such antibodies. Alternatively, a low β2GPI concentration (possibly related to the presence of the Gly^{306} or the Ser^{316} mutations) could result in a reduction in β2GPI binding to anionic phospholipids, thereby affecting the occurrence of aPL and/or anti-β2GPI antibodies.

Among the stroke patients who were tested for ACLA, the majority did not have a significant level of either IgG or IgM ACLA, and the few individuals who were tested positive for IgG ACLA did not present major differences in genotype or allele frequencies at any of the four tested codons when compared to IgG ACLA-negative individuals. This is in line with a previous study (Kamboh *et al*, 1999a) except for the mutation at codon 316, since we did not find the Ser^{316} mutation to be significantly more frequent in our patients with stroke who were aPL-negative compared to aPL-positive patients.

If the mutation at codon 88 had some significance on its own, it may have resided in the expression of a new antigenic epitope. Due to the J-shape configuration of the β2GPI molecule (figure 1.4), codon 88 is situated far away from the β2GPI-phospholipid interaction site and thus from the phospholipid surface, at a site allowing easy interaction with potentially pathogenic circulating β2GPI-dependent aPL and/or anti-β2GPI antibodies. However, all the stroke patients tested for ACLA were homozygous non-carriers of the Asn^{88} mutation, as was the only individual who was tested positive for anti-β2GPI antibodies. Thus, although our sample was relatively small, these results somewhat contradict a previously published finding that the Asn^{88} allele presents comparable distributions between aPL-positive and aPL-negative individuals (Kamboh *et al*, 1999a).

At position 247, the substitution of a valine to a leucine may alter hydrogen bridging between the adjacent lysine at position 246 and the nearby fourth domain, thus possibly exposing one or more otherwise cryptic epitope(s). However, it is most likely that the amino acid substitution results in only a negligible conformational change in the protein structure, as the two amino acids differ only by a methylene group and thus share very similar structural features and hydrophobicity. Moreover, the amino acid at position 247 is located in an area of β sheet formation that does not include the current proposed binding sites of known monoclonal anti-β2GPI antibodies (mapped in other positions of the 5th domain (Hunt *et al*, 1994; Igarashi *et al*, 1996), and more recently of the 1st (Iverson *et al*, 1998 & 2002; McNeeley *et al*, 2001), 3rd (Igarashi *et al*, 1996), and 4th (Igarashi *et al*, 1996; Koike *et al*, 1998) domains of β2GPI). Our data therefore did not reinforce the finding from other researchers that Val^{247}Val homozygosity conferred a higher risk for developing an anti-β2GPI response (Atsumi *et al*, 1999; Hirose *et al*, 1999; Prieto *et al*, 2003; Yasuda *et al*, 2005). However, the extremely small number of patients in our sample (n=11) makes it difficult to draw a definite conclusion.

At positions 306 and 316, among the stroke patients who were tested ACLA-positive, all were homozygous non-carriers of the Gly^{306} and Ser^{316} mutations, except one patient who was heterozygote at codon 316. The limited number of individuals who were IgG ACLA-positive does not allow us to draw any conclusion or provide further support to the finding from other researchers that the Ser^{316} mutation can affect the production of β2GPI-dependent aPL (Kamboh *et al*, 1998 & 1999a). Caution must however be exercised when comparing results from these studies as our group of patients contained individuals who suffered from stroke and not SLE and the prevalence of the mutation might be significantly different between the two groups of patients. It is also possible that the Ser^{316} mutation actually conferred some protection against the production of β2GPI-dependent aPL but not against β2GPI-independent aPL, and this distinction could not be made with the ACLA ELISA used in this study. Moreover, among the IgG ACLA-positive stroke patients, only one individual had anti-β2GPI antibodies and this patient did not carry the Gly^{306} or the Ser^{316} mutation. These results thus do not lend weight to the hypothesis that the allele products of the mutations at codons 306 or 316 may preclude the production of aPL and/or anti-β2GPI antibodies (Kamboh *et al*, 1995; Sanghera *et al*, 1997a&b).

Therefore, from our results, none of the mutations at codons 88, 247, 306 and 316 was strongly associated with a predisposition to or protection from the presence of ACLA and/or anti-β2GPI antibodies, and no relationship was observed between any of the mutations and serum concentrations of ACLA and/or anti-β2GPI antibodies. Furthermore, as reported earlier, serum β2GPI concentrations in the few individuals who were tested positive for IgG and IgM ACLA did not significantly differ from those tested negative (P=0.3422 and 0.4004, respectively), nor did they differ between individuals positive and negative for anti-β2GPI antibodies (P=0.8381). However, the extremely small number (n=11) of patients who were tested for anti-β2GPI antibodies makes these findings difficult to interpret, and the absence of data from healthy controls makes our results for the stroke group quite speculative.

4.6 Summary and conclusion

In this study, we have compared the frequency of four single-nucleotide polymorphic (SNP) variations in the β2GPI (APOH) gene in unrelated thrombotic patients and healthy controls, tested for the co-occurrence of the four point mutations with thrombotic diseases (namely stroke, early onset acute coronary syndrome (ACS) and recurrent foetal loss (RFL)), and analysed their impact on β2GPI, aPL, and anti-β2GPI concentrations. To do so, we used PCR-RFLP and ELISA methods and performed extended haplotype, genotype and allele analyses for each polymorphic variant.

- The allele and genotype frequencies observed for the four tested β2GPI mutations in the Irish population did not present any significant or consistent deviation from those observed in other European or American populations.
- The mutation at codon 306 was almost twice as frequent in patients with ACS. Its impact is unlikely to be due to its effect on circulating level of β2GPI as the mutation at position 316 is also associated with reduced level of the protein but not with an increased risk of ACS.
- Heterozygosity for the mutation at codon position 247 was found to be associated with a lower risk of ACS. Given that the 247 mutation has no significant impact on circulating level of β2GPI, it is not surprising that it might have different phenotypic consequences in comparison to the 306 mutation. Nevertheless, it is somewhat surprising that they act in opposite directions. While no significant associations were seen between the 247 codon and the other phenotypes, it is striking that the heterozygous state at position 247 is associated with higher risk of stroke, such that the confidence intervals for stroke and ACS are not overlapping. This suggests the hypothesis that whatever mechanism of 247 action protects against ACS, this mechanism may confer risk of stroke. The pattern of association for codon 306 with stroke does not show the same striking reversal of risk between ACS and stroke.
- Although the mutations at codon positions 88 and 316 were found to be somewhat associated with reduced incidence of thrombotic disorders, this did not reach statistical significance, nor was this association confirmed by measurements by allele analysis or homozygous carrier genotype frequencies.
- Our results confirmed the previously reported very significant correlation between reduced circulating levels of β2GPI and polymorphism at codon positions 306 and 316.
- Although our numbers were very small, we observed no association between the four studied mutations and the presence or level of ACLA and/or anti-β2GPI antibodies.

Thrombosis is a multifactorial process in which many different genetic and environmental risk factors are involved. Because of the late onset of the majority of thromboses, genetic changes are unlikely to be their sole determinant. It is thus most likely that gene-environment interactions have great importance in the susceptibility to, and onset and possibly progression of these pathologies. Mutations in the β2GPI (APOH) gene could thus have a modulating effect on the interactions between genetic variations and acquired influences such as smoking, diet, and metabolic changes and/or other external environmental risk factors, thereby influencing the final phenotype. The genetic control of β2GPI thus has to be considered together with environmental factors and efforts should be made in defining the mechanism(s) of these interactions and their role(s) in determining the risk of thrombotic disease. However, such studies are difficult to perform and need extremely large number of patients to study the possible interrelated effect of polymorphisms in β2GPI and polymorphisms in other proteins (e.g. factor V Leiden) and the wide variety of environmental dispositions (e.g. smoking, diet, exercise, oral contraceptive use, etc) to which individuals may be exposed.

5 β2GPI synthesis

5.1 Introduction

5.1.1 Inflammation

Inflammation is defined as a fundamental and stereotyped integrated complex of cytologic and chemical reactions that occur locally and systemically to counteract challenges in response to many different stimuli that include bacterial, viral, and parasitic infection, trauma, surgery, burns, tissue infarction, advanced cancer, as well as various immunologically mediated and crystal-induced inflammatory conditions (Zweifach *et al*, 1965; Janeway *et al*, 1994; Dirckx, 1997).[1] The term "inflammation" is in itself purely descriptive, as it refers to the cardinal signs *rubor*, *calor*, *dolor*, and *tumor* (Latin for redness, heat, pain, and swelling) that are associated with it (in *De Medicina*, Celsus, ~AD40). These local reactions are part of a set of reactions commonly referred to as the acute phase response (Kushner, 1987; Janeway *et al*, 1994). This response follows a sequence of events in which a series of inflammatory cells are recruited and activated, mediators are released, and adjacent cells of the stroma are stimulated to secrete chemotactic peptides so that cells of the immune system accumulate in the affected tissue. As a result, local vascular and systemic multi-organ effects are initiated, including biosynthetic changes, particularly pronounced in the liver, and a large number of behavioural, biochemical and physiologic changes (Janeway *et al*, 1994; Gabay *et al*, 1999). All these reactions are considered as part of the early-induced immune response, which accompanies the non-inducible innate immunity and precedes the specific adaptive T-cell dependent immunity (Janeway *et al*, 1994).

Initiation of the inflammatory cascade is most commonly associated with the activation of tissue macrophages or blood monocytes (figure 5.1).[2] Upon activation, these cells release a first range of cytokines (Dinarello, 1996; Ramadori *et al*, 1999), which act both locally and distally on other cells, including adjacent stromal cells such as fibroblasts and endothelial cells, and cause them to further secrete cytokines (Kohase *et al*, 1987; Taga *et al*, 1993). Some of these cytokines (such as interleukin (IL)-8) are highly chemotactic for granulocytes (Matsushima *et al*, 1989; Ohno *et al*, 1992). These secreted cytokines combined with the expression of endothelial cell surface adhesion molecules (such as E-selectin and intercellular adhesion molecule-1)

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[1] Numerous other conditions, such as strenuous exercise, heat stroke, childbirth, stress and some psychological disorders have also been found to cause inflammatory responses, on a much more moderate scale (Majno *et al*, 1996; Maes *et al*, 1997).

[2] However, other cell types, including neutrophils, mast cells (Gordon *et al*, 1990; Marshall *et al*, 1993) and aggregation-induced platelets (Janeway *et al*, 1994; Terr, 1994) can also prompt the process at the site of tissue damage, and pathogens, as well as by-products of opsonins, can also directly activate monocytes and macrophages (Janeway *et al*, 1994).

promote the migration of leukocytes into inflamed sites (Janeway *et al*, 1994; Parslow, 1994) where they accumulate and in turn release further pro-inflammatory cytokines (Xing *et al*, 1993; Cassatella, 1995).

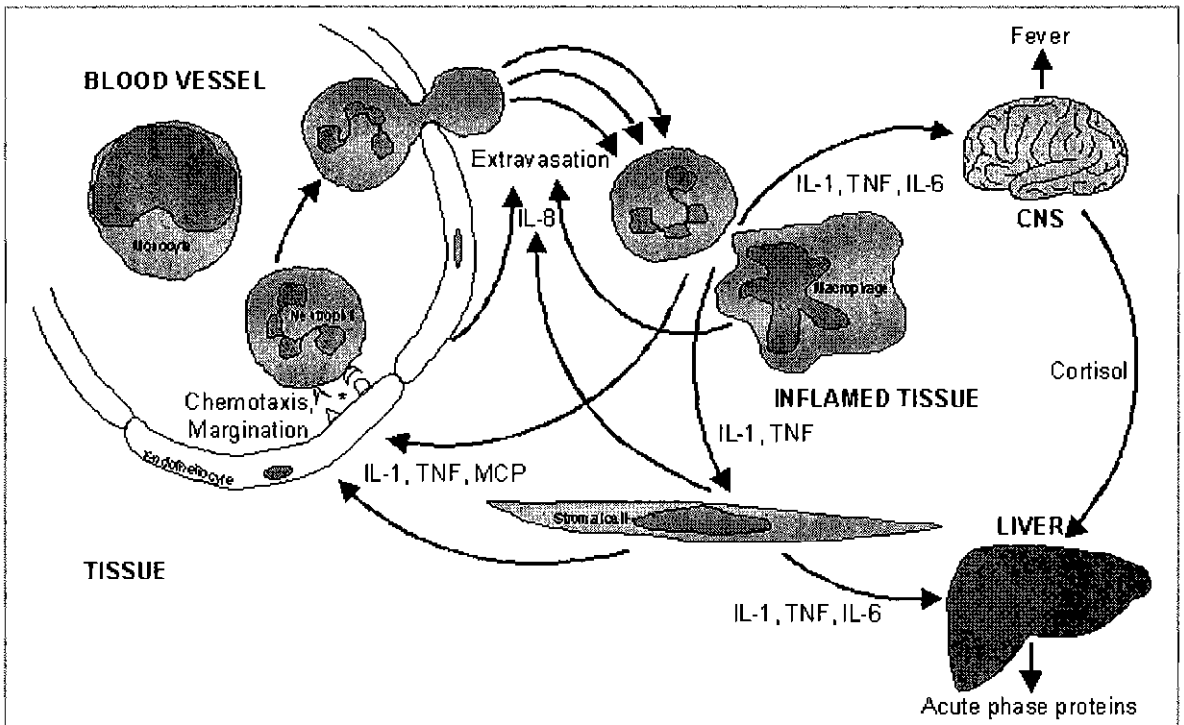

Figure 5.1: Schematic representation of cell and cytokine interaction in the acute phase response. IL: interleukin; TNF: tumour necrosis factor; MCP: monocyte chemoattractant protein; CNS: central nervous system; *: cell adhesion molecules. Modified from Baumann *et al.* (1994).

The combined action of the cytokines also induces coagulation and an increase in vascular permeability with the action of, among other agents, prostaglandins and leukotrienes (Janeway *et al*, 1994). Acting alone or synergistically, they also induce a number of effects that are mediated through the hypothalamus, e.g. fever (Dinarello *et al*, 1988 & 1991) and the secretion of corticotropin-releasing factor, which stimulates the release of adrenocorticotropic hormone from the pituitary gland and thus induces glucocorticoid production by the adrenal gland.

Inflammation is usually life-preserving, as reflected by the increased risk of acute infections in people with genetic deficiencies in principal components of the inflammatory process (Biesma *et al*, 2001; Bunting *et al*, 2002). However, not all inflammation-associated phenomena are uniformly beneficial, as its excessive or inappropriate maintenance over the longer term - in cases of chronic inflammation - may have deleterious clinical consequences (Means, 1995; Wheeler *et al*, 1999; Buxbaum *et al*, 2000).

5.1.2 The acute phase proteins

The acute phase response modifies the profile of circulating plasma proteins. Those that present a significantly modified plasma concentration as a consequence of the inflammatory process are referred to as acute phase proteins (APP). Approximately forty proteins are currently considered as APP (Gabay *et al*, 1999). For most of them, the major site of synthesis is the hepatic parenchymal cell, but some APP have also been shown to be produced in other cell types - such as monocytes, endothelial cells, fibroblasts and adipocytes - and expressed in extra-hepatic tissues (such as the choroids plexus, the yolk sac, the placenta or the seminal vesicles) (Pepys *et al*, 1983 & 1985; Ramadori *et al*, 1985; Aldred *et al*, 1987a&b).

Most of the known APP can be classified according to their functions (table 5.1).[3] On a general level, these functions are to isolate, neutralize and destroy inflammatory agents (infective organisms or harmful molecules), while minimizing tissue damage and activating tissue regeneration processes, thus restoring homeostasis. More specifically, APP are involved in blood clotting and fibrinolysis, transport of a variety of molecules, inhibition of proteases, opsonization, neutralization and clearance of a variety of inflammatory agents, and modulation of the immune responses against these agents. With such missions, APP are part of a first non-specific line of defence, which can immediately be mobilised after an insult when the specific immune response is still ineffective.

Table 5.1: Major positive acute phase proteins in humans.

Involved system	Acute phase protein
Coagulation and fibrinolysis	Fibrinogen, plasminogen, plasminogen activator inhibitor 1, protein S, tissue plasminogen activator, urokinase, von Willebrand factor, vitronectin.
Metal binding	Ceruloplasmin (Goldstein *et al*, 1979), ferritin, haptoglobin, haemopexin, manganese superoxide dismutase.
Protease inhibition	α_1-antichymotrypsin, α_1-anti-plasmin, α_1-antitrypsin, α_1-protease inhibitor, heparin cofactor II, inter-α-trypsin inhibitors, pancreatic secretory trypsin inhibitor.
Inflammatory responses	CRP (Mold *et al*, 1981; Wolbink *et al*, 1996; Abiij *et al*, 2002), C1q, C2, C3, C4, C9, factor B, C1-inhibitor, C4b-binding protein, factor H, granulocyte-colony-stimulating factor, interleukin-1 receptor antagonist, lipopolysaccharide-binding protein, mannose-binding lectin, secreted phospholipase A_2, tumour necrosis factor receptor 1 (McDermott *et al*, 1999).
Other	α_1-acid glycoprotein, angiotensinogen, fibronectin, lipoproteins, SAA (Uhlar *et al*, 1999).

Compiled from Baumann *et al*, 1994; Majno *et al*, 1996; Gabay *et al*, 1999.

[3] Many APP are multifunctional and have the potential to influence one or more stages of the inflammatory process. For instance, haptoglobin is a major transporter of (haemo)globin - that is found in increased amounts during tissue degradation - and is also considered as a significant angiogenic factor (Cid *et al*, 1993). On the other hand, no particular function has been precisely assigned to some APP, such as SAA (Kisilevsky *et al*, 1992; Malle *et al*, 1993; Banka *et al*, 1995).

A protein is considered as an APP when its concentration varies by at least 25 % during inflammatory disorders; this variation can be an increase (for "positive APP") or a decrease (for "negative APP") ([Morley *et al*, 1982]). Variations in concentration differ significantly from one APP to another, ranging from 25 % (e.g. ceruloplasmin and complement components) to as much as 1,000-fold variations (e.g. CRP and SAA) - hence the sub-classification as minor/weak or major/strong APP.

The intracellular mechanisms that regulate the expression of APP genes are not completely understood. There is however *in vivo* and *in vitro* evidence from primary hepatocyte and hepatoma cell line cultures, as well as from murine and rat model systems, that the synthesis and release of plasma APP from the liver are regulated by inflammatory mediators ([Baumann *et al*, 1987; Andus *et al*, 1988; Kushner, 1993]) via transcriptional ([Birch *et al*, 1986; Fey *et al*, 1987 & 1990; Goldberger *et al*, 1987; Andus *et al*, 1988; Morrone *et al*, 1988]) and post-transcriptional mechanisms ([Morrone *et al*, 1989; Rogers *et al*, 1990]).

There has been much speculation on the functional advantages of decreased plasma concentration of negative APP.[4] A common hypothesis is that reduced production stems from the need to divert available amino acids to the production of positive APP that are more important for host defence. In another hypothesis, the reduced concentration could rather represent a pro-inflammatory mechanism, as some negative APP can inhibit the production of pro-inflammatory cytokines when present at normal concentration (e.g. impact of transthyretin on IL-1β production ([Borish *et al*, 1992])).

[4] Examples of negative APP: albumin, pre-albumin, transferrin, apoAI, apoAII, α-foetoprotein, factor XII, inter-α-trypsin inhibitor, histidine-rich glycoprotein, insulin-like growth factor I, thyroxin-binding globulin.

5.1.3 β2GPI synthesis and inflammation

As mentioned earlier, inflammation is known as a significant risk factor for thrombosis (Cicala *et al*, 1998; Dhainaut *et al*, 2001), and there have been contrasting observations concerning β2GPI's behaviour during inflammatory events. Although serum β2GPI concentration seems to remain unchanged in a series of inflammatory disorders (Cleve, 1968; Cohnen, 1970), more recent studies have found β2GPI to behave as a negative APP under the influence of certain inflammatory mediators (Mehdi *et al*, 1991; Sellar *et al*, 1993; Lin *et al*, 2006). Understanding how serum β2GPI level is regulated and how it changes during an inflammatory process would thus throw a new light on β2GPI's potential role in inflammation-associated thrombosis.

In order to evaluate the impact of inflammation on β2GPI production, we examined β2GPI synthesis (through mRNA production) in a mouse model of severe sepsis. Of course, there is a species-dependent qualitative and quantitative variability in the stimulation of APP gene expression. However, there is also a strong similarity in the general pattern of hepatic acute phase response between species; this evolutionary conservation suggests that this mechanism is a significant advantage for survival. A good example of this is haptoglobin, which is a major APP in both humans (Fey *et al*, 1990) and mice. Owing to the fact that β2GPI and haptoglobin are expressed in humans and mice and that the liver is the main producing organ of both proteins (Averna *et al*, 1997; Chamley *et al*, 1997; Caronti *et al*, 1999), we used haptoglobin as a comparative protein. In this study we presuppose that the regulation mechanisms of both transcripts are similar in both species. If this hypothesis is easily acceptable for haptoglobin, which has been recognised as a significant APP in both humans and mice, it must be taken with care for β2GPI, especially since its exact functions in both species are still unknown.

5.2 Aim of chapter 5

As very few *in vivo* data are available concerning β2GPI expression during inflammation, we investigated β2GPI expression at the mRNA level in an *in vivo* mouse model of intra-abdominal sepsis-type inflammation using the LightCycler™[5] (Roche Diagnostics GmbH, Mannheim, Germany) with probe hybridisation and resonance energy transfer technologies.

[5] LightCycler is a trademark of a member of the Roche Group.

5.3 Materials and methods

5.3.1 Mouse model of inflammation

Following the approval from local ethical committees, 30 male (CD1) mice were used in this study. 20 of them underwent caecal ligation and puncture (CLP) under neuroleptic anaesthesia. Half of these surgically treated mice (S+) were pre-treated with Danazol® (a derivative of the synthetic steroid 17-ethyl testosterone) before CLP, while the other half (S0) received a placebo (distilled water). The remaining mice were used as controls (C0). They underwent the same protocol of pre-treatment with Danazol® or placebo but no surgery. Buprenorphine (Temgesic, R & C, Hull, UK) was administered for pain when necessary and any animal that suffered unduly was killed. During the observation period all animals were allowed rodent chow and water *ad libitum*. All mice were sacrificed by CO_2 inhalation 24 hours post CLP. Livers of sacrificed animals were extracted, snap frozen in liquid nitrogen and stored until RNA extraction. All samples were coded and experiments and analyses were done in a blinded fashion.

This section of the thesis was part of a wider study that analysed the modulating effect of Danazol® on sepsis-type inflammatory reactions ([Beddy *et al*, 2006]).

5.3.2 Statistics

Comparisons between cohorts were performed by means of the Mann-Whitney test. All analyses were performed with InStat GraphPad Software, Version 2.03, 1994 (PK Wallace, Wally Word) or SPSS Inc., Version 11.0 for Windows, 2001. P values under 0.05 were considered statistically significant.

5.3.3 mRNA detection techniques

5.3.3.I Introduction

Five methods are commonly used for the quantification of mRNA: *in situ* hybridisation and northern blotting ([Parker *et al*, 1999]), RNase protection assays ([Hod, 1992, Saccomanno *et al*, 1992]), cDNA arrays ([Bucher, 1999]), and the reverse transcription PCR (RT-PCR) ([Weis *et al*, 1992]). Of all methods, RT-PCR is the most sensitive and flexible as it requires less RNA and sequence information ([Wang *et al*, 1999]). It can be used to compare mRNA level and to analyse RNA structure. It is an *in vitro* method that enzymatically amplifies defined sequences of RNA ([Rappolee *et al*, 1988 & 1989]) and that circumvents time-consuming and technically demanding cloning steps. Practically, RT-PCR combines cDNA synthesis with PCR amplification ([Veres *et al*, 1987]). The exponential nature of the PCR allows, in theory, to calculate the amount of starting

material from the amount of product at any point in the reaction. In practice, however, reaction conditions (including limitation and interaction/overlapping of reagents) can interfere with the exponential amplification and affect product concentration (Hildebrandt *et al*, 1999). Standard PCR and RT-PCR work best, therefore, as a qualitative technique.

Instead of measuring "end-point" product amplification, modern techniques that monitor product formation as it progresses in the thermal cycler are now available and widely used. Real-time RT-PCR techniques show high sensitivity, good reproducibility, and wide quantification ranges. Nowadays, they can be considered as the most sensitive and rapid methods for the detection and quantification of gene-expression levels. These techniques are based on the real-time fluorescence monitoring of the accumulation of PCR-amplified reverse-transcribed mRNA during the exponential (log) phase of the reaction. During this phase, the fractional number of PCR cycles that are required to generate enough fluorescent signal to reach a predetermined threshold (background) value is defined, hence the denomination "threshold cycle" or C_T (Roche Molecular Biochemicals, 1998 & 2000; Bustin, 2000; Rasmussen *et al*, 2001; Ginzinger, 2002).

Several real-time RT-PCR protocols allow the detection of product amplification with about the same sensitivity and specificity. The simplest method of all uses intercalating fluorescent dyes, such as SYBR™[6] Green I and SYBR™ Gold, that bind to double-stranded-DNA (figure 5.2). However, despite being both inexpensive and generic, these dyes are less specific than sequence-specific probes (as they may bind, for instance, to primer dimers), they depend on the mass of double-stranded DNA produced in the reaction, and they also cannot be used in multiplexed assays. Protocols that rely on the hybridisation of fluorescence-labelled sequence-specific probes to the correct amplicons do not have such drawbacks. These detecting probes differ primarily in their specificity and do not require post-PCR Southern analysis or sequencing to confirm the identity of the amplicons. They include the use of hairpin probes (such as molecular beacons and Scorpion probes), hydrolysis probes or hybridisation probes (figure 5.3) (Roche Molecular Biochemicals, 1998 & 2001; Caplin *et al*, 1999; Landt *et al*, 1999).

[6] SYBR is a trademark of Molecular Probes Inc, Eugene, OR, USA.

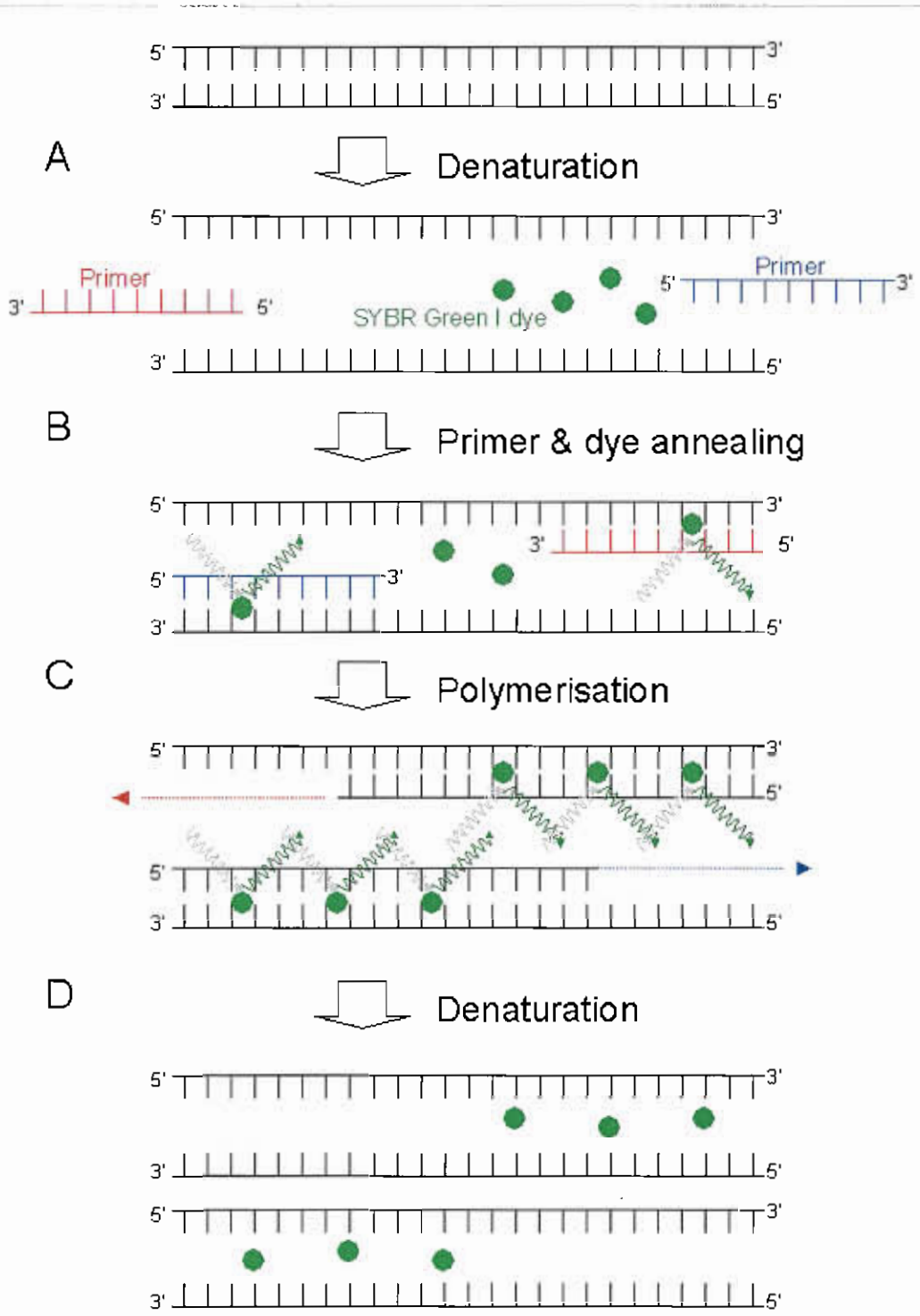

Figure 5.2: Double-stranded DNA dye incorporation method. The RT step has been omitted. (A) During the denaturation step, unbound SYBR™ Green I dye exhibits little fluorescence. (B) At annealing temperature, a few dye molecules bind to the double-stranded primer/target, resulting in light emission upon excitation. (C) During the polymerisation step, more and more dye molecules bind to the newly synthesised DNA, and the increase in fluorescence is monitored in real-time. (D) During the next denaturation step, the dye molecules are released and the fluorescence signal returns to background. Adapted from Roche Manual.

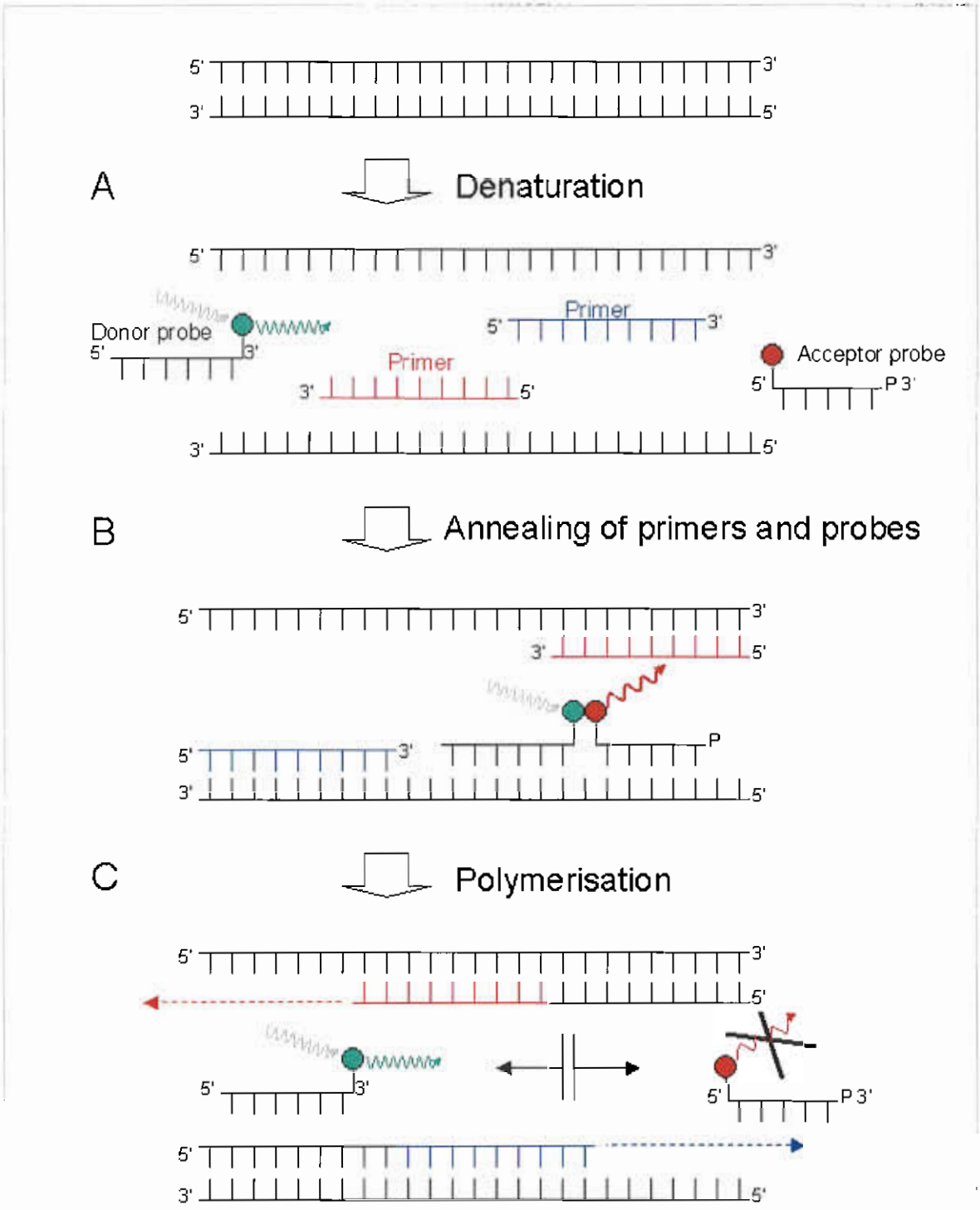

Figure 5.3: Hybridisation probe method. The RT step has been omitted. (A) During the denaturation step, both hybridisation probes remain in solution and separate. Any emission from the donor fluorophore (namely fluorescein) at 530 nm is disregarded by the detector. (B) During the annealing step, the probes hybridise in a head-to-tail arrangement, thereby bringing the two fluorophores in close proximity. Fluorescence resonance energy transfer (FRET) occurs during which fluorescein emits energy that excites the acceptor (detector) fluorophore which in turn emits red fluorescent light at a longer wavelength. (C) At polymerisation temperature, both probes return into solution, ceasing FRET and acceptor (detector) fluorophore emission. Any emission from fluorescein is ignored. Adapted from Roche Manual.

Numerous real-time thermocyclers are available on the market ([Pray, 2004 & 2005]). Their primary structure consists of an integrated thermal cycler with an optical excitation and detection device (fluorometer) that allows very rapid and accurate microvolume PCR cycling with real-time fluorescence monitoring. The LightCycler™ from Roche Molecular Biochemicals (Mannheim, Germany) ([Roche LightCycler™ Manual; Roche Molecular Biochemicals, 1998; Rasmussen *et al*, 2001]) was used in this study with either SYBR™ Green or sequence-specific hybridisation probes (Figure 5.4).

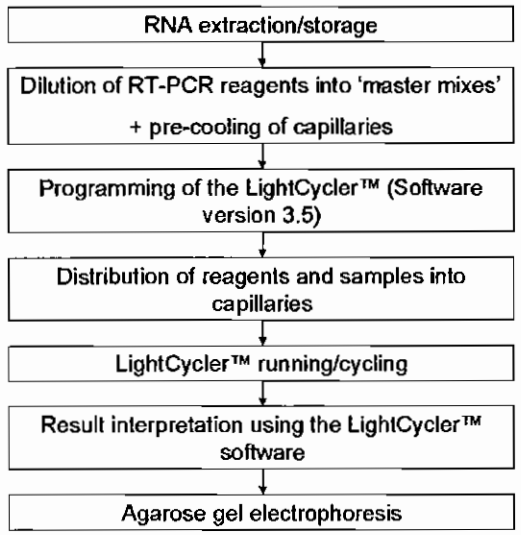

Figure 5.4: Schematic outline of the main steps of the LightCycler™ procedure.

In order to estimate β2GPI gene expression during an inflammation process, the level of β2GPI mRNA was measured and compared to the one of an acute phase protein, namely haptoglobin, in an animal model of sepsis-type inflammation using the LightCycler™ technology. The ultimate objective of this study was to quantify β2GPI and haptoglobin in duplex with a housekeeping gene (i.e. a non-regulated reference gene), using dual LightCycler™-Red640 together with LightCycler™-Red705 colour detection. Unfortunately, the study was shortened for technical reasons and as a consequence duplex design was not achieved. Nevertheless, we could still indirectly compare target and reference transcripts among samples through amplification ratios using concentrations estimated on the same run (but in separate tubes) in relation to proper standard curves.

5.3.3.II Extraction of total RNA from mouse liver tissues

Total RNA was extracted from mouse liver tissues using either the single-step Tri-Reagent® (Molecular Research Center, Inc; Cincinnati; Ohio; USA) as prescribed by the manufacturer or the single-step guanidium thiocyanate-phenol-chloroform extraction procedure (slightly modified) from Chomczynski and Sacchi ([1987]) (appendices M, N & K).

5.3.3.III LightCycler™ modus operandi

Primers and probes were designed by Genset SA, now Proligo Primers and Probes (Paris, France) using mouse exon sequences obtained from GenBank (accession N° D10056 for β2GPI, NM_017370 for haptoglobin, NM_008084 for glyceraldehydes-3-phosphate dehydrogenase (GAPDH), and M26689 for actin) (figures 5.5 to 5.8). The design of the primers (table 5.2) and probe pairs (table 5.3) followed generally accepted rules (size, distance of binding, G/C content, melting temperature -T_m- values, complementary sequences, secondary structures, etc) while keeping the amplicon lengths short (i.e. below 300 bp), and considering that the two probes of a set must anneal between 1 and 5 bases away from each other on the amplified cDNA target. The probes required some modifications (a fluorescein was attached at the 3' end of the "donor" probes, while one of the two LightCycler™ dyes (LCRed640 or LCRed705) was attached at the 5' end of the acceptor probes which were also phosphorylated at their 3' end) (Hildebrandt *et al*, 1999).

Reagents of the LightCycler™-RNA "master hybridisation probe" kit (Cat.N°.3018954) are ready-to-use reagents (unlike those of other LightCycler™ kits that require some preparatory dilutions). Since only template RNA, primers, hybridisation probes, and (if necessary) additional $Mn(OAc)_2$ have to be added such kits provide convenience and minimise contamination risk. The following description of the protocol is valid only for the LightCycler™-RNA "master hybridisation probe" kit.

Two master mixes were prepared, one containing the enzymes and the buffer (table 5.4), and the other containing the sequence-specific primers and probes (table 5.5). Each of the test transcripts (β2GPI or haptoglobin) was tested in parallel with a housekeeping gene (GAPDH or actin). The volume of both master mixes depended on the total number of reactions to be performed. The following tables present the volume and final concentration of each reagent for 10 µL reactions:

Figure 5.5: Partial nucleotide sequence coding for mouse β2GPI exon. Primer sequences are in bold, probe sequences are underlined.
GenBank accession N° D10056 MUSB2GLP Mouse mRNA for β2GPI.

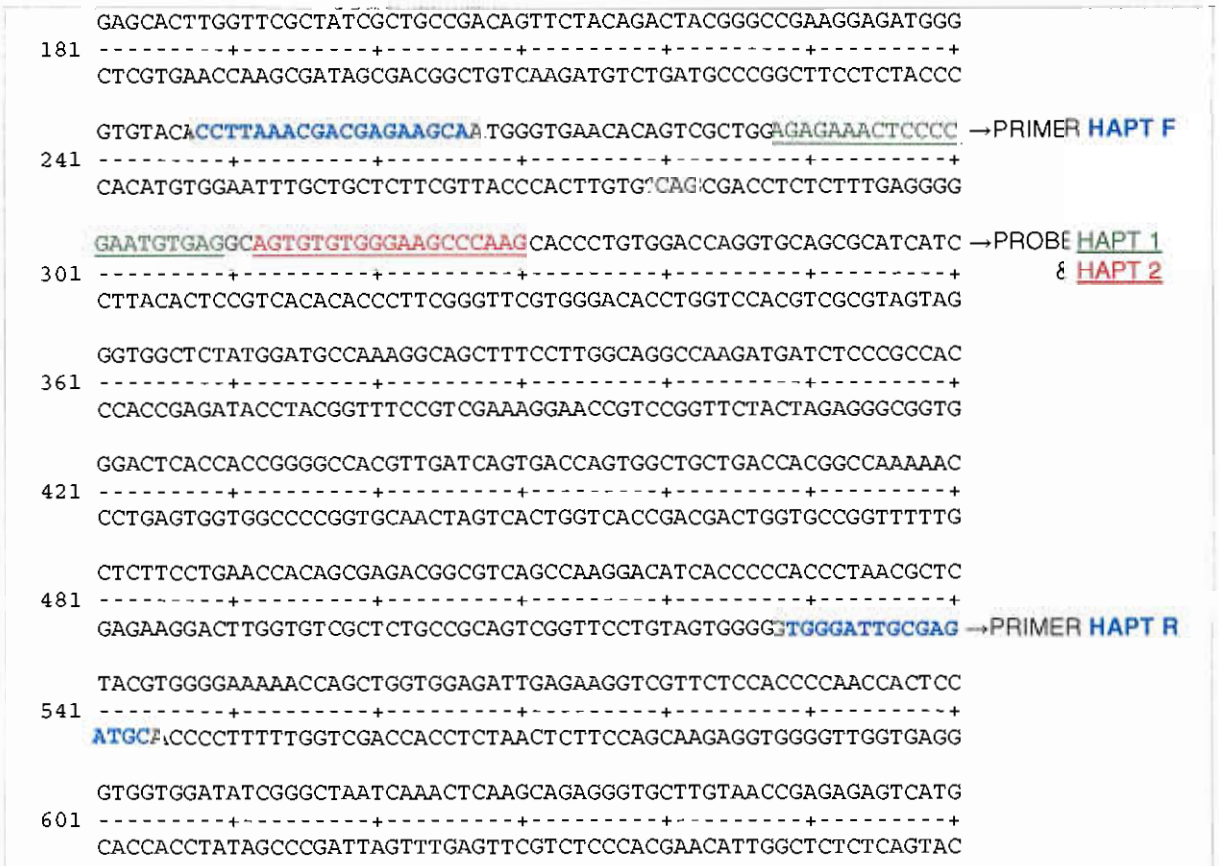

Figure 5.6: Partial nucleotide sequence coding for mouse haptoglobin exon. Primer sequences are in bold, probe sequences are underlined.
GenBank accession N° NM_017370 Mus musculus haptoglobin, mRNA.

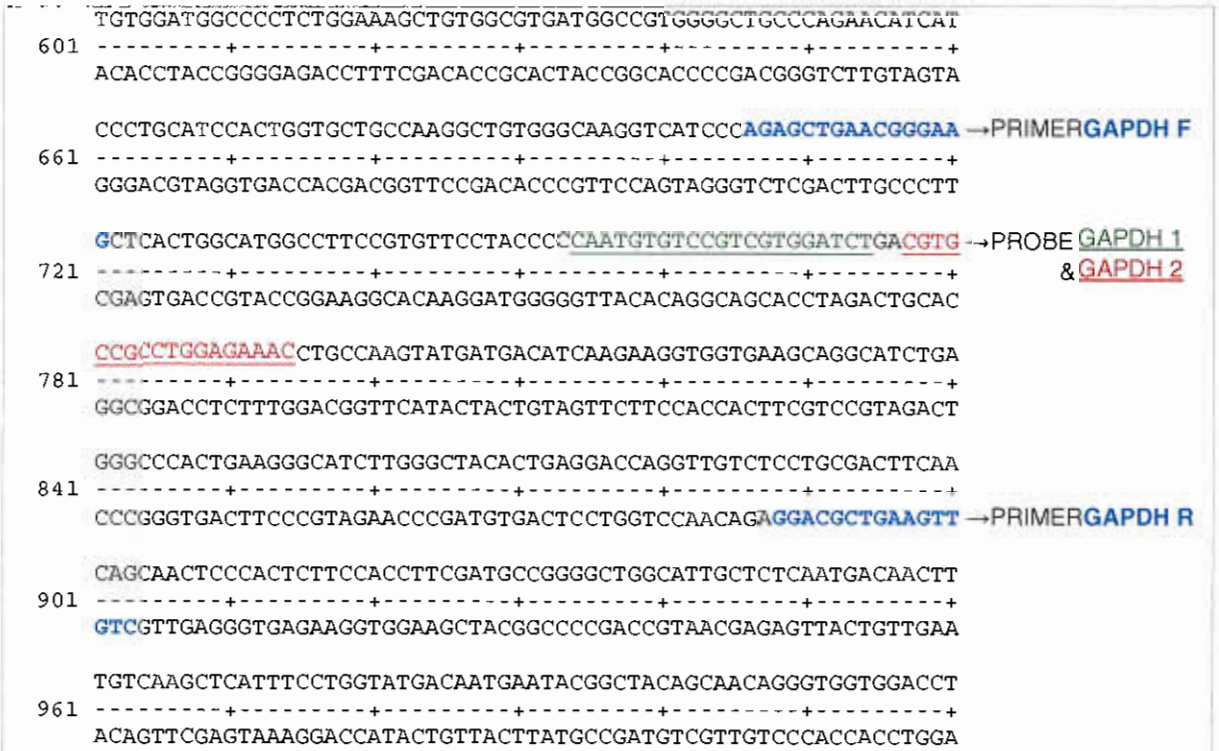

Figure 5.7: Partial nucleotide sequence coding for mouse GAPDH exon. Primer sequences are in bold, probe sequences are underlined.
GenBank accession N° NM_008084 Mus musculus glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA.

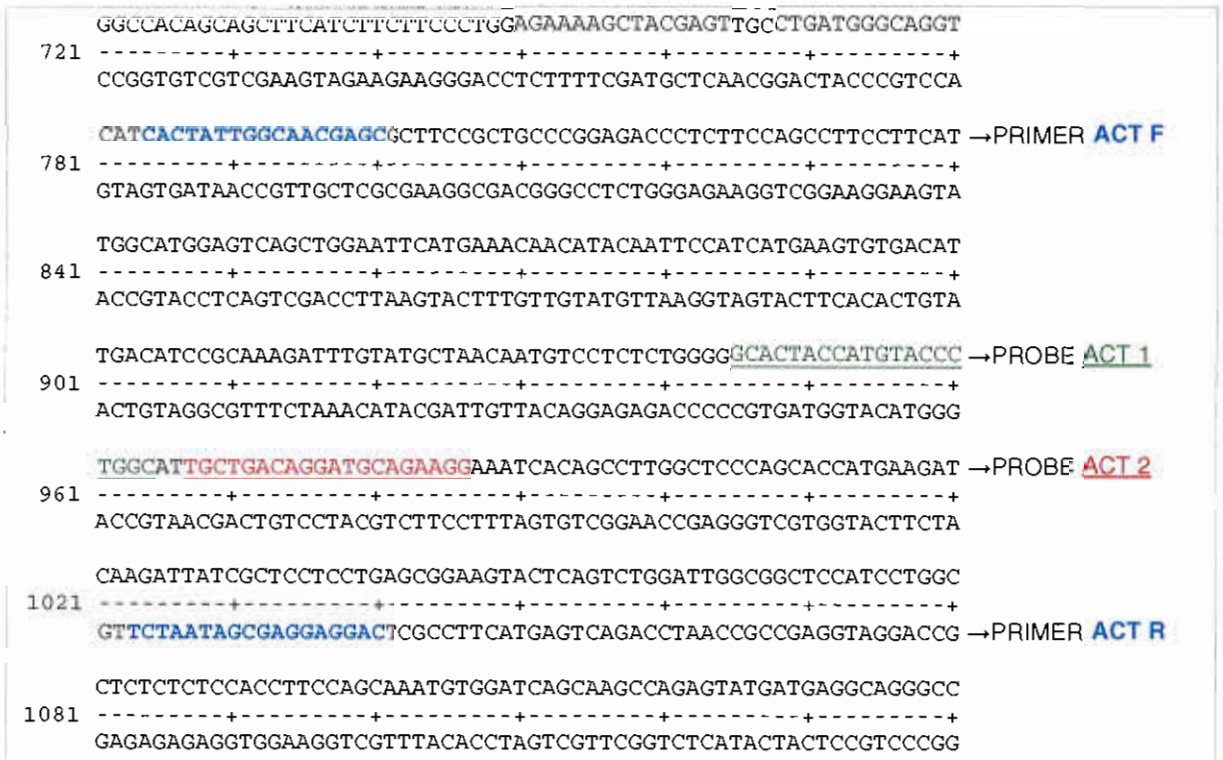

Figure 5.8: Partial nucleotide sequence coding for mouse actin exon. Primer sequences are in bold, probe sequences are underlined.
GenBank accession N° MUSGAAC M26689 Mus musculus smooth muscle γ-actin mRNA.

Table 5.2: Primer sets used to reverse transcribe mRNA and PCR amplify the cDNA sequences of β2GPI, haptoglobin, GAPDH, and actin.

Name	Orientation	Label	Sequence (5'→3')	T_m (°C)	Product size
β2GPI	Forward	APOH F	Cgg ATC TgT CCg AAg C	59.7	237 bp
	Reverse	APOH R	ACT CgT gTA gCg TAC AAT	60.4	
Haptoglobin	Forward	HAPT F	CCT TAA ACg ACg AgA AgC A	59.5	297 bp
	Reverse	HAPT R	CgT AgA gCg TTA ggg T	59.5	
GAPDH	Forward	GAPDH F	AgA gCT gAA Cgg gAA g	59.6	198 bp
	Reverse	GAPDH R	CTg TTg AAg TCg CAg g	60.0	
Actin	Forward	ACT F	CAC TAT Tgg CAA CgA gC	59.8	257 bp
	Reverse	ACT R	CAg gAg gAg CgA TAA TCT	59.4	

Table 5.3: Probe sets used to quantify PCR-amplified cDNA sequences of β2GPI, haptoglobin, GAPDH, and actin.

Name	Label	Sequence (5'→3')	g/mole	T_m (°C)
β2GPI	APOH1	TgT ggC CCA TCA ACA CCC T (Fluo)	5,706	65.1
	APOH2	(Red640) gAT gTg TCC CCA gAg TAT gTC CT (P)	7,027	65.2
Haptoglobin	HAPT1	AgA gAA ACT CCC CgA ATg TgA g (Fluo)	6,774	64.1
	HAPT2	(Red640) AgT gTg Tgg gAA gCC CAA g (P)	5,915	65.0
GAPDH	GAPDH1	CAA TgT gTC CgT CgT ggA TCT (Fluo)	6,425	64.6
	GAPDH2	(Red705) CgT gCC gCC Tgg AgA AAC (P)	5,507	65.3
Actin	ACT1	gCA CTA CCA TgT ACC CTg gC (Fluo)	6,035	64.3
	ACT2	(Red705) TgC TgA CAg gAT gCA gAA gg (P)	6,228	64.2

Table 5.4: Buffer/enzyme master mix for a single 10 µL RT-PCR reaction.

Reagent	Volume (µL)	Final concentration
H_2O, sterile, filtered and PCR grade	1.60	
$Mn(OAc)_2$, stock solution 50mM	0.65	3.25 mM
LightCycler™ mix (enzyme + dNTP + buffer)	3.75	1x

Table 5.5: Sequence-specific master mix for a single 10 µL RT-PCR reaction.

Reagent	Volume (µL)	Final concentration
H_2O, sterile, filtered and PCR grade	1.00	
Primer mix * (see table 5.2)	1.00	0.5 µM each
Hybridisation probe mix * (see table 5.3)	1.00	0.2 µM each

* The primer mix corresponds to 100 µg (~0.5 µM) of each sense (forward) and antisense (reverse) primer for a particular sequence, while the probe mix corresponds to 0.2 µM of each fluorescein (donor) and fluorophore (acceptor) probe for the same particular sequence.

Both master mixes were added into glass capillaries and 1 µL of template RNA (diluted 1 in 125) was subsequently added. Following a 2-second centrifugation at 3,000 rpm, the capillaries were placed into the LightCycler™ instrument for cycling, after which the capillaries were emptied into Eppendorf tubes and the content was immediately checked by electrophoresis on a 1.0 % agarose gel or stored at -20°C until electrophoresis. Each run included a "no template" control (i.e. with all reagents but no RNA template) and a "no RT" control (i.e. with the RNA template and all reagents except the reverse transcriptase).

5.3.3.IV Programming of the LightCycler™ (software version 3.5)

The four successive steps that constitute the experimental real-time RT-PCR protocol were as follows:

1. Reverse transcription of template RNA into cDNA (20 minutes of incubation at 61°C, temperature transition rate (TTR): 20°C/second);
2. Denaturation of the cDNA/RNA hybrid (incubation at 95°C for 2 minutes, TTR: 20°C/second);
3. Amplification of cDNA by 45 cycles of denaturation (95°C, 5 seconds, TTR: 20°C/second), annealing (55°C, 15 seconds, TTR: 20°C/second) (step during which fluorescence was acquired), and elongation (72°C, 13 seconds, TTR: 2°C/second);[7]
4. Cooling of the rotor and the thermal chamber (decrease in incubation temperature at a rate of 20°C/second until reaching 40°C, 30 seconds of incubation).

The PCR reaction performed by the LightCycler™ can be described as $C_T = C_0 * E^n$, where C_T is the threshold cycle, C_0 the starting concentration of the sample, E the overall reaction efficiency, and n the crossing point value (CP) expressed in number of cycles. The LightCycler™ software offers two methods of threshold cycle determination, which use slightly different algorithms: the "threshold cycles" or "fit points" method and the "second derivative maximum" method. Both methods generate linear regression lines through the data points (of the standard curves) by plotting the CP versus the logarithm of the concentration for each standard, allowing to extrapolate the concentration of any given sample via its CP. The "second derivative maximum method" was used in this study (Higuchi *et al*, 1993; Rasmussen *et al*, 2001; Roche LightCycler™ software guide).

Our purpose was to show changes in gene expression according to the inflammatory state. We were thus only interested in the relative quantification of both β2GPI and haptoglobin transcription, and had little to gain from accurately determining the absolute copy number of mRNA from both β2GPI and haptoglobin. Hence, we only drew relative standard curves. They consisted of a calibrator, which was used to create dilution series with arbitrary units for each individual amplicon against which the target C_T in the sample was directly compared during the RT-PCR assay. Thus, four standard curves (one for each

[7] Single fluorescence was acquired once per sample, just before extension when the probes were hybridised, in order to obtain the strongest signal. Colour compensation (although optional when performing single colour detection experiments) was activated.

amplicon) were made with a pool of RNAs (extracted from the liver of five healthy untreated mice) serially diluted in a series of 5-fold dilutions. From the CP and their assigned dilution factor, the LightCycler™ software calculated the slope for each standard curve and the overall PCR efficiency was estimated according to the equation: $E = 10^{(-1/slope)}$. The LightCycler™ software also automatically set the crossing line in a way that the mean squared error of the standard curve data points was minimized, requiring no manual setting (Bustin, 2000; Pfaffl, 2001; Rasmussen *et al*, 2001; Roche LightCycler™ software guide).

The relative expression ratio of the target genes was normalized with the expression of an endogenous (housekeeping) reference RNA standard in order to compensate variations in the amount of starting material between samples and inter-run variations. GAPDH and actin were chosen in this study because they are ubiquitously expressed in all nucleated cell types while being necessary for basic cell survival and presumably invariant (Haberhausen *et al*, 1998; Karge *et al*, 1998; Thellin *et al*, 1999). There are a number of different mathematical ways to measure the relative expression ratio of the investigated transcripts, but all equations include PCR efficiency (E) and crossing point difference (ΔCP). In this study, unknown samples were tested versus controls using the formula (Pfaffl *et al*, 2001 & 2002):

$$(E_{target})^{\Delta CP\ target\ (control\text{-}sample)} / (E_{reference})^{\Delta CP\ reference\ (control\text{-}sample)}$$

It can be seen from the above equation that even if $E_{reference}$ is not equal to E_{target}, endogenous mRNAs can be used to compare relative target level.

To generate the data basis for the determination of PCR efficiency for each transcript, standard curves for each transcript were created using a pool of RNAs from non-treated control mice, ensuring a relatively good estimate of the PCR efficiency. The standard curves that were the most appropriate following the optimization procedure were stored and used in subsequent runs.

It has to be noted that the LightCycler™ software performs some signal normalization when using single colour hybridisation probe experiments. This is performed by setting the Y-axis display of the fluorescence graph to channels F2/F1 or channels F3/F1, which causes the signal of the reporter dye (LightCycler™-Red640 measured in channel 2, LightCycler™-Red705 measured in channel 3) to be divided by the signal of the donor dye (fluorescein measured in channel 1) providing an internal reference for the displayed data.

5.3.3.V Optimization of the real-time-PCR method

The very first set of optimization experiments consisted in confirming the primer specificity. Because LightCycler™ kits were relatively expensive, the different primer sets were first tested on Hybaid PCR-thermocycler blocks with two-step RT-PCR protocols, and subsequently on the LightCycler™ device with the LightCycler™ kits.

On Hybaid PCR-thermocycler blocks reverse transcription was analysed in a total reaction volume of 20 µL with 100 µg (~0.5 µM) of each sense and antisense primer, 1 unit of enhanced avian reverse transcriptase (Sigma), 2 units of ribonuclease inhibitor (Sigma), 500 µM of dNTPs, and in a buffer containing 50 mM Tris-HCl, pH 8.4, 1 mM DTT, 40 mM KCl, and 8 mM $MgCl_2$. Reverse-transcribed RNAs were then PCR amplified using a protocol similar to the one used in chapter 4. Tested annealing temperature and $MgCl_2$ concentration ranges were respectively 45 to 60°C and 0.5 to 5.0 mM. Primer sets were tested separately and in duplex (in four combinations: β2GPI-GAPDH, β2GPI-actin, haptoglobin-GAPDH, and haptoglobin-actin). Duplex amplification was estimated by gel electrophoresis on 1.0 % agarose gel. All four combinations gave good duplex amplification when proportions of the primer sets were adjusted.

Primers were then tested on the LightCycler™ using the LightCycler™-RNA "SYBR™ Green I amplification" kit (Cat.N°.2015137) following the manufacturer's one-step RT-PCR protocol, except the total reaction volume that was halved to 10 µL (modification based on personal results from F. Ryan, Department of Biological Sciences, DIT, Dublin). Transposing the optimal test conditions for two-step protocols on the thermocycler blocks to one-step protocols using the LightCycler™-RNA "SYBR™ Green I amplification" kit was not effective, the latter requiring a much higher $MgCl_2$ concentration. Optimal test conditions were therefore empirically examined for each amplicon separately. $MgCl_2$ and template concentrations were tested at concentrations ranging from 3 to 9 mM and 100 to 200 nM respectively. A volume of 1 µL of the template RNA samples was tested for the four amplicons, and each run included a "no template" and a "no RT" control. The protocol included five successive steps:

1. Reverse transcription of template RNA into cDNA (20 minutes of incubation at 55°C, TTR: 20°C/second);
2. Denaturation of the cDNA/RNA hybrid (incubation at 95°C for 30 seconds,

TTR: 20°C/second);

3. Amplification of cDNA by 45 cycles of denaturation (95°C, 0 second, TTR: 20°C/second), annealing (55°C, 10 seconds, TTR: 20°C/second), and extension (72°C, 13 seconds, TTR: 2°C/second) during which fluorescence was recorded;
4. Melting curve (a three-step cycle with a first incubation at 95°C, 0 second, TTR: 20°C/second, a second step at 65°C, 10 seconds, TTR: 20°C/second, and a third step with a TTR at 0.1°C/second until reaching 95°C and during which fluorescence was continuously acquired;
5. Cooling step (decrease in incubation temperature at a rate of 20°C/second until reaching 40°C, 30 seconds of incubation).

A melting curve analysis was performed at the end of the PCR stage and involved slowly heating the reaction (at 0.1°C/second) while continuously monitoring the fluorescence. This allowed the detection of primer dimers or unspecific products (Roche Molecular Biochemicals, 1999). In this study, no primer dimers formed when $MgCl_2$ concentration was adjusted around 5 mM. RT-PCR amplification products were subsequently subjected to agarose gel electrophoresis and visualized by staining with ethidium bromide for amplicon size confirmation (Figure 5.9).

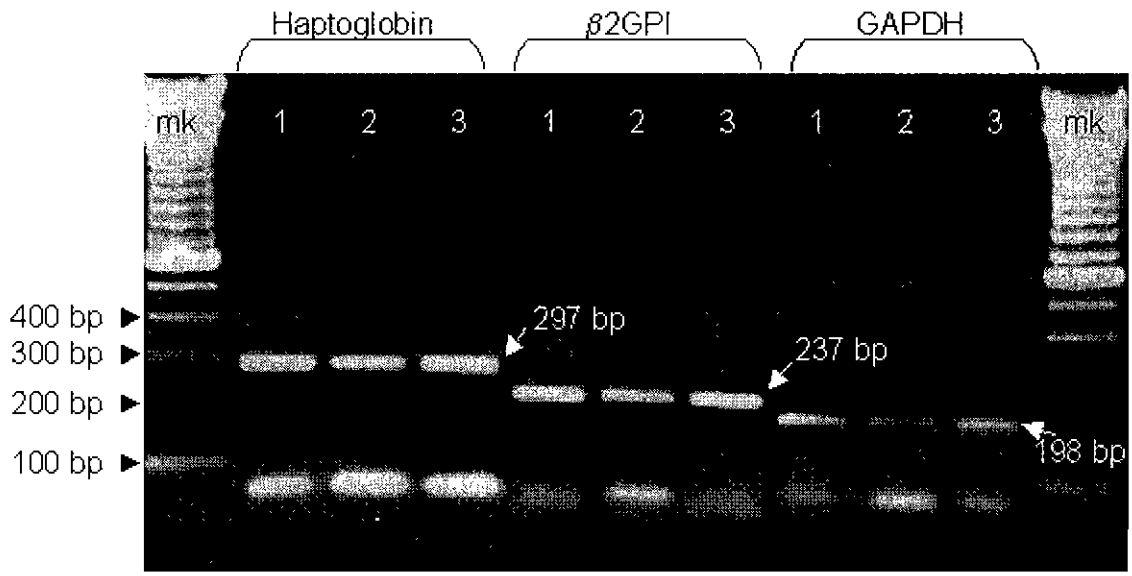

Figure 5.9: Gel electrophoresis sizing of RT-PCR amplified sequences of haptoglobin, β2GPI, and GAPDH mRNA extracted from the liver of three control mice (1 to 3). RT-PCR was performed with total RNA on the LightCycler™ device with the LightCycler™-RNA "SYBR™ Green I amplification" kit.

Duplex amplification was then optimized using two-step RT-PCR protocols on Hybaid thermocycler blocks and one-step protocols on the LightCycler™ device with the LightCycler™-RNA "SYBR™ Green I amplification" kit. GAPDH, but not

actin, was found to be satisfactorily co-amplified with the target transcripts.

Since the ultimate objective was the co-amplification of β2GPI and haptoglobin with a housekeeping gene using dual colour detection, hybridisation probes were then analysed (through polyacrylamide gel electrophoresis) and tested with the LightCycler™-RNA "hybridisation amplification" kit (Cat.N°.2015145) and eventually with the LightCycler™-RNA "master hybridisation probe" kit. One-step RT-PCR were performed for each amplicon to test probe specificity. Optimal test conditions were, again, empirically analysed, especially since the LightCycler™-RNA "master hybridisation probe" kit uses aptamers©,[8] a different buffer containing $Mn(OAc)_2$[9] (and not $MgCl_2$) and the thermostable *Tth* DNA polymerase that has a RNA-dependent reverse transcriptase activity together with a DNA-dependent polymerase activity. The use of the LightCycler™-RNA "master hybridisation probe" kit was found to be very convenient, requiring little optimization from the standard protocol proposed by the manufacturer. To increase sensitivity ([Don *et al*, 1991]), a touchdown segment was added to the early stages of the amplification program (i.e. cycling begun with an annealing temperature of approximately 5°C above the estimated T_m and was incrementally decreased by 1°C every cycle until the optimal annealing temperature was reached). The use of additional compounds (such as DMSO) was not thoroughly tested, and neither were modifications in the number or duration of cycles, or in the concentrations of dNTPs, DNA polymerase, primers or probes. The possibility of amplification of contaminating genomic DNA was excluded with the inclusion of a negative control (i.e. RNA samples subjected to PCR without prior reverse transcription), which gave no product. As a double check, all amplified products were run on agarose gels.

The real-time PCR efficiency (E) in the cycle's exponential phase was calculated by the formula: $E = 10^{(-1/slope)}$ ([Bustin, 2000, Pfaffl, 2001]) for each amplicon. Investigated transcripts showed mean real-time amplification efficiency rates of 2.046, 2.164 and 2.120 for respectively β2GPI, haptoglobin and GAPDH in the investigated range of 0.20 to 120 ng RNA input. These values resulted from the average of three standard curves performed in duplicate. High linearity was observed for all standard curves (Pearson correlation coefficient $r > 0.95$).

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[8] Aptamers are under license from Gilead Sciences Inc, USA. They are oligonucleotides that obstruct polymerase activity until the optimal temperature of the *Tth* enzyme is reached, minimizing mis-priming during the initial phases of the reaction.

[9] Which minimises problems caused by amplification of re-annealed DNA fragments ([Bauer *et al*, 1997]).

Since the probes proved to be specific and adequately concentrated, and since all four amplicons could be analysed separately, duplex combinations that worked best on thermocycler blocks and with the LightCycler™-RNA "SYBR™ Green I amplification" kit (i.e. with GAPDH) were tested with the LightCycler™-RNA "master hybridisation probe" kit. Unfortunately, the lack of time and the excessive cost of the kits' reagents prevented us from fully optimizing duplex RT-PCR measurements using the latter kit. Nevertheless, indirect quantification of β2GPI and haptoglobin remained possible through standard curve and ratio calculations.

The real-time RT-PCR assay reproducibility using the LightCycler™-RNA "master hybridisation probe" kit was evaluated by measurements of intra- and inter-assay coefficients of variation (CV). For intra-assay CV, three replicates of a sample at two different input concentrations were tested within one experimental run. For inter-assay CV, three standard curves performed on three different runs were compared to each other and the mean values of duplicates of two different dilutions of a RNA sample tested on five different experimental runs performed on five different days were analysed and compared to each other. Three runs were analysed versus full standard curves and two runs were analysed versus imported standard curves. Intra- and inter-assay CV were calculated using the crossing point value (CP) and the respective estimated concentration (table 5.6).

Table 5.6: Intra and inter-assay coefficients of variation (CV) for RT-PCR on the LightCycler™ device for haptoglobin, β2GPI, and GAPDH.

	Haptoglobin		**β2GPI**		**GAPDH**	
Intra-assay CV	CP	cc	CP	cc	CP	cc
dilution 1	0.50%	8.62%	0.45%	6.12%	0.62%	11.31%
dilution 2	0.59%	11.35%	0.42%	6.27%	0.33%	6.64%
Inter-assay CV	CP	cc	CP	cc	CP	cc
dilution 1	0.71%	12.20%	0.84%	11.36%	0.56%	10.37%
dilution 2	0.72%	13.33%	0.78%	11.67%	0.62%	12.48%
Standard curve *E*	3.08%		1.91%		2.72%	

The observed CP variations were found to be minimal within each run and between runs made on different days. The corresponding estimated mRNA concentrations were also very reproducible (maximum intra and inter-assay CV of 12.2 % and 13.3 % respectively). Repeated runs of the same standard curve gave variations of 2.7 to 3.1 % in the slope, which correlated with variation in the unknowns. Although not thoroughly tested during the optimization procedures, the target input load and number of PCR cycles were found to give the best results when kept as small as possible for the former and around 45 cycles for the latter.

5.4 Results

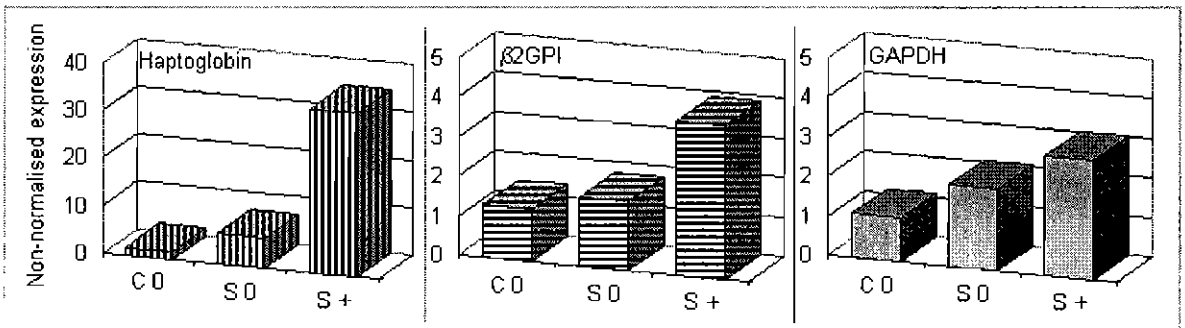
5.4.1 Concentration, purity, and integrity of extracted RNA

The used protocol provided high yield of purified non-degraded RNA preparations. Yield and purity of the RNA extract were estimated by absorption spectroscopy considering that 44.2 µg/mL of RNA give an OD value of 1.0 at a wavelength of 260 nm and that a ratio of absorbance at wavelengths of 260 nm and 280 nm (A_{260nm}/A_{280nm}) over 1.8 suggests that RNA is almost pure. Diluted samples (at 1 in 100) that presented A_{260nm} below 0.1 or a A_{260nm}/A_{280nm} ratio below 1.8 were discarded and the corresponding livers were retreated for RNA extraction. The mean ratio for all (accepted) extracted RNA was 1.871 (range: 1.802 - 1.975).

Small aliquots of each RNA extract were run by electrophoresis on 1.0 % agarose gels and stained with ethidium bromide to estimate their integrity. Non-degraded total RNA gave sharp bands of large and small ribosomal RNA in an approximate (staining) ratio of 2 to 1.

5.4.2 Regulation of the investigated gene transcripts

Without normalisation by the reference gene, the expression of β2GPI and haptoglobin presented relatively similar variations between the three tested groups with all three transcripts (haptoglobin, β2GPI and GAPDH) presenting consistent up-regulation in surgically treated mice (with or without Danazol® medication) when compared to controls (Figures 5.10, 5.11 & 5.12).

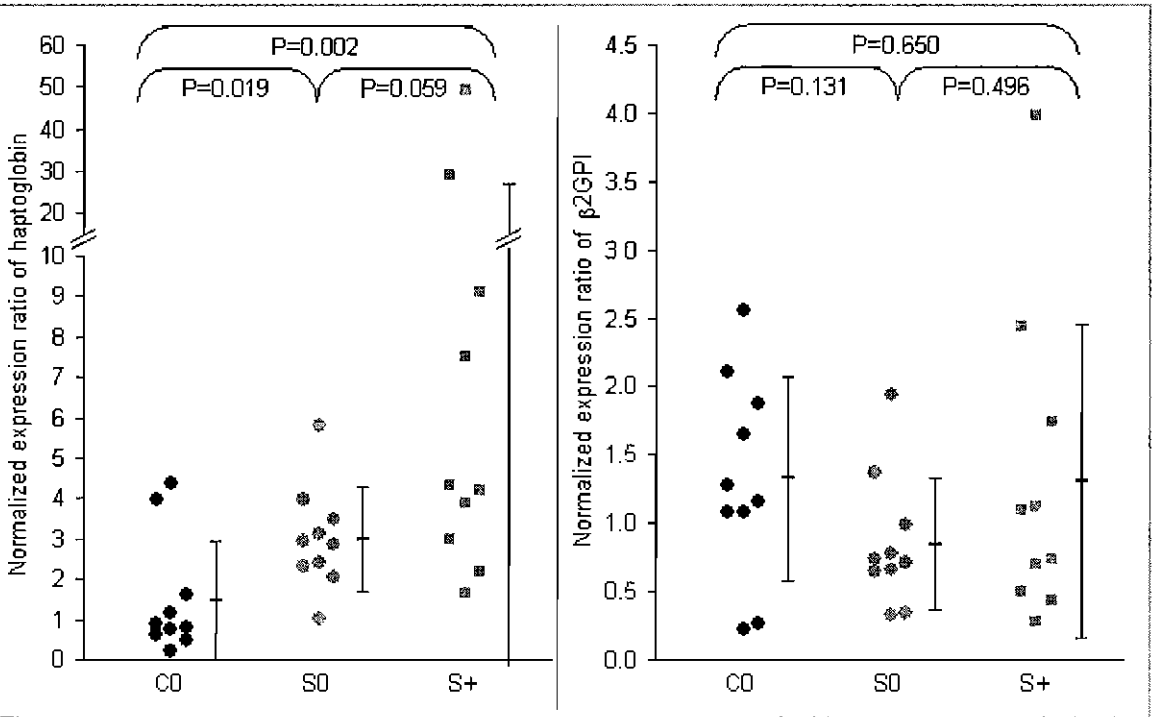


Figures 5.10, 5.11 & 5.12: Non-normalized mean expression level of haptoglobin, β2GPI, and GAPDH. C0: control mice, S0 and S+ surgically treated mice respectively without and with Danazol® medication.

However, such variations could not be attributed exclusively to the differences in transcript expression since technical variations (especially regarding RNA loads) could not be considered as rigorously equivalent between tested samples. In order to circumvent this inconvenient, the expression of both investigated transcripts was normalized versus GAPDH expression. As can be seen from the following

figures (5.13 & 5.14), the expression of β2GPI and haptoglobin seemed to be regulated in a divergent way. Not only did the expression of haptoglobin rise in reaction to the surgical treatment (2.0-fold increase when compared to controls; P=0.0191) but it increased even further when CLP was accompanied by Danazol® treatment (7.7-fold increase when compared to controls; P=0.0025). By contrast, the expression of β2GPI seemed to decline in reaction to the surgical treatment (1.6-fold decrease when compared to controls). However, this decline was not found to be statistically significant (P=0.1306). Interestingly, the administration of Danazol® was found to be accompanied by the resumption of β2GPI expression (back to the same level as controls). The normalized β2GPI expression ratio between Danazol®-treated and control mice presented a difference of only 1.9 % and was not statistically significant (P=0.6501).

The normalized expression of haptoglobin mRNA 6 hours post CLP matched plasma protein concentration (results not shown). In fact, there was an identical logarithmical relationship[10] between the expression ratio of haptoglobin mRNA and plasma concentration in placebo versus control, Danazol®-treated versus control and Danazol®-treated versus placebo.



Figures 5.13 & 5.14: Ratio of expression level of haptoglobin and β2GPI normalized by GAPDH expression (using the actual mean real-time amplification efficiency rate). C0: control mice, S0 and S+: surgically treated mice 6 hours post CLP without and with Danazol® respectively.

[10] This relationship could be empirically determined as log (A/B * 4) / 0.7, where A/B represents placebo/control or Danazol®-treated/placebo data for haptoglobin plasma concentration or mRNA normalized expression.

5.5 Discussion and conclusion

In patients with severe sepsis or septic shock, a series of systemic and tissue-based reactions that are in part attributed to the action of acute phase proteins (APP) are induced and may lead to abnormalities in coagulation and fibrinolysis. Systemic activation of coagulation together with early activation and subsequent inhibition of fibrinolysis are common features of sepsis and septic shock ([Voss *et al*, 1990; Fourrier *et al*, 1992; Lorente *et al*, 1993; Kidokoro *et al*, 1996; Vervloet *et al*, 1998]). The most extreme clinical manifestation of these alterations is disseminated intravascular coagulation, which is a frequent complication of sepsis with major implications for morbidity and mortality ([Fourrier *et al*, 1992]).

Some evidence indicates that in severe sepsis, an adequate hepatic acute phase response is important for survival ([Dominioni *et al*, 1987]). Accordingly, modulations of this response by therapeutic strategies may have some benefit and improve the outcome of sepsis, reducing morbidity and aiding survival and recovery ([Patti *et al*, 2005a&b]). In this regard, APP are known to affect the binding of several drugs to proteins, and hence their distribution in blood and their therapeutic availability in tissues and organs ([Kremer *et al*, 1988; Tagawa *et al*, 1994; Son *et al*, 1996]).

The fates of haptoglobin and β2GPI were shown to be radically different from each other. As expected ([Bauman *et al*, 1994; Majno *et al*, 1996; Gabay *et al*, 1999]), haptoglobin was found to be uniformly increased in surgically treated mice at the levels of both mRNA and plasma protein production. On the contrary, β2GPI presented lower normalized mRNA production in mice with sepsis in comparison to controls, but this decrease did not reach statistical significance. The number of mice used in this study is relatively small and examining figure 5.14, it is possible that a larger number of studied mice would have shown a significant reduction in β2GPI induced by inflammation. However, it is clear from figures 5.13 and 5.14 that in mice, haptoglobin and β2GPI behave in opposite ways following an inflammatory stimulation, i.e. that in mice, the mRNA for haptoglobin appears to rise and the mRNA for β2GPI appears to fall. These findings support our observation made in chapter 3, which showed a negative correlation between β2GPI level and the degree of inflammation and suggest that at least some of the reduction in the observed level of β2GPI is due to diminished synthesis. In addition, these findings are in line with other studies, which showed that β2GPI behaves as a negative APP ([Mehdi *et al*, 1991; Sellar *et al*, 1993]). However, Sellar *et al.* ([1994]) have also shown no change in β2GPI mRNA level during the early acute phase response in mice ([Sellar

et al, 1994), thus suggesting that β2GPI may not be a true negative APP, at least not as clearly as albumin and transferrin. Methodological differences may partially explain the differences between the findings of Sellar *et al.*'s study and our own findings. Interestingly, this study also suggests that anabolic steroids are able to enhance APP synthesis and may have a role as therapeutic agent in augmenting acute phase responses. This is the subject of a separate research project.

6 General discussion

6 General discussion

Atherosclerosis in general, and atherothrombosis in particular, are multifactorial chronic processes that result from intricate interactions between different cellular elements (including macrophages, lymphocytes, endothelial cells, and smooth muscle cells) and serum-derived molecules (such as inflammatory proteins, lipoproteins, antibodies, complement and coagulation factors). The pathogenesis of these processes is generally accepted as being mediated in part by inflammatory and immunological reactions ([Libby *et al*, 1991; Hansson, 1993; Ross, 1993 & 1999; Wick *et al*, 1995]) against a background of inherited and acquired risk factors (appendix A) (figure 6.1). The hypothesis that β2GPI may have a significant role to play in the aetiology of atherothrombosis has been developed in several studies, and our results tend to corroborate this suggestion.

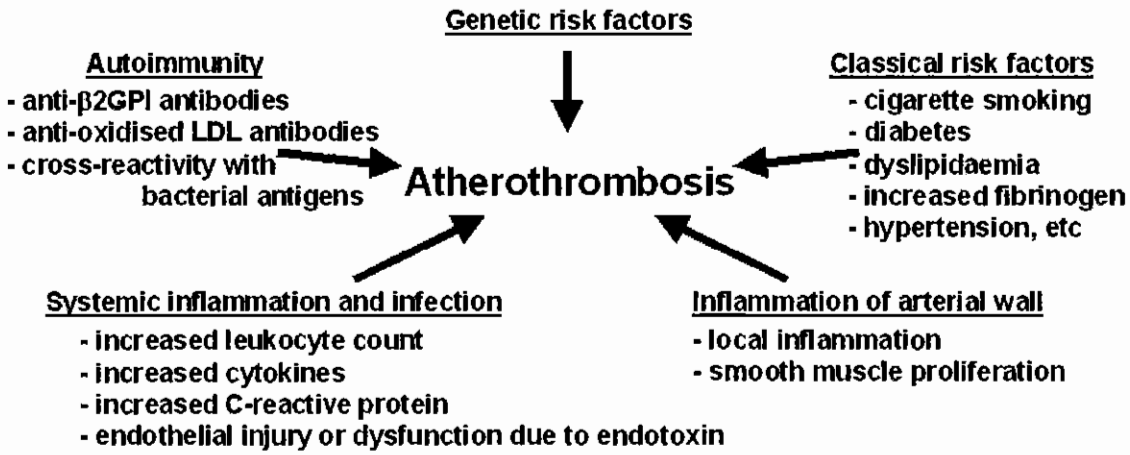


Figure 6.1: Postulated mechanisms linking inflammation, autoimmunity, and classical and genetic risk factors with vascular diseases.

Atherosclerosis is an inflammatory disorder...

Although the association between markers of inflammation and atherosclerosis is not entirely understood, there is growing evidence to indicate a key role for pro-inflammatory factors in the pathogenesis of coronary artery disease and other manifestations of atherosclerosis.[1] The most convincing evidence that atherosclerosis is an inflammatory process, and not merely a corollary of deposits of lipids in the arterial wall, is the consistent cellular infiltration and accumulation of monocyte-derived macrophages and T lymphocytes in fatty streaks and advanced atherosclerotic lesions. Such cellular invasion is known to result in a chronic inflammatory process that is characterised by an increased production of pro-inflammatory cytokines ([Ridker *et al*, 2000]). This process leads to increased oxidative

[1] Numerous publications on that matter have been published: Hansson *et al*, 1989; van der Wal *et al*, 1994; Ridker *et al*, 1997 & 1998a&b; Frostegård *et al*, 1999, Ross, 1999; Glass *et al*, 2001; Libby *et al*, 2002a&b; Leitinger, 2003

capacity ([Kramer *et al*, 1995; Chisolm *et al*, 1999]) that causes peroxidation of lipids, which induces the expression of chemotactic agents and increased expression of endothelial adhesion molecules for monocytes and lymphocytes, thereby further potentiating the vascular inflammatory reaction ([Frostegård *et al*, 1991 & 1993; Klouche *et al*, 1999; Shih *et al*, 1999]).

... with evidence of autoimmune reactivity

An oxidative modification of LDL[2] has been incriminated in foam-cell formation through the uptake of this modified LDL by the unregulated scavenger receptors on macrophages ([Steinberg *et al*, 1989; Witztum *et al*, 1994]), but also in the formation of many neo-antigens ([Palinski *et al*, 1990]) and hence in the induction of autoimmune responses. T lymphocyte clones derived from atherosclerotic plaques have been shown to be reactive against oxidatively modified LDL ([Frostegård *et al*, 1992; Stemme *et al*, 1995]), and an elevated level of circulating autoantibodies to these self-proteins is associated with active atherosclerotic processes ([Salonen *et al*, 1992; Puurunen *et al*, 1994; Wu *et al*, 1997]). These autoantibodies, which enhance the accumulation of LDL into macrophages *in vitro* ([Lopes-Virella *et al*, 1997]), constitute a heterogeneous group of autoantibodies with respect to their specificity.

Potential role of β2GPI in the atherosclerotic process

β2GPI and aPL/anti-β2GPI antibodies may potentially fill a gap in this complex jigsaw of interconnected cells, antigens and antibodies. On the one hand, not only has β2GPI been shown in numerous independent studies to bind to major constituents of atherosclerotic plaques (such as endotheliocytes, oxidatively modified LDL or apolipoprotein (a), see chapter 1.5.3), but the protein has also been shown *in vivo* to be incorporated into these atherosclerotic lesions ([George *et al*, 1999b]) and *in vitro* to prevent endocytosis of oxidized LDL by macrophages via scavenger receptors ([Hasunuma *et al*, 1997]). On the other hand, aPL/anti-β2GPI antibodies have been shown to cross-react with oxidized LDL ([Vaarala *et al*, 1993; Hörkkö *et al*, 1997 & 2000; Wu *et al*, 1999]) and to increase its uptake by macrophages when in the presence of β2GPI ([Hasunuma *et al*, 1997]), and the presence of these autoantibodies is strongly associated with the presence of anti-oxidized LDL antibodies ([Becarevic *et al*, 2005]) and enhanced early atherosclerosis ([George *et al*, 1998b]). In addition, some sub-populations of autoantibodies specifically directed against endothelial cells have

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[2] Modifications of LDL by other mechanisms than oxidation, such as aggregation, glycation, immune complex formation, proteoglycans complex formation or conversion to cholesterol-rich liposomes may also potentiate its atherogenic nature ([Hazell *et al*, 1996; Torzewski *et al*, 1998b; Tabas, 1999]).

been shown to cross-react with oxidized LDL and β2GPI (Wu *et al*, 1999) and to be associated with early atherosclerosis (Frostegård *et al*, 1998). Recently, circulating oxidized LDL/β2GPI complexes and IgG antibodies to these complexes have been associated with arterial thrombosis in patients with SLE and the APS (Lopez *et al*, 2005 & 2006). These observations thus indirectly support the concept that β2GPI displays anti-atherogenic properties in active atherosclerotic plaques, and that it may also serve as an important target antigen for an immune-mediated attack.

β2GPI behaves as a negative acute phase protein

One of the most important observations that emerges from this thesis is that β2GPI behaves as a negative acute phase protein. Its level is reduced during acute inflammation (Lin *et al*, 2006) and in patients with ACS and stroke. Furthermore, our observations from the mouse model of inflammation support the concept that synthesis of β2GPI is reduced during inflammation. How this reduction in serum β2GPI concentration could have an impact on individuals with inflammation or thrombosis is an open question. It is also unclear whether the reduction in β2GPI is due to decreased synthesis, increased consumption or both.

β2GPI serum concentration: a cause, a consequence, or both?

Compared to age-matched controls, elderly patients with stroke and acute coronary syndrome presented a lower serum β2GPI concentration that did not change during follow-up over six months after the thrombotic event. Several hypotheses can be drawn from these observations:

- First, these patients suffered from a thrombotic event that was in part related to a decreased concentration in serum β2GPI. The observed reductions in β2GPI in this case were thus causative.
- Second, the decreased concentration of β2GPI measured after the stroke event was induced by the stroke event itself, and the reduction was thus in this case consequential.
- Third, other mechanisms, independent from the thrombotic event, might also decrease serum β2GPI concentration. For instance, because β2GPI presents a particular tropism for atherosclerotic lesions, and since the burden of atherosclerosis in elderly individuals can be expected to be high compared to younger counterparts (Benditt *et al*, 1994), it is conceivable that elderly patients with stroke and acute coronary syndrome had β2GPI that was in some ways "consumed" by the atherosclerotic lesions distributed throughout their circulatory system. In this case, the observed reductions in serum β2GPI level

would thus indicate a pre-existing consumptive condition, and were coincidental to the thrombotic event.

At present, there is no argument to exclude the possibility that the aforementioned non-exclusive hypotheses are all relevant and actually occur concomitantly *in vivo*. This could be one of the causes for the large range of β2GPI concentrations observed in the general population (and diseased groups) as the amplitude of these mechanisms can vary from one individual to another. Although a low level of serum β2GPI concentration in elderly patients with acute coronary syndrome tends to support the "consumptive" hypothesis (given their high atherosclerotic burden ([Benditt *et al*, 1994])), and although the absence of difference in serum β2GPI concentration between young patients with acute coronary syndrome and age-matched controls tends to exclude the "consequential" hypothesis, our (retrospective) study, as it has been designed, does not allow to exclude either hypothesis, and the question remains open.

However, we believe that β2GPI may have a significant beneficial role in maintaining prothrombotic atherosclerotic lesions under control through its anti-atherogenic and anticoagulant properties. As atherosclerotic lesions progress with age, β2GPI may be under increasing demand and thus produced in greater quantities, hence the more elevated β2GPI level observed in healthy elderly individuals. In an exacerbated atherosclerotic environment, β2GPI may be steadily and continuously moved towards lesions and its function influenced by autoimmune-mediated reactions, resulting in lowered quantities of efficient β2GPI. This might generate an additional predisposing element for thrombosis.

Concurrent compensatory mechanisms regulating β2GPI concentration

Some concurrent compensatory phenomena could also take place and significantly modulate serum β2GPI level. Such phenomena include adsorption, *de novo* synthesis or release of β2GPI by specific cells (such as endotheliocytes ([Caronti *et al*, 1999])) or from complexed forms (including any β2GPI-containing complexes, such as lipoprotein-β2GPI and β2GPI-containing immune complexes). For reasons that remain to be clarified, the effectiveness of these compensatory mechanisms could vary a lot between young and elderly individuals, and thus be a cause of the difference in serum β2GPI concentration observed between young and elderly controls. Moreover, one cannot exclude the importance of other

haemostatic regulatory pathways that may also either compensate or exacerbate the prejudiced situation related to modulated β2GPI concentration.

Atherosclerosis, complement and β2GPI

Although there is evidence of complement activation in atherosclerotic lesions (Hansson *et al*, 1984; Vlaicu *et al*, 1985; Niculescu *et al*, 1987; Torzewski *et al*, 1998a&b; Yasojima *et al*, 2001a&b), little is known about how complement is activated. The most common assumption is that, following the diffusion of complement components from the serum into the arterial wall, the complement system is activated through circulating immune complexes (Niculescu *et al*, 1987), immunoglobulin directed against altered arterial structures (Hansson *et al*, 1984; Niculescu *et al*, 1987), modified oxidized LDL deposits (Torzewski *et al*, 1998b) or CRP (Volanakis, 1982; Wolbink *et al*, 1996; Torzewski *et al*, 1998a; Bhakdi *et al*, 1999; Nijmeijer *et al*, 2003). CRP has been reported to opsonise native LDL (Pepys *et al*, 1985) and to enhance its uptake by macrophages (Hatanaka *et al*, 1995; Zwaka *et al*, 2001). However, the exact ligand mediating such binding is still unknown. Although phosphocholine, a constituent of the phospholipids of cell membranes and plasma lipoproteins, is often mentioned in the literature as the major ligand for CRP (Volanakis *et al*, 1971 & 1979; Thompson *et al*, 1999), β2GPI could represent another ligand of CRP on LDL within atherosclerotic plaques.[3] CRP has indeed been shown to present a calcium-independent binding capacity for cationic polymers (DiCamelli *et al*, 1980; Potempa *et al*, 1981), and β2GPI, with its high content in lysine and arginine residues, can be considered as one. Furthermore, CRP, which is known to bind sugar moieties (such as on microbes) (Ganrot *et al*, 1969; Heidelberger *et al*, 1972; Pepys *et al*, 1977), could possibly also adhere to the high carbohydrate content of β2GPI (estimated to approximately 20% of the protein's molecular weight). Thus, not only can the binding of β2GPI to atherosclerotic elements and the subsequent binding of aPL/anti-β2GPI antibodies (or other cross-reacting antibodies) to β2GPI influence the functional properties of cellular components within the atheroma and result in fixation and activation of complement (Davis *et al*, 1992; Stewart *et al*, 1997; Odorczuk *et al*, 1999; Pierangeli *et al*, 2005), but the potential binding reactivity of CRP molecules to β2GPI could also represent a mechanism through which the former opsonise to native LDL (Zwaka *et al*, 2001) and thereby activate complement.

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[3] In the same line of thought, Gershov *et al*, (2000) reported that CRP could bind to the surface membrane of apoptotic cells and subsequently promote their clearance but did not characterise the ligand for CRP on these cells. β2GPI, which avidly binds to membranes of senescent/apoptotic cells (Price *et al*, 1996; Balasubramanian *et al*, 1997; Levine *et al*, 1998; Pittoni *et al*, 2000), could represent a ligand for CRP on these cells.

Using mRNA and protein-based methods, it was previously shown that CRP and complement components are not only secreted from atherosclerotic plaques, but also at a higher level than from the liver (Yasojima *et al*, 2001b). From these observations, it was proposed that the dramatic increase in plasma CRP concentration observed following a thrombotic event (e.g. a heart attack or a stroke) might result from the release of CRP from injured tissue rather than from an increased liver synthesis. This would in part explain the mild but persistent increase in CRP concentration that seems to predict a subsequent thrombotic event,[4] as it would be related to the atheromatous burden in the vasculature. This assumption, if β2GPI actually interacts with CRP, would also explain the decrease in serum β2GPI concentration observed in elderly patients with stroke and acute coronary syndrome as opposed to younger patients since more β2GPI molecules in the former would form complexes with CRP. This assumption could also partially explain the negative correlation between β2GPI and CRP concentrations observed in patients with inflammatory disorders.

Mutations in the β2GPI (APOH) gene associated with lowered serum β2GPI concentration

In this study, we also confirmed that two mutations in the β2GPI (APOH) gene locus, namely at codons 306 and 316, were associated with lower serum β2GPI concentration, and this in an additive fashion. It is worth noting that the mutation at codon 306 was about twice as frequent in patients with thrombotic disorders than in healthy subjects.[5] This suggests a role for β2GPI deficiency in the pathogenesis of hypercoagulable states. Pushing this reasoning further, we can hypothesize that in old age, functional deficiency of β2GPI (whether congenital or acquired) could contribute to a prothrombotic tendency when it occurs together with conditions associated with increased generation of procoagulant surfaces and/or exhausted, yet to be identified, compensatory mechanisms. This partially contradicts two previous studies (Bancsi *et al*, 1992; Takeuchi *et al*, 2000) that reported that congenital β2GPI deficiency *per se* does not seem to be strictly associated with thrombotic disorders. However, the characteristics of the populations tested in these studies are different from ours, especially since they studied fewer individuals. Furthermore, even though there are examples of β2GPI-deficient individuals

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[4] Numerous publications on that matter have been published: Mach *et al*, 1997, Ridker *et al*, 1998a&c & 2000; Koenig *et al*, 1999; Lloyd-Jones *et al*, 2003.

[5] For data from all patients (with stroke, ACS, RFL and other thrombotic disorders) grouped together (P=0.0394; OR=1.829; 95%CI: 1.022-3.273).

without thromboembolic complications (Bancsi *et al*, 1992; Takeuchi *et al*, 2000), the subjects reported in the other studies were all in their thirties. We cannot exclude that the absence of β2GPI in their serum might represent a significant risk factor if additional prothrombotic threats occur, especially in older age.

Thrombotic diseases and β2GPI genetics

Concerning the potential link between the presence of the mutations in the β2GPI (APOH) locus and the occurrence of thrombotic diseases, we observed that the relative risk for ACS was a little less than twice as high among carriers of the Gly^{306} allele as among carriers of the Cys^{306} allele (P=0.0470; OR: 1.859; 95%CI: 1.000-3.457). This risk was a little less than twice as low among heterozygotes at position 247 as among $Val^{247}Val$ homozygous subjects (P=0.0088; OR=0.646; 95%CI: 0.466-0.896). However, in the latter case, this observation was not confirmed in homozygous carriers of the mutation (in the former case, no individual with such genotype was observed). It is possible that when these β2GPI variants are present together with other concurrent genetic and conventional risk factors, they significantly modify prothrombotic susceptibility.

No definite association between mutations in the β2GPI (APOH) gene and the presence of ACLA and/or anti-β2GPI antibodies

aPL have been implicated in atherogenesis (Vaarala *et al*, 1995; Levine *et al*, 1997; Wu *et al*, 1997) particularly with ischaemic cerebrovascular disease (Asherson *et al*, 1989b; Briley *et al*, 1989; Levine *et al*, 1990; APASS, 1993; Zielinska *et al*, 1999) although there is controversy on that matter (Ginsburg *et al*, 1992; Sletnes *et al*, 1992; Ahmed *et al*, 2000). Similarly to Hess *et al.* (1991a), Montalban *et al.* (1991), and de Jong *et al.* (1993), we found in this study that most of our elderly patients with stroke had both IgG and IgM ACLA below the cut-off value of the manufacturer for positivity. Moreover, we did not find any correlation between IgG ACLA and anti-β2GPI antibody titres, in contradiction to a previous report (Martinuzzo *et al*, 1995), but the small numbers of patients tested for anti-β2GPI antibodies precluded the observed difference from being statistically significant. There was no significant difference either in the levels of ACLA or anti-β2GPI antibodies among genotypes for any of the four tested mutations. This part of our study with patients with stroke thus does not reinforce the finding made in patients with SLE that carriers of the Ser^{316} mutation could be protected from production of aPL (Kamboh *et al*, 1999a), and the observation made in patients with the APS that the Val^{247} mutation seems to be associated with anti-β2GPI auto-reactivity (Atsumi *et al*,

1999; Hirose *et al*, 1999; Yasuda *et al*, 2000a & 2005; Prieto *et al*, 2003).[6] However, as stated above, the number of individuals we tested for aPL and anti-β2GPI antibodies is probably too small to obtain reliable results.

The fact that we did not observe any definite association between the studied mutations and the presence of ACLA and/or anti-β2GPI antibodies suggests that none of the β2GPI variants and their presumed resulting conformational changes are specifically associated with auto-reactivity. This implies that mechanisms other than simply the expression of variant forms of the molecule are involved in the induction of an aPL and/or an anti-β2GPI reactivity. Added to the absence of correlation between IgG ACLA and β2GPI concentrations and to the positive correlation between IgG ACLA level and the age of the stroke patients, these findings also suggest that ACLA may arise from mechanisms that are not related to serum β2GPI concentration. However, they do not exclude the possibility that β2GPI activity can be altered by these aPL, when present, without any demonstrable variation in the serum protein concentration. Furthermore, the generation of aPL directed against β2GPI may independently influence the development of atherothrombosis by altering the haemostatic balance towards hypercoagulation. However, our findings on the relationship between β2GPI polymorphisms of β2GPI and the presence of absence of aPL and/or anti-β2GPI antibodies need to be interpreted with caution due to the low number of subjects studied and also to the fact that they are limited to healthy subjects and individuals with stroke.

[6] Care must be taken when comparing data across ethnic groups, particularly when major disparities exist in prevalence of given mutations. For instance, Val^{247} is the most common allele in Caucasians, while Leu^{247} is the most common one in Asians.

7 Conclusion and future work

7 Conclusion

A number of interesting conclusions can be derived from this study:

1. It is possible to measure circulating level of β2GPI in a reliable way using an ELISA assay.
2. β2GPI behaves as a negative acute phase protein.
3. Patients with stroke and acute coronary syndrome have lowered levels of serum β2GPI.
4. β2GPI polymorphisms may contribute to genetic risk of stroke and acute coronary syndrome.
5. The association between the mutations at codons 306 and 316 and lowered serum β2GPI was confirmed.
6. In a mouse model of sepsis, hepatic synthesis of β2GPI is reduced.

In summary, we have produced significant new evidence supporting a role of β2GPI in inflammation and common thrombotic disorders.

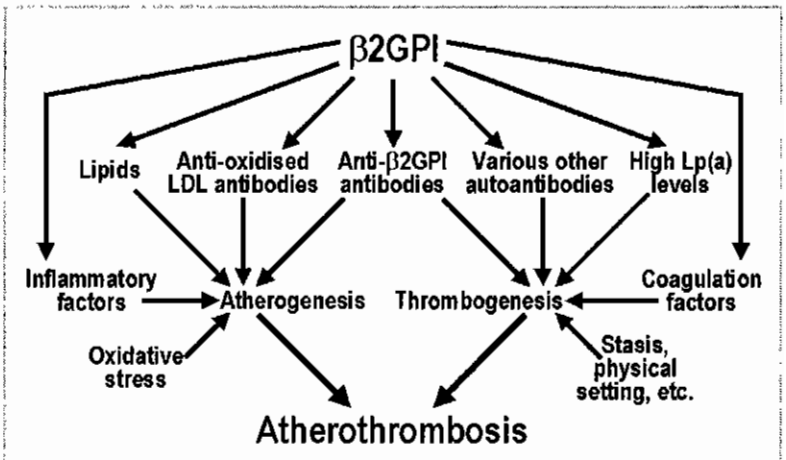

Figure 7.1: Central position of β2GPI and anti-β2GPI antibodies among atherogenic and thrombogenic factors.

It is clear that genetic variation, differences in exposure to environmental influences and the mass of inflammation-producing tissue (e.g. atherosclerotic plaques) can all influence responses to an inflammatory or a thrombotic event. The importance of β2GPI as an antigen for certain aPL, and its role in the pathogenesis of aPL-associated clinical manifestations via mechanisms involving immune complexes, particularly at locations such as atherosclerotic plaques, lead us to suggest that β2GPI is at the crossroads of inflammation, autoimmunity, and atherosclerosis (figure 7.1).[1] A better understanding of these mechanisms may contribute to novel therapeutic strategies to decrease the morbidity and mortality of thrombotic disorders potentially associated with β2GPI.

[1] By analogy with the APS, which has been previously described as the "crossroads of autoimmunity and atherosclerosis" (George *et al*, 1997b).

Future work

Our sampling of hospitalised patients with thrombotic and inflammatory disorders has enabled us to highlight selective areas in which more extensive investigation and larger serial studies may be of clinical benefit. To fully comprehend the exact function of β2GPI in normal and disease states, especially thrombotic disorders, it is clear that further studies are required. The following paragraphs summarise some of the future works that would be interesting to undertake.

1. Since all tested individuals in this study were exclusively Irish citizens, our findings require confirmation in other ethnic groups. A larger prospective study testing individuals with predisposing risk factors for thrombosis and patients with common thrombotic conditions similar to stroke and acute coronary syndrome (ACS) is also required to fully establish which of our two hypotheses is true (with a lower serum β2GPI concentration as a cause or a consequence of the thrombotic conditions) and whether a reduction in serum β2GPI concentration actually represents a risk factor for atherosclerosis and/or thrombosis. Knowing that the level of plasma proteins can fluctuate very rapidly and radically during a disease process, such a prospective study should pay particular attention to time intervals between blood samplings around the occurrence of a thrombotic event.

Since premature atherosclerosis is a considerable clinical problem in patients with SLE (Manzi *et al*, 1997; Ward, 1999; Bruce *et al*, 2000; Roman *et al*, 2001 & 2003; Asanuma *et al*, 2003), and patients with lupus have substantially increased morbidity and mortality from cardiovascular diseases (Jonsson *et al*, 1989; Manzi *et al*, 1997; Ward, 1999; Doria *et al*, 2005), it would also be worth measuring serum β2GPI level in larger-scale prospective groups of such patients to determine the validity of the "consumptive" hypothesis. This would be especially interesting with regard to the commonly associated presence of aPL and anti-β2GPI antibodies in the serum of these patients. A number of case-control studies have previously tested serum β2GPI level in patients with lupus (Cohnen, 1970; Ichikawa *et al*, 1992, Kamboh *et al*, 1999a; Matsuda *et al*, 1993a; McNally *et al*, 1995b) but the reported findings were contradictory, possibly because the tested groups or individuals were at different stages of the atherosclerotic process.

A similar analysis in patients with venous thrombosis would also add to the understanding of the potential role of β2GPI in prothrombotic diseases, as generally speaking thrombi arising in veins are more localised and much larger than those arising in arteries. A previous study reported no decrease in circulating

β2GPI in patients with either venous or arterial thrombosis ([Brighton *et al*, 1996]), but that study involved only 21 (4 venous and 17 arterial) thrombotic patients, half of whom were receiving thrombolytic therapy while being tested. A new, larger-scale study would thus be very valuable.

2. To date, β2GPI binding to CRP has never been tested. Although highly speculative, the potential binding reactivity between the two proteins with subsequent activation of the complement system is worth being elucidated. If actually taking place, such β2GPI-CRP complex formation could take place in two non-exclusive locations: in the circulatory system and within CRP-containing tissues such as atherosclerotic plaques ([George *et al*, 1999b]). A number of parameters, including the avidity and rate of binding of β2GPI to CRP, may significantly modulate gross serum β2GPI concentration. The observation of many individuals with significantly elevated levels of both serum β2GPI and CRP allows us to postulate that, as our in-house ELISA seems to be suitable for the detection of β2GPI in its putative CRP-complexed form, there seems to be neither a lack of specificity for (supposedly) CRP-bound β2GPI nor interference of complexed CRP on the detection of β2GPI. If there is an actual increased binding of β2GPI to tissue-based CRP (accompanied or not by an increased serum CRP level), the detection of lowered β2GPI by our in-house ELISA would represent a major indicator of the consumptive hypothesis, and possibly a risk marker for thrombosis.

3. It is unfortunate that we did not manage to fully optimise duplex real-time RT-PCR measurements of β2GPI and haptoglobin mRNAs. A greater understanding of β2GPI's origin would be possible if this experiment was repeated and brought to completion, as well as if tissues representing potential sources of β2GPI other than liver were tested (for instance endothelia from atherosclerotic lesions and normal arteries for comparison).

4. Concerning the association between a reduced serum β2GPI concentration and the mutations at codons 306 and 316, our study reinforces previous reports ([Ruiu *et al*, 1997; Sanghera *et al*, 1997b; Kamboh *et al*, 1999a; Mehdi *et al*, 1999]) without establishing the exact strength of this association. Since it has been proposed that the two mutations are not functional but are rather in linkage disequilibrium with one or several other functional mutation(s) that are yet to be identified - possibly located in the

promoter region (Mehdi *et al*, 2003), further studies should focus on these other mutations[2] that might directly affect the synthesis of the protein.

An interesting parallel can be drawn between current knowledge of β2GPI and protein C deficiency. Although the heterozygous state of protein C deficiency is relatively common (1 in 60 healthy adults), it is generally not associated with a history of thrombotic disease (Miletich *et al*, 1987). However, within kindred, virtually all protein C-deficient individuals eventually experience thrombosis (Bovill *et al*, 1989, Tabernero *et al*, 1991). Even though severe β2GPI deficiency is relatively rare, in order to establish whether (genetic or acquired) β2GPI deficiency is actually an additional risk factor for atherosclerosis and thrombosis, especially in the long term, further investigation is needed on the haemostasis of clotting-triggered plasma from subjects with a significantly lowered serum β2GPI concentration of various ages, both carriers and non-carriers of mutations at codons 306 and 316, and from their relatives.

A weakness of our analysis of β2GPI genetics in patients with stroke and ACS was the difficulty to obtain DNA samples from age-matched controls. Although we obtained some relevant results using healthy subjects from the general population as controls, these individuals cannot be considered as absolutely exempt from a risk of thrombosis. It would be interesting to compare our findings with genuinely healthy elderly age-matched controls.

If the studied mutations in the β2GPI (APOH) gene are in linkage disequilibrium with an actual disease-causing polymorphism, one cannot exclude that the degree of linkage (disequilibrium) may vary from population to population. This may explain some of the discrepancies observed between studies that have found the same gene variations to be risk factors for the production of aPL/anti-β2GPI antibodies. Better estimates of the prevalence of these antibodies in the general population and in individuals at increasing risk of thrombosis are therefore needed. As a start, it would be interesting to add the measurements of ACLA and anti-β2GPI antibodies in the above-mentioned prospective studies.

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[2] Very recently, DNA sequence variations in the entire β2GPI (APOH) gene encompassing a 20.3 kb region have been reported with a total of 150 single nucleotide polymorphisms (SNPs) and one tri-allelic polymorphism identified (Chen *et al*, 2006).

5. The above-mentioned suggestions do not obliterate a number of other aspects of β2GPI that have not been tackled in this study and remain to be studied, particularly structural β2GPI modifications including cleavage (Hagihara *et al*, 1997; Ohkura *et al*, 1998; Horbach *et al*, 1999; Matsuura *et al*, 2000; Shi *et al*, 2005), glycosylation (Gambino *et al*, 1997 & 1999b; de Laat *et al*, 2006), and oxidation (Arvieux *et al*, 2001; Buttari *et al*, 2005), as well as β2GPI's interaction with lipids and its role in lipid/lipoprotein metabolism. Furthermore, knowing that infectious agents are potential contributing factors to autoimmunity and atherosclerosis[3] and that β2GPI binds protein from pathogenic organisms, it also seems justified to consider that infections could provide a possible stimulus for the production of aPL/anti-β2GPI antibodies. Infections may indeed induce the processing of exogenous epitopes that share structural similarities with native β2GPI (Blank *et al*, 2002, 2004a&b), which may result in an effective presentation of peptide-MHC complexes that stimulate auto-reactive T cells (Luo *et al*, 1993) and thereby break immune tolerance (Mamula *et al*, 1992).[4] Further studies are also required on the role of cellular immunity against β2GPI in the progression of atherosclerosis (Visvanathan *et al*, 1999; Hattori *et al*, 2000; Ito *et al*, 2000; Yoshida *et al*, 2002; Kuwana *et al*, 2004 & 2005).

[3] Numerous papers have been published on that matter: Lopes-Virella *et al*, 1985; Danesh *et al*, 1997; Libby *et al*, 1997; Epstein *et al*, 2000 & 2002; Espinola-Klein *et al*, 2002a&b.

[4] Some anti-β2GPI antibodies that were found to recognize epitopes on β2GPI have been shown to cross-react with native epitopes present in viruses, bacteria, and parasites (Blank *et al*, 2002); rabbit immunization with lipids found on the outer surface of gram-negative bacteria have been shown to induce β2GPI-dependent ACLA and LA (Goloh *et al*, 1996); and mice immunization with synthetic viral and bacterial peptides analogous to the putative phospholipid binding site of the 5th domain of β2GPI has led to the production of ACLA and anti-β2GPI antibodies (Gharavi *et al*, 1999 & 2002).

Appendices

Appendix A. Classical and genetic risk factors

Table A1: Most commonly referenced classical risk factors for thrombosis[1]

Category	Risk factors
Personal	(Advanced) age
	(Male) gender[A]
	Family history and race[2A]
	Personal history: previous thrombotic event, pregnancy/puerperium[V], post-operative state[V], trauma
	Socio-economic class
Lifestyle	Cigarette smoking[A]
	Alcohol consumption and illegal drug abuse[A]
	(Imprudent) diet - obesity
	Sedentary lifestyle - lack of exercise - excess of stress[A] / immobility[V]
	Synthetic oestrogen (oral contraceptive, hormone replacement therapy)[V]
Physiological	High blood lipid levels (cholesterol, triglycerides, lipoprotein (a), etc)[A]
	Hormonal status (menstruation, menopause)[V]
	Coagulation factors (such as a high fibrinogen level)
	Elevated blood pressure[A]
	Underlying diseases (see below)

Most of the classical risk factors are common to both arterial and venous thromboembolic diseases, but some are more specific to one vascular system than to the other. ([A]): arterial, ([V]): venous.

[1] Compiled data from Anderson *et al*, 1991; Gerstman *et al*, 1991; Bendiff *et al*, 1994; Creager, 1994; Rubin *et al*, 1994; Carter, 1996; Kraft *et al*, 1996; Rabkin, 1996; Danesh *et al*, 1998; van Lennep *et al*, 2002

[2] Genetics as part of racial and family backgrounds is considered as a classical risk factor.

Table A2: Candidate gene variations predisposing to thrombotic disease[1,2&3]

	Polymorphism	Action
Procoagulant factors		
Factor II	G→A 20210	Altered level but unclear
Factor V	G→A 1691 (Arg506Gln = factor V Leiden and HR2 haplotype)	Resistance to activated protein C inactivation of factor Va
Factor VII	Various mutations (e.g. Arg353Gln)	Altered level
Factor XIII (α-subunit)	G→T (Val34Leu)	Increased enzyme activity rate
Fibrinogen (α-chain)	Thr312Ala	Altered clot stability
Fibrinogen (β-chain)	Several different mutations	Altered level
Tissue factor	Several polymorphisms	Altered level
Anticoagulant proteins		
Antithrombin III	>250 different mutations	Altered level (deficiency)
Protein C	>160 different mutations	Altered level (deficiency)
Protein S	>130 different mutations	Altered level (deficiency)
Tissue factor pathway inhibitor	4 different mutations	Altered level
Anticoagulant proteins / endothelial receptors		
Endothelial cell protein C/activated PROC receptor	23-bp repeat insertion	Altered expression on cell surface (predicted)
Thrombomodulin	Several mutations	Unclear
Fibrinolytic proteins		
Tissue-type plasminogen activator	Numerous mutations (e.g. the Alu insertion/deletion)	Unclear
Anti-fibrinolytic proteins		
Plasminogen activator inhibitor-1	Various mutations	Altered level
Platelet membrane glycoproteins		
Glycoprotein Ia/IIa complex	Several mutations among which C→T 807 and G→A 873	Altered surface expression of receptor and collagen receptor activity
Glycoprotein Ib/V/IX complex	Various mutations among which a variable number tandem repeat and C→T 3550 (Thr145Met)	Unclear
Glycoprotein IIb/IIIa complex	Several mutations including Ile843Ser for glycoprotein IIb and T→C 1565 (Leu33Pro) for glycoprotein IIIa	Increased sensitivity to activation
Vascular homeostasis		
Angiotensin II receptor 1	C→A 1166	Unknown
Angiotensin-1-converting enzyme	Insertion/deletion polymorphism	↑ Angiotensin-1-converting enzyme
Angiotensinogen	M235T	↑ Angiotensinogen
Endothelial nitric oxide synthase	A/b	Unknown
Metabolic factors		
Cysthationine-β-synthase	Various mutations	Altered level resulting in ↑ homocysteine
Methylene-tetra-hydrofolate reductase	C→T 677 (Ala226Val)	Altered level resulting in ↑ homocysteine
Lipids and associated factors		
Apo (a)	Various mutations	↑ Lipoprotein (a)
Apo AI-CIII-AIV	Few mutations	↓ HDL, ↑ triglyceride
Apo AII	Various mutations	↓ HDL
Apo B100	Few mutations	↑ LDL, ↑ VLDL
ApoE (C1, CII)	Various mutations	↑ LDL, ↑ VLDL
Cholesteryl ester transfer protein	KIV repeats	↓ HDL
Hepatic lipase	D9N	↓ HDL
Lecithin cholesteryl acyl-transferase	Few mutations	↓ HDL
Lipoprotein lipase	Several mutations (including Asn291Ser, Asp9Asn, Gly188Glu)	↓ HDL, ↑ triglyceride
Low-density lipoprotein receptor	Various mutations	↑ LDL
Paraoxonase	Gln92Arg	Increased enzyme activity rate

[1] Compiled data from De Stefano *et al*, 1996; Marian, 1998; Rosendaal, 1999; Lane *et al*, 2000; Franco *et al*, 2001; Reiner *et al*, 2001

[2] A number of these *genetic* risk factors are common to both arterial and venous thromboembolic diseases, but most of them seem to be specific to one vascular system rather than to both.

[3] ↑/↓: increased / reduced plasma concentration.

Appendix B. Antiphospholipid antibodies (aPL)

Antiphospholipid antibodies (aPL) are extremely heterogeneous circulating autoantibodies that cross-react with the majority of the negatively charged phospholipids[1] (Harris *et al*, 1985; Gharavi *et al*, 1987; Pengo *et al*, 1987; Loizou *et al*, 1990) and that show reactivity to a number of phospholipid-binding proteins, among which β_2-glycoprotein I (β2GPI) (Galli *et al*, 1990; McNeil *et al*, 1990), vitamin K-dependent proteins (such as prothrombin, protein C, protein S and thrombomodulin) (Puurunen *et al*, 1996; Galli *et al*, 1999; Roubey, 1999), on occasions DNA (Lafer *et al*, 1981; Rauch *et al*, 1984), and possibly annexin II (Ma *et al*, 2000) and V (Rand *et al*, 1997, 1998, 1999 & 2000), factor X, high molecular weight kininogen, factor XI, and the protein core of heparan sulfate (Shibata *et al*, 1994c).

Serum-derived aPL have been commonly identified in a variety of clinical settings including autoimmune conditions (particularly systemic lupus erythematosus (SLE),[2] the antiphospholipid syndrome (APS, appendix C), Sjögren's syndrome, mixed connective tissue disease, rheumatoid arthritis, systemic sclerosis, ankylosing spondylitis, and Crohn's disease), as well as non-autoimmune conditions, in particular acute and chronic infections[3] (Loizou *et al*, 1996), lymphoproliferative diseases, and during the administration of drugs[4] (McNeil *et al*, 1991). aPL associated with thrombosis, thrombocytopenia, and recurrent foetal loss are usually of the IgG isotype, show high persistent titres and are cross-reactive with all negatively charged phospholipids. In contrast, aPL appearing secondary to infection and drug exposure tend to be of the IgM isotype, are present at lower transient titres and are generally not cross-reactive with other phospholipids (Gharavi *et al*, 1987; Levy *et al*, 1990; Bernard *et al*, 1990; McNeil *et al*, 1991).

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[1] such as cardiolipin, phosphatidic acid, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, and phosphatidylserine.

[2] Selection of articles on SLE-associated aPL: Harris *et al*, 1983; Asherson *et al*, 1989b&c; Khamashta *et al*, 1990; Long *et al*, 1991; Abu-Shakra *et al*, 1995, Aho *et al*, 1996; Horbach *et al*, 1996; Swadzba *et al*, 1997

[3] due to viruses (e.g. HIV-1 (Naimi *et al*, 1990; Sorice *et al*, 1994; Constans *et al*, 1998), hepatitis A virus (Colaco *et al*, 1989), hepatitis B virus (Martinuc Porobic *et al*, 2005), hepatitis C virus (Munoz-Rodriguez *et al*, 1999b; Cojocaru *et al*, 2005), B19 parvovirus (Colaco *et al*, 1989), cytomegalovirus (Mengarelli *et al*, 2000), ruebella (Colaco *et al*, 1989), etc.), to bacteria (e.g. *Treponema pallidum* (Pedersen *et al*, 1987; Loizou *et al*, 1990; Versalovic *et al*, 1990; Hunt *et al*, 1992; Bernard *et al*, 1990), Streptococcus, Salmonella, E.*coli* (Vaarala *et al*, 1988) etc.), or to parasites (e.g. *Plasmodium* (Facer *et al*, 1994)).

[4] such as hydralazine, phenothiazines (Zarrabi *et al*, 1979; Canoso *et al*, 1988; Lillicrap *et al*, 1990), phenytoin, quinidine, and procainamide (Merrill *et al*, 1997).

Appendix C. International consensus statement on preliminary criteria for the classification of the antiphospholipid syndrome (APS)

The antiphospholipid syndrome (APS) is an autoimmune disease defined as the occurrence of recurrent venous and/or arterial thromboses, and/or recurrent foetal loss frequently accompanied by a moderate thrombocytopenia in the presence of antiphospholipid antibodies (aPL), namely lupus anticoagulant (LA), anticardiolipin antibodies (ACLA), or both (Hughes *et al*, 1983 & 1986; Asherson *et al*, 1989a; Sammaritano *et al*, 1990; Cervera *et al*, 2002). The syndrome is called primary APS when it occurs without underlying disorder (Asherson *et al*, 1989a), and secondary APS in patients with coexistent diseases such as autoimmune diseases (particularly SLE) or malignant diseases (Asherson *et al*, 1989a; Vianna *et al*, 1994). At the present time, the APS is considered a common cause of acquired hypercoagulability in the general population and a major cause of morbidity in pregnancy (Hochfeld *et al*, 1994; Lockshin, 1999; Cervera *et al*, 2002). A diagnosis of definite APS requires the presence of at least one of the clinical criteria and at least one of the laboratory criteria in the following lists.[1]

Clinical criteria

- Vascular thrombosis
  - One or more clinical episodes of arterial, venous, or small-vessel thrombosis, occurring within any tissue or organ.
- Complications of pregnancy
  - One or more unexplained deaths of morphologically normal foetuses at or after the 10th week of gestation; or
  - One or more premature births of morphologically normal neonates at or before the 34th week of gestation (due to severe preeclampsia, eclampsia or placental insufficiency); or
  - Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation (with maternal anatomic or hormonal abnormalities and parental chromosomal causes excluded).

Laboratory criteria[2]

- Anticardiolipin antibodies (ACLA)
  - IgG or IgM ACLA present at moderate or high levels in the blood on two or more occasions at least six weeks apart, measured by standardized β2GPI-dependent ACLA ELISA (Harris *et al*, 1990b & 1998).
- Lupus anticoagulant antibodies (LA)
  - LA detected in the blood on two or more occasions at least six weeks apart, according to the guidelines of the International Society on Thrombosis and Haemostasis (Brandt *et al*, 1995).

[1] No limits are placed on the interval between the clinical event and the positive laboratory findings. However, it is recommended that an aPL test be positive on at least two occasions more than six weeks apart.

[2] Two methods have been used to characterise aPL: solid-phase immunoassays, which use microtitre plates coated with phospholipid, most commonly cardiolipin (hence, the term "anticardiolipin antibody (ACLA)" used to name any antibody detected via solid-phase immunoassays) (Harris *et al*, 1983, Gharavi *et al*, 1987), and functional coagulation assays, which identify aPL with *in vitro* lupus anticoagulant (LA) activity (i.e., an inhibitory effect on *in vitro* phospholipid-dependent coagulation tests (Shapiro *et al*, 1981, Pengo *et al*, 1987)). Despite the frequent concordance between ACLA and LA activity, the two are not identical (Exner *et al*, 1988; McNeil *et al*, 1988 & 1989; Galli *et al*, 1992b), and the more general term "antiphospholipid antibodies (aPL)" has often been used to denominate these autoantibodies. Although they have been detected in many patients with the APS, the following antibodies are currently not included in the laboratory criteria (Pengo *et al*, 2005): IgA ACLA, anti-β_2-glycoprotein (β2GPI) antibodies, aPL directed against phospholipids other than cardiolipin (e.g., phosphatidylserine and phosphatidylethanolamine) (McNeil *et al*, 1991; McIntyre *et al*, 2000) and against phospholipid-binding proteins other than cardiolipin-bound β2GPI (e.g., prothrombin, annexin V, protein C, or protein S) (Roubey, 1996; Galli, 2000), as well as anti-mitochondrial (M5 type), anti-endothelial cell, anti-platelet, anti-erythrocyte, and anti-nuclear antibodies.

Appendix D. Clinical manifestations associated with the APS

The clinical picture of the APS is characterised by venous and arterial thromboses ([Munoz-Rodriguez *et al*, 1999a]), obstetrical complications ([Faden *et al*, 1997; Cervera *et al*, 2002]), thrombocytopenia ([Munoz-Rodriguez *et al*, 1999a; Cervera *et al*, 2002; Amoroso *et al*, 2003]), cardiovascular abnormalities ([Hojnik *et al*, 1996; Nesher *et al*, 1997; Cervera *et al*, 2002]), central nervous system manifestations ([Levine *et al*, 1987 & 1990; Zielinska *et al*, 1999; Cervera *et al*, 2002; Katzav *et al*, 2003]), and osteoarticular, cutaneous and ophthalmologic manifestations ([Cervera *et al*, 2002; Durrani *et al*, 2002]). Single vessel or multiple vascular occlusions may give rise to a wide variety of presentations. Any combination of vascular occlusive events may occur in the same individual, and the time interval between them varies considerably from weeks to even years. Rapid chronological occlusive events, occurring over days to weeks, have been termed the "catastrophic" APS ([Asherson *et al*, 1996 & 2000; Triplett *et al*, 2000]).

Table D1: Cumulative clinical features during the evolution of disease in 1,000 patients with the APS ([Cervera *et al*, 2002 ; Asherson *et al*, 2003]).

Peripheral thrombosis (total: 63.7 %) Deep vein thrombosis (38.9 %) Superficial thrombophlebitis in the legs (11.7 %) Arterial thrombosis in the legs (4.3 %) Venous thrombosis in the arms (3.4 %) Arterial thrombosis in the arms (2.7 %) Other (2.7 %)	**Cardiac manifestations (total: 26.9 %)** Valve thickening / dysfunction (11.6 %) Myocardial infarction (5.5 %) Myocardiopathy (2.9 %) Angina (2.7 %) Vegetations (2.7 %) Coronary bypass rethrombosis (1.1 %) Intracardiac thrombus (0.4 %)
Neurologic manifestations (total: 65.8 %) Migraine (20.2 %) Stroke (19.8 %) Transient ischaemic stroke (11.1 %) Epilepsy (7.0 %) Multi-infarct dementia (2.5 %) Chorea (1.3 %) Acute encephalopathy (1.1 %) Transient amnesia (0.7 %) Cerebral venous thrombosis (0.7 %) Cerebellar ataxia (0.7 %) Other (0.7 %)	**Pulmonary manifestations (total: 19.7 %)** Pulmonary embolism (14.1 %) Pulmonary hypertension (2.2 %) Pulmonary microthrombosis (1.5 %) Fibrosing alveolitis (1.2 %) Other (0.7 %)
Osteoarticular manifestations (total: 68.2 %) Arthralgia (38.7 %) Arthritis (27.1 %) Avascular necrosis of the bone (2.4 %)	**Intraabdominal manifestations (total: 6.9 %)** Renal manifestations (2.7 %) Gastrointestinal manifestations (1.5 %) Splenic infarction (1.1 %) Pancreatic infarction (0.5 %) Hepatic manifestations (0.7 %) Addition's syndrome (0.4 %)
Cutaneous manifestations (total: 39.6 %) Livedo reticularis (24.1 %) Leg ulcers (5.5 %) Pseudovasculitic lesions (3.9 %) Digital gangrene (3.3 %) Cutaneous necrosis (2.1 %) Splinter haemorrhages (0.7 %)	**Obstetric manifestations (n = 590 pregnant women) (total: 16.4 %)** Preeclampsia (9.5 %) Abruptio placentae (2.0 %) Postpartum cardiopulmonary syndrome (0.5 %)
Haematologic manifestations (total: 39.3 %) Thrombocytopenia (29.6 %) Haemolytic anaemia (9.7 %)	**Foetal manifestations (n = 1,580 pregnancies)** Embryonic loss (<10 weeks) (35.4 %) Foetal loss (≥10 weeks) (16.9 %) Premature birth (n = 753 live birth) (10.6 %)
	Ophthalmologic manifestations (total: 8.8 %)
	Ear, nose, and throat manifestations (total: 0.8 %)

Appendix E. CCP superfamily

β2GPI is a non-complement member of the complement control protein (CCP) or short consensus repeats (SCR) superfamily characterised by repeating CCP modules. β2GPI has five of these modules. Each module is composed of some 60 amino acids with the cysteine residues arranged in a characteristic C1-3, C2-4 disulphide bonding pattern ([Janatova *et al*, 1989; Kato *et al*, 1991; Steinkasserer *et al*, 1992a]). The CCP superfamily comprises more than 140 complement[1] and non-complement[2] proteins with the number of SCR motifs in each member varying from one to up to 30 (in the case of complement receptor 1) ([Kristensen *et al*, 1987; Reid *et al*, 1989; Norman *et al*, 1991; Day *et al*, 1992; Bork *et al*, 1996]) (Figure E1). Little information is available on the structural properties of SCR in general. Intriguingly, most of the members of the CCP superfamily that are involved in the control of C3b and/or C4b or in the proteolytic activation of the complement system contain the usual four conserved cysteine residues, while the members involved in cell adhesion (GMP-140, ELAM-1, MEL-14) contain six conserved cysteine residues ([Reid *et al*, 1989]).

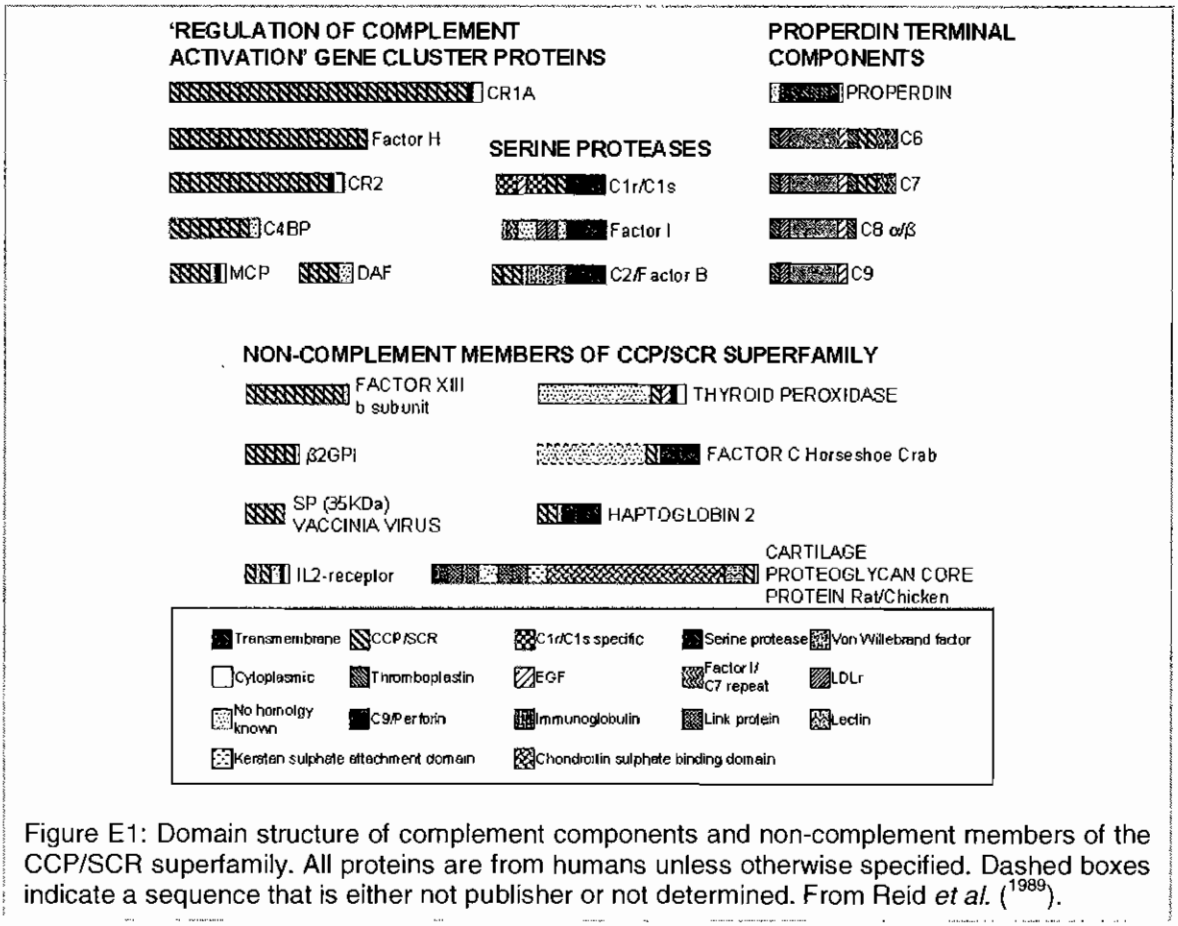

Figure E1: Domain structure of complement components and non-complement members of the CCP/SCR superfamily. All proteins are from humans unless otherwise specified. Dashed boxes indicate a sequence that is either not publisher or not determined. From Reid *et al.* ([1989]).

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[1] Examples of complement members include C1s ([MacKinnon *et al*, 1987, Tosi *et al*, 1989; Hess *et al*, 1991b]), C2 ([Ishii *et al*, 1993]), factor B ([Mole *et al*, 1984; Campbell *et al*, 1987]), factor H ([Vik *et al*, 1988; Ripoche *et al*, 1988]), C4b-binding protein (α and β chains) ([Rodriguez *et al*, 1991; Hillarp *et al*, 1993]), complement receptor 1 (CR1, CD35) ([Wong, 1990; Vik *et al*, 1993]) and 2 (CR2, CD21) ([Fujisaku *et al*, 1989]), decay accelerating factor (DAF, CD55) ([Post *et al*, 1990]), membrane cofactor protein (MCP, CD46) ([Lublin *et al*, 1988; Liszewski *et al*, 1991]), C6 and C7 ([Hobart *et al*, 1995]).

[2] Example of non-complement members include interleukin-2 receptor ([Leonard *et al*, 1985]), granule membrane protein (GMP-140) ([Bevilacqua *et al*, 1989, Johnston *et al*, 1989]), coagulation factor XIII (β subunit) ([Bottenus *et al*, 1990]), thyroid peroxidase ([Kimura *et al*, 1989]), haptoglobin (α-chain) ([Kurosky *et al*, 1980; Bork *et al*, 1996]), lymph node homing receptor (MEL-14, leukocyte adhesion molecule 1) ([Siegelman *et al*, 1989a&b; Tedder *et al*, 1989]), and endothelial leukocyte adhesion molecule 1 (ELAM-1) ([Bevilacqua *et al*, 1989; Collins *et al*, 1991]).

Appendix F. β2GPI-ELISA materials and reagents

Table F1: Reagents for the β2GPI ELISA

Reagent	Manufacturer
Disodium carbonate (Na_2CO_3)	BDH (Laboratory Supplies, Poole, UK)
Sodium hydrogen carbonate ($NaHCO_3$)	BDH
Sodium chloride (NaCl)	BDH
Potassium chloride (KCl)	Merck
Disodium hydrogen phosphate (Na_2HPO_4)	Merck
Potassium dihydrogen phosphate (KH_2PO_4)	BDH
Tween®20 (polyoxyethylene sorbitan monolaurate)	Sigma Chemical C° (St Louis, MO, USA)
1,2-phenylenediamine dihydrochloride (OPD)	Dako A/S (Glostrup, Denmark)
30% Hydrogen peroxidase (H_2O_2)	BDH
Sulphuric acid (H_2SO_4)	BDH

Table F2: Plastic sources

Plate	Manufacturer	Catalogue#	Lot #
Nunc F96 MaxiSorp™	Nunc A/S (Roskilde, Denmark)	4-42404	40205 40985 42576 47444 49001 52953
Nunc F96 MaxiSorp™ certified	Nunc A/S	4-39454	49091 61830
Immunolon™ 1	Dynatech Laboratories (Chantilly, VA, USA)	011-010-3350	CM510120
Immunolon™ 2	Dynatech Laboratories	011-010-3455	CS520636

Table F3: ELISA antibodies

Antibody	Manufacturer	Catalogue#	Lot #	Quantity
Mouse monoclonal anti-human β2GPI	Chemicon International Inc. (Temecula, CA, USA)	MAB 1066	18090602 19040694 19091017 19100196 20030997 20031232 21041262 21060663 22031533	100 µg at 1 mg/mL
Horseradish peroxidase-conjugated rabbit polyclonal anti-human β2GPI	Dako A/S	PE 854	053 1003 1203	1 mL at 1.3 g/L
Rabbit polyclonal anti-human β2GPI	Dako A/S	O 9152	066 (101-4)	1 mL at 0.6 g/L
Horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins	Dako A/S	P 0217	037	2 mL at 1.3 g/L
Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins	Dako A/S	P 0161	107	2 mL at 1.3 g/L
Horseradish peroxidase-conjugated swine anti-mouse immunoglobulins	Dako A/S			

Table F4: Other antibodies

Antibody	Manufacturer	Catalogue#	Quantity
Mouse anti-human CD3-FITC labelled	Becton Dickinson (Cowley, Oxford, UK)	349201	100 µg/mL
Mouse anti-human CD14-FITC labelled	Becton Dickinson	347493	25 µg/mL
Mouse anti-human CD14-PE labelled	Becton Dickinson	347497	50 µg/mL
Mouse anti-human HLA DP-pure	Becton Dickinson	347730	25 µg/mL
Mouse anti-human HLA DR -PE labelled	Becton Dickinson	347367	12.5 µg/mL

Table F5: Blocking agents

Reagent	Manufacturer	Catalogue#
Bovine albumin liquid *	DiaMed (Cressier, Switzerland)	E106000
Bovine albumin solid **	Sigma	A-7906
Chicken albumin **	Sigma	A-5253
Fish skin gelatin *	Sigma	G-7765
Gelatin powder **	Merck	4078.0500
Gelatin **	Sigma	G-7765
Carrageen **	Sigma	C-3889
Glycine **	Sigma	G-7126
Casein ***	Sigma	C-5890

* : diluted directly in phosphate-buffered saline (PBS)
**: purified protein powders dissolved in PBS
***: 2 g of purified casein powder in 100 mL water while adding 0.1 M NaOH dropwise to maintain pH around 7.0 (between 6.8 and 7.2). When pH was stabilized, the suspension was stirred overnight at room temperature, then filtered through standard-grade paper. This saturated casein (turbid) solution contained about 1.5% protein.

Table F6: Other reagents

Reagent	Manufacturer	Catalogue#	Lot #	Quantity
Apolipoprotein H	Crystal Chem Inc. (Chicago, Illinois, USA)		PQ-8	1 mg at 8.1 mg/mL
Apolipoprotein H	Calbiochem-Novabiochem Ltd (Nottingham, UK)	362225	B25650	100 µg
Cardiolipin	Sigma	C1649	21K5210	10 mg

Table F7: Test related to acute phase proteins

Reagent	Manufacturer	Catalogue#
Multi-species serum amyloid A EIA kit	Tridelta Development Limited (Wicklow, Republic of Ireland)	TP-802

Appendix G. Optimization of the β2GPI ELISA protocol

The extraordinary affinity, specificity and variety of antibody-antigen binding reactions have made immunoassays one of the most widespread analytical procedures, used for routine applications and for research purposes throughout biological and medical sciences since the end of the '60s ([Gosling, 2000]). The number of available immunoassay formats is astonishing. The choice depends on the nature and amount of samples that is to be tested, on the availability of reagents, and on the required specificity, sensitivity, speed and cost. A common configuration when reagents are used in excess is the enzyme-linked immunosorbent assay (ELISA), which commonly uses polystyrene as solid-phase support. In order to measure the quantity of β2GPI in serum samples using commercially available monoclonal and polyclonal anti-human β2GPI antibodies, we have designed a sandwich capture ELISA in which, for convenience, the polystyrene solid support surface was coated by direct passive adsorption. All the parameters of this β2GPI ELISA needed to be optimised to improve assay consistency and reproducibility. Among these parameters, several were found to be critical, especially the brand and batch of the plastic support, the coating antibody concentration, and the blocking step.

G.1 Developing the "initial protocol": direct rather than indirect sandwich assay

Since a directly labelled polyclonal anti-β2GPI antibody was available, both direct (figure 3.1) and indirect (figure G1) sandwich β2GPI ELISA protocols were compared.

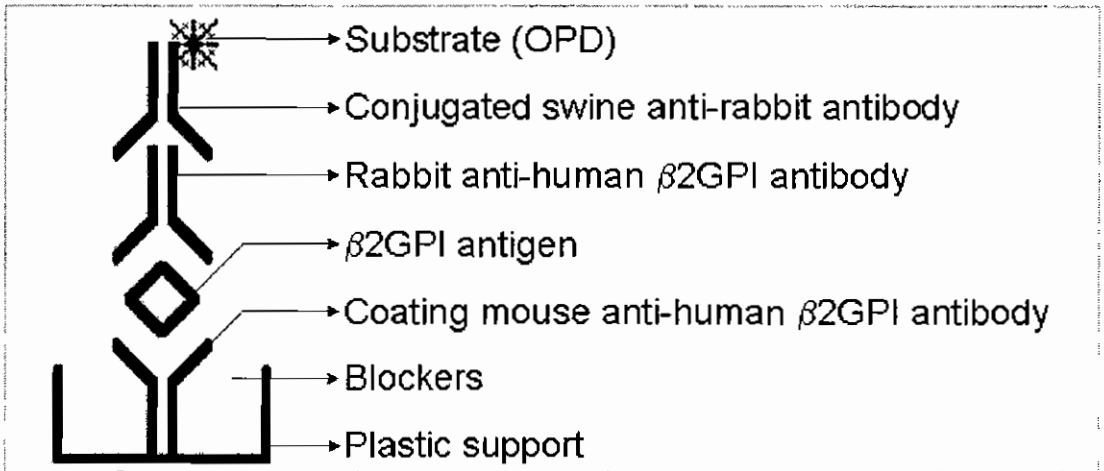

Figure G1: Diagram illustrating the succession of reagents in the β2GPI indirect ELISA. Mouse monoclonal anti-β2GPI antibodies are attached to the solid phase by passive adsorption. After washing, serum samples or standards containing β2GPI antigen are added. After an incubation period of an hour and washing, secondary rabbit polyclonal anti-β2GPI antibodies are added, and let for incubation for an hour. After washing, the detecting swine polyclonal enzyme-labelled anti-rabbit immunoglobulin antibodies are added. Following another incubation period of an hour and washing, the OPD/H_2O_2 substrate is added. Colour is allowed to develop an then stopped with sulphuric acid and measured spectrophotometrically.

The indirect sandwich test protocol involved two antibodies after the addition of the serum samples on the plate: a rabbit polyclonal anti-human β2GPI antibody (Dako A/S, Glostrup, Denmark) as the secondary antibody, followed by a horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin antibody (Dako A/S) as the revealing

antibody. On the other hand, the direct sandwich test protocol involved just one antibody after the addition of the serum samples on the plate: a directly labelled horseradish peroxidase-conjugated rabbit polyclonal anti-human β2GPI antibody (Dako A/S, Glostrup, Denmark). All these antibodies were used at a dilution of 1 in 1,000 (0.6, 1.3 and 1.3 µg/mL respectively) in PBST, and were added to the plate following a washing step with PBST. For optimum comparison, both protocols were tested on the same plate with the same standard and control serum samples.

Slight differences were found between the overall ranges and shape of the constructed standard curves from the direct and the indirect protocols, even after adjusting the duration of the last OPD/H_2O_2 substrate step. However, these differences were not statistically significant as there was strong correlation between the straight portions of both standard curves ($r=0.9983$, $P<0.001$). Moreover, the detection limits of both protocols were similar, as was the range of the differences between the β2GPI concentration of the three control samples estimated from the respective standard curves. Background binding was slightly lower with the direct protocol. The indirect sandwich assay protocol did not significantly increase the sensitivity of the assay. Since a directly labelled rabbit polyclonal anti-β2GPI antibody was readily available, hence allowing a more rapid procedure with no significant additional cost, we subsequently only used the direct protocol for optimization. This direct protocol is referred to as "initial protocol" in the rest of this chapter.

G.2 Potential cross-reactivity with phospholipids

β2GPI is known to bind negatively charged surfaces avidly, including phospholipids ([Polz *et al*, 1979b; Schousboe, 1983b; Wurm, 1984]), with a significant proportion of plasma β2GPI being conjugated to lipoproteins in the bloodstream ([Polz *et al*, 1979c]). There was a risk that such binding might significantly affect the accuracy of the β2GPI assay, especially if the antibodies that were used did not recognise the analyte in its bound form. We therefore tested whether cardiolipin (CL, Sigma), used as a source of phospholipids, could influence the β2GPI assay. This was performed by pre-incubating various dilutions of CL up to a

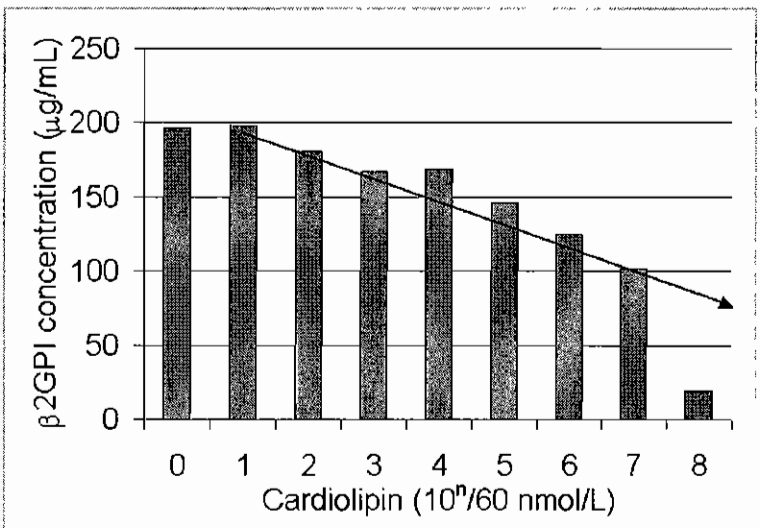

Figure G2: Cardiolipin test. β2GPI concentration measured in a serum with an intermediate level of β2GPI using the β2GPI ELISA protocol with an additional pre-incubation step with cardiolipin at increasing concentrations. Bars represent the mean of three measurements.

final concentration of 1.7 mmol/L in a serum sample with an estimated intermediate level of β2GPI. The system thus reached a molar CL/β2GPI ratio equivalent to 1,350,000/1. Since the commercialised CL (composed of >80% polyunsaturated fatty acid, primarily linoleic acid) is dissolved in an ethanol solution, we also controlled the effect of ethanol on the assay in a separate experiment.

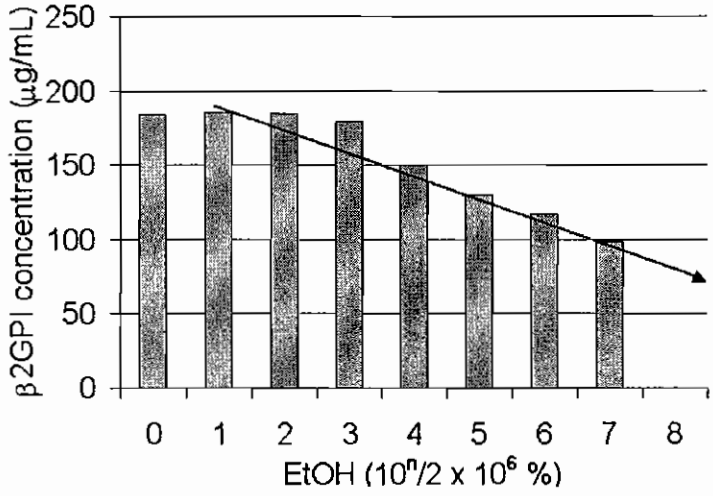

Figure G3: Ethanol test. β2GPI concentration measured in a serum with an intermediate level of β2GPI using the β2GPI ELISA protocol with an additional pre-incubation step with ethanol alone at increasing concentrations. Bars represent the mean of three measurements.

Up to a concentration of 16.67 nmol/L of CL (equivalent to 14 molecules of CL per molecule of β2GPI), no significant perturbation could be seen, as shown in figure G2. Above that concentration, further increases in CL concentration gradually lowered β2GPI detection. However, this relative declining effect fully correlated with the quantity of ethanol in which CL was diluted (figure G3).

G.3 Potential interference with serum anti-β2GPI antibodies

As only a small number of the patients were tested positive for anti-β2GPI antibodies (see chapter 3) and since it has been demonstrated that there is no difference between β2GPI concentration in patients that are positive for anti-β2GPI IgG antibodies and those than are negative (Martinuzzo *et al*, 1995), this potential source of variation was not systematically investigated.

G.4 Specificity of commercially available anti-β2GPI antibodies

Only one source of coating mouse monoclonal anti-human β2GPI antibody (Chemicon International Inc.) was used in this study. The specificity of this antibody for β2GPI had been previously defined and positively referred to in several publications (Mehdi *et al*, 1999; Kamboh *et al*, 1999a); it had also been used satisfactorily by Jan Guerin (PhD) (who tested its specificity by Western-blotting). We had thus no hesitation in using that product. It showed strong stability and high availability throughout the project. Nine lots of the antibody were used, and all worked satisfactorily.

As regards the specificity of the horseradish peroxidase (HRP)-conjugated and the non-conjugated rabbit polyclonal anti-human β2GPI antibodies, two experiments were performed. Firstly, the conjugated antibody was tested on isoelectric focusing (IEF)-

immunoblotting and gave IEF-immunoblotting patterns that corresponded to those published by Kamboh *et al.* ([1988]) (see chapter 4) Secondly, a series of experiments were performed using an indirect sandwich assay and both antibodies. The non-conjugated anti-human β2GPI antibody was added at various dilutions just after the serum sample step and incubated for one hour. The conjugated anti-human β2GPI antibody was then added, revealing any remaining β2GPI antigen not "covered" by the non-conjugated anti-human β2GPI antibody. From this experiment, it appeared that such pre-incubation significantly inhibited the conjugated antibody binding. It must be added here that, as tested with "antigen blanks" (i.e. all reagents present without β2GPI antigen, none of the two polyclonal antibodies added on coated plates in PBST could adsorb to the plate surface or bind to the capture antibody. Thus, in other words, the specificity of the two polyclonal antibodies for human β2GPI was confirmed and their non-specific adsorption onto the solid phase or onto coated plates was virtually negligible.

G.5 Nature of the plastic support and plate brand

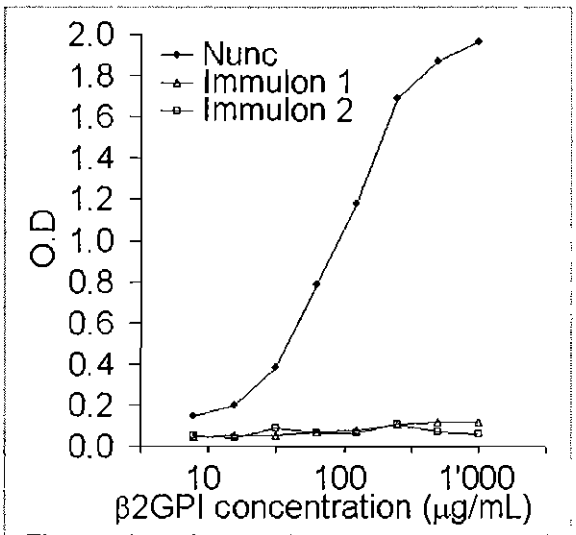

Figure G4: Standard curves constructed using the "initial" direct sandwich β2GPI ELISA protocols on three different plastic plates: Nunc A/S' F96 MaxiSorp™, and Dynatech Laboratories' Immulon 1 and Immulon 2. Points represent the mean of three measurements.

Using the "initial protocol", several brands of 96-flat bottom-well polystyrene ELISA plates from two different manufacturers (appendix F) were tested as potential solid support for the β2GPI assay. A sample of each brand was assessed by analysing the precision with which a standard curve of the β2GPI assay could be reproduced. As can be seen in figure G4, Dynatech plates did not allow proper binding of the coating antibody. Moreover, when signal was detectable, plate-to-plate variation was considerable. On the contrary, Nunc A/S plates always allowed proper coating of the capture antibody, and plate-to-plate variation was significantly reduced when using these plates, especially γ-irradiated Nunc MaxiSorp™ plates. These plates were selected primarily for economical reasons, as they were commonly used for various routine immunoassays in the Department of Immunology, St James's Hospital, Dublin. They consist of a highly charged polystyrene surface with a high affinity to molecules with mixed hydrophilic/hydrophobic domains such as proteins, like globular antibodies ([Esser, 1997d]). Thus, this brand of γ-irradiated plates offered very high sensitivity in double antibody sandwich tests. They subsequently proved to be perfectly suitable for the β2GPI ELISA, as soon as batches were checked for consistency in adsorption of protein.

G.6 Non-specific binding of β2GPI to the plastic support

With the "initial protocol", the assay showed reasonably good accuracy and reproducibility for a period of time. A normal range for β2GPI concentrations, measured with sera from one hundred healthy individuals, was consistent (in mean value and range) with values found in the literature. However, a certain degree of variation was observed from time to time. All materials and reagents were checked, but none was tagged responsible for this variability. With the "initial protocol", the assay was actually subjected to non-specific binding of some β2GPI antigens present in serum samples. Although the assay was able to differentiate sera with low β2GPI concentration from others with high β2GPI content, this differentiation was not the result of the sole specific recognition of the β2GPI antigen by the coating antibody, but was primarily due to the non-specific binding of β2GPI present in these serum samples onto the solid surface. The more β2GPI antigens in the sample, the more antigens bound to the plate and were subsequently detected by the conjugated antibody. This non-specific binding rendered the "initial protocol" more susceptible to artefacts, such as the blocking behaviour of other protein constituents potentially present in the serum or the possibility that different β2GPI variants might react differently with the solid support. It is also possible that the non-specific adsorption of β2GPI might result in some denaturation of the protein that could reveal unexpected "non-native" antigenic determinants (Friguet *et al*, 1984). These artefacts may render result interpretation uncertain.

G.7 Testing of blockers

In order to overcome non-specific binding of β2GPI to the plastic support, it was decided to add a blocking step just after the overnight coating step to obstruct possible vacant solid support surface. Serial dilutions from 1.0% of five protein-based blocking substances (chicken albumin (Sigma), casein (Sigma), gelatine (Merck and Sigma), carrageen (Sigma), and glycine (Sigma)) were added onto uncoated and coated plates to test their blocking capacity and/or reactivity with the β2GPI assay reagents. Despite testing numerous dilution protocols (especially for the non water-soluble casein) in various combinations with coated and uncoated plates, none of these five tested blockers were considered suitable as blocking agent for the β2GPI assay. Either they were not able to properly block non-specific binding of β2GPI to the plate or they not only blocked non-specific binding of β2GPI but also interfered with the specific recognition of β2GPI by the coating antibody.

Bovine serum albumin (BSA) was also tested as a potential blocking substance but it had to be eliminated since some commercial preparations of BSA have been shown to present significant amounts of bovine β2GPI (Roubey, 1994; personal comment from R. Sim, MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford)

that presents remarkable homology with human β2GPI (Steinkasserer *et al*, 1991; Matsuura *et al*, 1991).

Detergents, especially of a non-ionic type like Tween®20 (polyoxyethylene sorbital monolaureate), can also be used as stable blocking agents (Batteiger *et al*, 1982; Wedege *et al*, 1986; Esser, 1997b; Esser, 1997c; Matson, 2000) without affecting antigen-antibody interactions when used at a concentration lower than 1% (Dimitriadis, 1979). Following previous recommendations (Esser, 1997b; Mohammad *et al*, 1988),[1] we circumvented the absence of a protein-based blocker by fully saturating the plate with the coating antibody and by using Tween®20 in all dilution and washing solutions at a final concentration of 0.05%.

G.8 Binding rate of the mouse monoclonal anti-β2GPI antibody

In order to measure how well a Nunc MaxiSorp™ ELISA plate could be coated by the capture mouse monoclonal anti-human β2GPI antibody, horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin antibodies at a dilution of 1 in 1,000 (1.3 µg/mL) were added following a washing step on plates initially coated with an increasing concentration of the coating mouse monoclonal anti-β2GPI-antibody. As can be seen in figure G5, the antibody started to be fully detectable when coated at a minimum of 0.05 µg/mL. However, since the OD value at that coating concentration exceeded the technical limit of the apparatus, the concentration of 0.05 µg/mL did not indicate whether the plate was fully saturated.

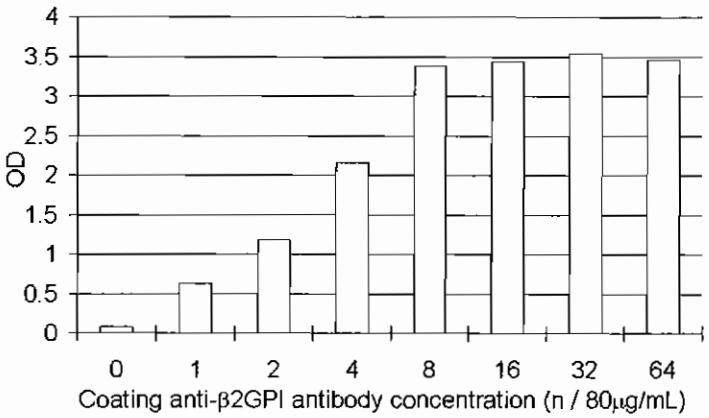

Figure G5: Binding rate of the mouse monoclonal anti-β2GPI antibody on Nunc MaxiSorp™ ELISA plates, tested by direct detection of increasing concentrations of the coated mouse antibody by horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins antibodies. Bars represent the mean of three measurements.

G.9 Testing plate saturation with irrelevant antibodies

In order to determine the minimal and optimal "saturating" concentration for the coating mouse monoclonal antibody on Nunc MaxiSorp™ plates, several experiments were performed using irrelevant mouse monoclonal antibodies (used as blocking agents on uncoated –"bare"- plates). Using coating and washing buffers, as well as incubation times and temperatures from the "initial protocol", these irrelevant antibodies, including mouse monoclonal anti-human CD3, CD14, HLA-DP or HLA-DR antibodies (Becton

[1] Nunc A/S has reported that problems can arise when blocking agents and detergents are used together and recommends that they should primarily be regarded as alternatives (Esser, 1997b).

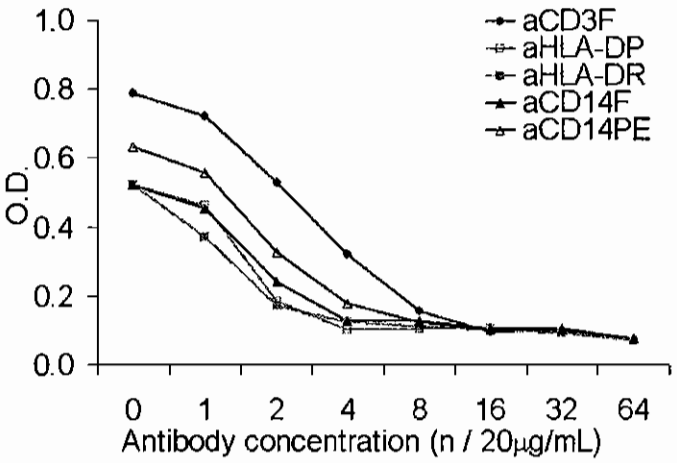

Figure G6: Blocking capacity of irrelevant antibodies on "bare" Nunc MaxiSorp™ plates. Non-specific binding of β2GPI was increasingly impeded by increasing concentrations of the irrelevant antibodies. Points represent the mean of three measurements.

Dickinson, San Jose, CA, USA), were tested at an increasing concentration up to 4.00 µg/mL on uncoated plates, followed by incubation of a serum with an intermediate level of β2GPI. As can be seen in figure G6, these irrelevant mouse monoclonal antibodies significantly reduced non-specific binding to the plate of β2GPI present in the serum sample when coated at or above 0.4 µg/mL, far below the theoretical saturating concentration calculated by Nunc A/S (see following paragraph).

G.10 Theoretical saturating concentration

The range of protein concentrations wherein the proportion of bound protein was independent of the amount added and at which there is no interference with binding to the plastic is called the zone of independent binding ([Cantarero *et al*, 1980]). In practice, this range extends from 1 to 10 µg/mL for ELISAs. Neither insufficient nor excess input concentration are recommended as the former can permit non-specific binding of assay reagents to the plastic support, and the latter can result in the formation of unstable secondary adsorption layers from which weakly associated proteins are often released during the assay ([Matson, 2000]). Interestingly, some ELISAs have been shown to perform better when the plate was not entirely saturated with the capture antibody ([Butler *et al*, 1997]). Nunc MaxiSorp™ plates contain 96 flat-bottom wells, each 33 mm^2 in diameter, arranged in 12 columns and 8 rows, and with a total volume capacity of 400 µL. According to the manufacurer ([Esser, 1985]), a liquid volume of 100 µL in a plate of Nunc MaxiSorp™ type covers an area of 94 mm^2 and forms a liquid height of 3.0 mm, which results in an area/volume ratio of 9.4 cm^2/cm^3. Using the plausible estimate that the MaxiSorp™ surface can adsorb, for geometrical reasons alone, 400 ng of IgG per cm^2, the approximate saturating concentration for such microtitre plates can be estimated at 3.76 µg/mL (0.4 x 9.4) ([Esser, 1997d]). This concentration is within the range of independent binding (the range of protein concentrations at which it is thought that there is no interference with binding to the plastic) ([Cantarero *et al*, 1980]).

However, the apparent full detection of the mouse monoclonal anti-β2GPI capture antibody at a concentration as low as 0.05 µg/mL, and the estimated saturating concentration of 0.4 µg/mL from the experiments with irrelevant antibodies did not allow

to conclude that the concentration of 0.4 µg/mL matched the ultimate activity of the coating antibody, as the spacing and the orientation of the capture molecules also matter. A titration of the coating anti-β2GPI antibody was thus needed.

G.11 Checkerboard titration: serum sample and conjugated detecting antibody screening dilution

Before making a titration of the coating antibody, we performed checkerboard titrations of β2GPI antigen against the detecting (conjugated) antibody on a plate coated with the capture antibody at the theoretical saturating concentration (3.76 µg/mL) in order to set the optimal serum and conjugated antibody screening dilutions. A serum with an estimated intermediate concentration of β2GPI was tested at dilutions ranging from 1 in 750 to 768,000 and the conjugated detecting antibody was tested at dilutions ranging from 1 in 250 (2.4 µg/mL) to 16,000 (0.0375 µg/mL). The same amount of coating capture antibody being present in all the wells, the resulting differences in OD values only depended on the quantity of β2GPI antigens and detecting antibodies in the system. The OD values of the wells that contained no β2GPI antigen were used as a measure of the background binding of the detection system.

The optimum concentration of the conjugated antibody was determined empirically. Since the assay consisted in a non-competitive protocol, the detector antibody needed to be used in excess. As can be seen in figure G7 (bracket A), dilutions of the conjugated antibody at 1 in 1,000 (0.6 µg/mL) or higher were ideal. Indeed, at these dilutions, no OD value corresponding to any of the tested serum dilutions exceeded 2.0 absorbance units, the physical limit of our detection system, and this was coupled with a background level of less than 0.1 absorbance unit. The presence of a plateau at one end of the curve gave an indication of how saturated the system was. When using the conjugated antibody at 1 in 1,000, the plateau height was at its maximum, while at higher dilutions, OD values in the plateau region -and thus the sensitivity of the assay- were significantly reduced.

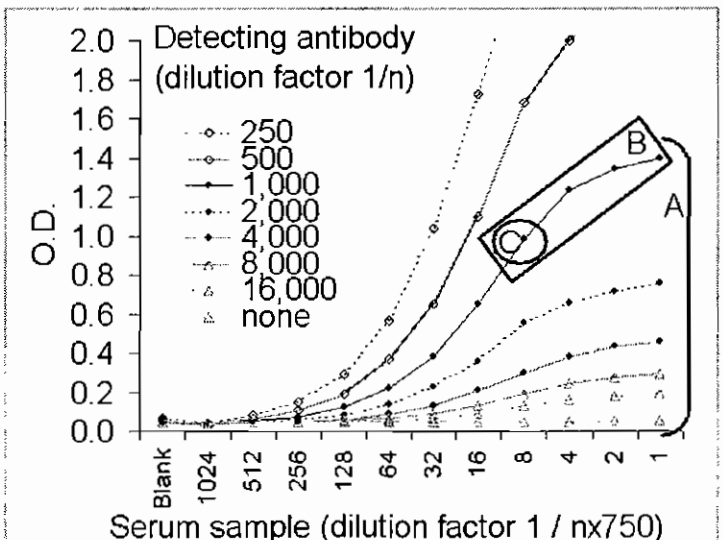

Figure G7: Titration curves relating the serum sample dilution with the detecting antibody dilution. Points represent the mean of three measurements.

Concerning the serum screening dilution, if a single dilution is to be used for quantitative determination of β2GPI, it is important to select a dilution that allows the OD values corresponding to a wide range of

β2GPI concentrations to remain on the linear section of the standard curve. i.e. the straight middle region of the sigmoid curve. Using the conjugated antibody at a dilution of 1 in 1,000 (0.6 µg/mL), the serum screening dilutions that gave, for a serum with an intermediate level of β2GPI, OD values ranging between 1.0 and 1.5, were at 1 in 6,000 or lower (figure G7-box B).

In order to determine which serum screening dilution was most suitable to test sera with not only intermediate, but also low and high β2GPI concentrations, we then measured β2GPI level in several samples with estimated low, intermediate, and high levels of β2GPI diluted from 1 in 750 to 1 in 96,000. A sample dilution of at least 1 in 3,000 was required in order to obtain minimal variation in β2GPI concentration calculated over the linear range of the standard curve. Results obtained with lower dilutions of serum (from 1 in 1,500 and lower) showed considerable variation (data not shown) when compared to those obtained with dilutions above 1 in 3,000 (figure G7-circle C for a serum with an intermediate level of β2GPI). The latter were relatively consistent with each other. This variation is most probably due to interferences resulting from the higher protein concentration present at these dilutions. Therefore, the 1 in 3,000 dilution was considered as the most suitable as serum screening dilution. It allowed as many as possible OD values corresponding to sera with low β2GPI concentration to remain on the linear section of the standard curve. For sera with an extremely high β2GPI concentration, it was decided to further dilute them when their corresponding OD values exceeded 2.0. In practice, no sera required such further dilution.

G.12 Coating antibody titration

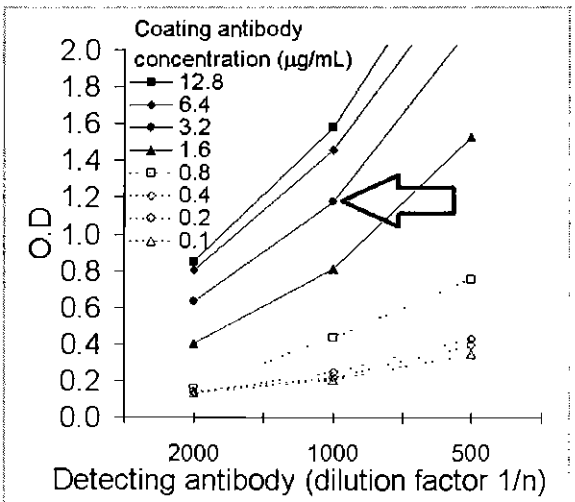

Figure G8: Titration curves relating the coating antibody concentration with the detecting antibody dilution factor. Points represent the mean of three measurements.

Using the assay with screening dilutions of 1 in 3,000 for the serum samples and 1 in 1,000 (0.6 µg/mL) for the conjugated antibody, we still needed to titrate the last major parameter, the coating antibody. As can be seen in figure G8, the tested concentration that gave the steepest dilution curve was 3.2 µg/mL, allowing optimal detection of β2GPI. The increase in OD values and steepness of the curve at concentrations higher that 3.2 µg/mL were not considered as substantial. The coating material being particularly precious, it was considered wiser to use as little of it as possible. Moreover, 3.2 µg/mL was a concentration close to the saturating plateau concentration. At lower concentrations, significantly lower OD values were observed, indicating that a certain amount of available antigen was not captured. We therefore

opted for a coating antibody concentration of 3.2 µg/mL, which is slightly lower than the theoretical saturating concentration calculated by Nunc A/S.

G.13 Modified "final protocol"

At this point, the design of the "initial protocol" had been significantly modified. This modified protocol will be called "final protocol" in the remainder of this document. ELISA plates from different manufacturers were then tested again, but no significant difference was found from the previous results (data not shown).

G.14 Adjustment of incubation and washing steps

Although this was not systematically evaluated for each step, a comparison of standard curves constructed at 37°C and room temperature (~22°C) for periods from 30 min to 2 hours showed that an incubation time of 1 hour was sufficient and 37°C more reproducible than room temperature. Although plate coating with the capture antibody can indifferently be performed for 1 hour at 37°C or overnight at 4°C, the latter method was chosen for reasons of convenience. Similarly, incubation with the horseradish peroxidase-conjugated rabbit anti-β2GPI antibody can be performed at room temperature, but because the reaction required longer incubation time to reach optimum potential and presented increased variability at this temperature, the secondary antibody was incubated for 1 hour at 37°C. Initially, the washing procedure was performed at room temperature and consisted of three successive washes (i.e. emptying and rinsing the wells three consecutive times). A protocol with an additional washing step reduced the background level, but a five-washing-step protocol did not bring further amelioration. We therefore used a four-washing step protocol.

G.15 Calibration of the standard

A standard was constituted and consisted in a pooled serum sample (from sera of healthy individuals). This pooled serum was calibrated against purified β2GPI (Crystal Chem Inc., Chicago, IL, USA; and Calbiochem-Novabiochem Ltd, Nottingham, UK) and its concentration was estimated at 176 µg/mL.

G.16 Finding the optimal standard curve

Various standard curves were performed using doubling to tripling serial dilutions of the pooled serum sample (figure G9). The best standard curve was the 8-point curve that used doubling dilutions of the pooled serum, with the top standard diluted at 1 in 320 (equivalent to a relative concentration of β2GPI of 0.55 µg/mL) (figure G10). This standard curve had the most characteristic sigmoid shape (i.e. with sharp plateau regions, and the straightest middle linear section) with the top OD plateau not exceeding 2.0 absorbance units, a background level of less than 0.1 absorbance unit,

and a linear section ranging from around 40 to around 400 µg/mL. A remarkable feature of the three tested standard curves is their strong parallelism.

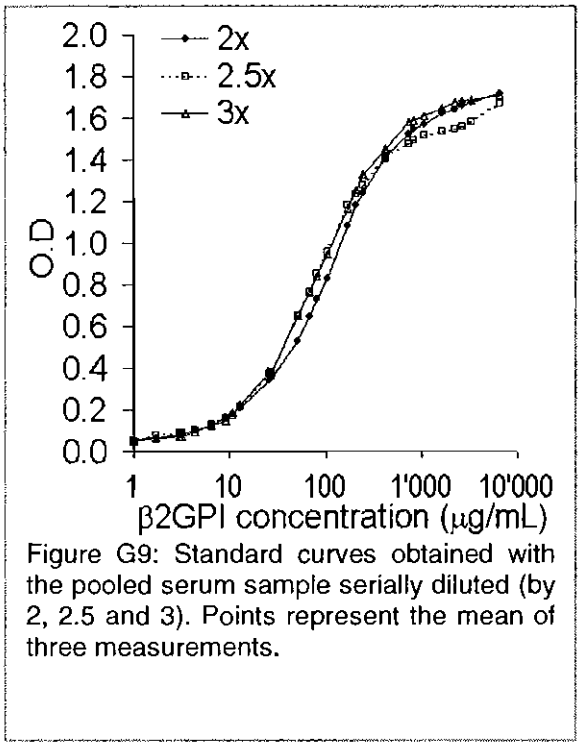

Figure G9: Standard curves obtained with the pooled serum sample serially diluted (by 2, 2.5 and 3). Points represent the mean of three measurements.

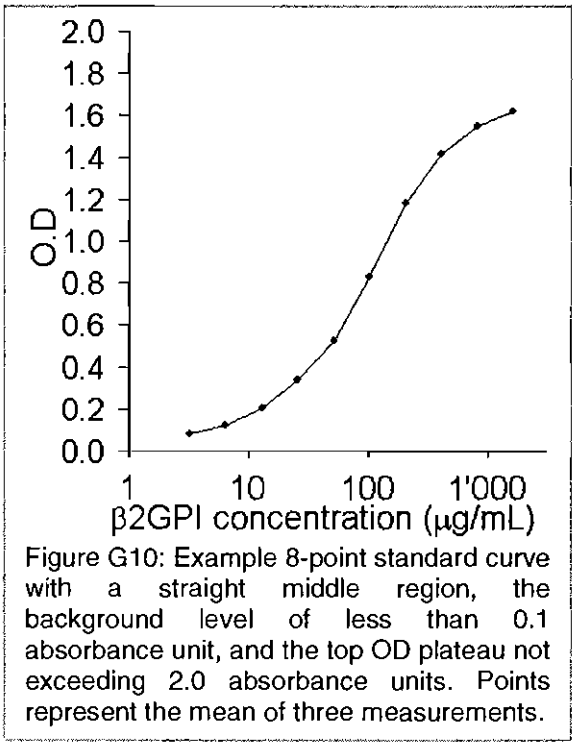

Figure G10: Example 8-point standard curve with a straight middle region, the background level of less than 0.1 absorbance unit, and the top OD plateau not exceeding 2.0 absorbance units. Points represent the mean of three measurements.

G.17 Conjugated detecting antibody titration and OPD step incubation time

The conjugated antibody was then titrated again, using dilutions of 1 in 500, 1,000, and 2,000 (1.2, 0.6, and 0.3 µg/mL respectively), and the duration of the OPD/H_2O_2 substrate step was also adapted by testing three durations (namely 5, 7 and a half, and 10 minutes).

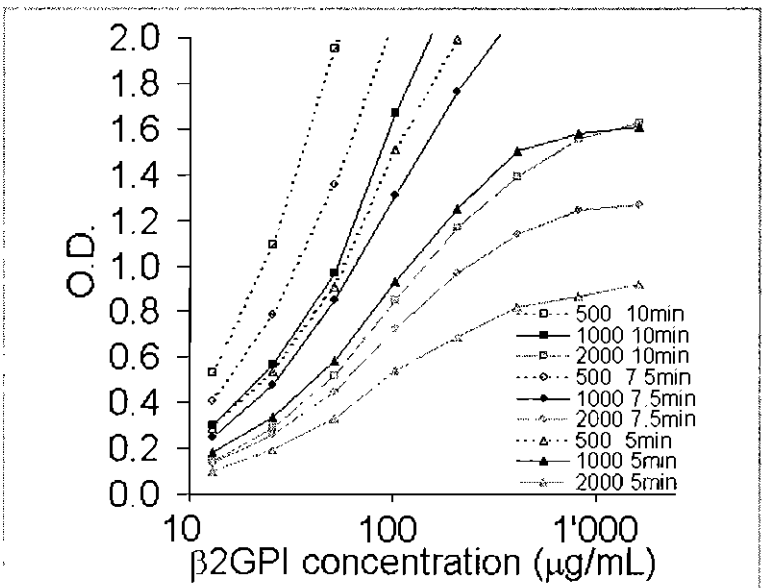

Figure G11: Titration curves relating the detecting antibody at three dilutions with three different incubation times for the OPD/H_2O_2 step. Points represent the mean of three measurements.

Firstly, the assay sensitivity decreased with a decreasing concentration of the conjugated antibody. Secondly, a dilution of 1 in 500 gave greater sensitivity but also showed greater background binding (figure G11). A dilution of 1 in 1,000 revealed to be adequate for the conjugated antibody, allowing elevated concentrations of β2GPI to give a maximum OD value that did not exceed 2.0 absorbance values. If the conjugated antibody was to be used at 1 in 1,000, the substrate step worked best when left for 5 minutes only. Because the OPD/H_2O_2 substrate step was temperature-dependent (depending on how warm (or cold) "room" temperature was, it required more

(or less) than 5 minutes to reach adequate OD signal) this step was visually adjusted by assessing colour development in both the blank and top standard.

G.18 Unexpected variation in the results with certain plate batches

From then on, the assay worked perfectly with the "final protocol" until a change in the Nunc MaxiSorp™ plate batch occurred that coincided with variability in the results. Subsequently, six different Nunc MaxiSorp™ plate batches were tested, paying particular attention to the optional certification provided by the manufacturer for consistency in adsorption of protein ([Esser, 1997a]) (appendix F). To compare the plates and the batches with each other, intra-assay coefficients of variation (CV) were calculated for every single plate and inter-assay CV were calculated for each plate batch using data from eight β2GPI concentration measurements in two serum samples (A and B) with estimated intermediate and high levels of β2GPI (respectively 147.7 ± 20.6 µg/mL and 244.2 ± 35.7 µL) made on five plates of each plate batch.

Significant differences were found between certified and uncertified batches. Firstly, non-specific binding of β2GPI to the plates without capture antibody was strikingly more variable among uncertified plates (compared to certified ones). Secondly, when tested on plates coated with 3.2 µg/mL of mouse monoclonal anti-β2GPI antibodies, the two control sera gave maximum intra and inter-assay CV values of 4.7% and 14.6% respectively for the certified batches and 23.4% and 36.1% respectively for the uncertified batches (table G1). Although some uncertified batches showed better inter-assay CV values, we preferred to use exclusively certified batches for the β2GPI assay, since the presence of one inadequate batch out of five uncertified batches that were tested rendered uncertified batches unreliable.

Table G1: Intra-assay coefficients of variation (CV) average and inter-assay CV of two serum samples (A and B) with estimated intermediate and high levels of β2GPI on 6 different Nunc Maxisorp™ ELISA plate batches coated with 3.2 µg/mL of mouse monoclonal anti-β2GPI antibody. Batches 1 to 5 are non-certified and batch 6 is certified by the manufacturer for homogeneity in adsorption of IgG.

		Plate batch					
	Sample	1	2	3	4	5	6
Intra-assay variation	A	8.3%	9.4%	8.8%	5.5%	23.4%	4.7%
	B	7.9%	5.7%	7.0%	6.2%	8.8%	3.4%
Inter-assay variation	A	14.9%	17.9%	15.1%	14.5%	36.1%	14.6%
	B	8.0%	6.6%	7.9%	9.8%	12.2%	13.9%

G.19 Sensitivity

Many laboratories calculate the detection limit of an ELISA (i.e. the minimal detectable level of product producing absorbance) as 1.5 to 2.0 times the background value or as the mean plus two standards of deviation of blank values. These calculations did not give a meaningful detection limit in the context of our β2GPI assay, as the background level of the assay was extremely low (rarely exceeding an OD value of 0.060). We therefore estimated the detection limit of the assay from the slope of the dose-response curve, using the concentration extrapolated from the absorbance of 20 replicates of the last standard dilution at 1 in 40,960 of the pooled serum to which 2 standards of deviation were added, which gave an approximate value of 13.80 µg/mL.

G.20 Linearity

Good linearity was visualised on a semi-logarithmic paper (figure G12). The intensity of colour generated was linear and covered a relatively large concentration range. Linearity was particularly pronounced in the middle portion of the standard curve.

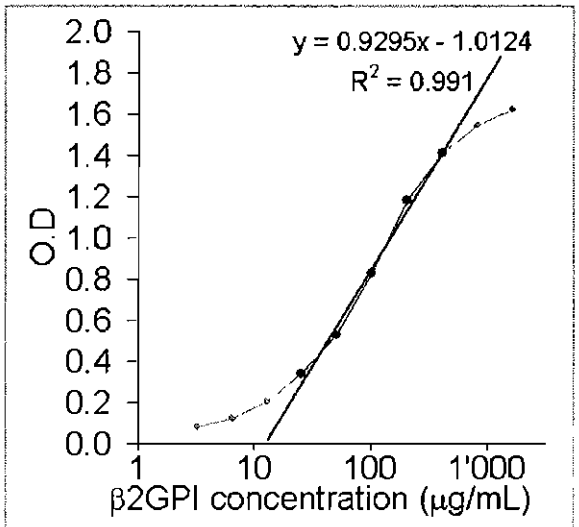

Figure G12: Standard curve for the β2GPI assay with both optical density and concentration expressed logarithmically.

To check whether the β2GPI assay was independent of β2GPI level in the sample, we tested three different sera with low, intermediate, and high levels of β2GPI at five different dilutions (from 1 in 750 to 1 in 12,000). The OD values of the various dilutions covered most of the straight portion of the standard curve. The final concentration, calculated by multiplying the measured concentration by the dilution factor, corresponded to each other.

G.21 Precision

Each plate contained a set of standards (8-point standard curve) and three control sera containing low, intermediate, and elevated levels of β2GPI. Results from the three quality control sera showed good plate-to-plate reproducibility, with the mean inter-assay CV for the low, intermediate, and high samples being at 13.4%, 14.0%, and 7.2% respectively. The intra-assay ("within-run") imprecision of the β2GPI assay, evaluated by assaying 20 duplicates of two serum samples (with high and low β2GPI levels), was estimated between 3.4% and 4.7%.

Serum dilutions were originally added to the plate in triplicates. The CV of triplicates rarely being above 5%, it was decided, in order to save reagents, to test the serum

dilutions in duplicates. Duplicates that showed CV above 10% (less than 5% of all samples that were tested) were systematically retested.

A particular well-to-well variation (sometimes called "edge effect" in the literature) was observed from time to time and depended on the location on the plate of the wells that were used. This variation was minimised by incubating the plates in a non-stacked fashion, sealed with a plastic adhesive, and in the dark, as well as by using all reagents adjusted to room temperature.

G.22 Recovery

Analytic recovery was assessed in two sera with an estimated low level of β2GPI, which were assayed before and after addition of 5, 10, and 20 µg of purified β2GPI (Crystal Chem Inc., Chicago, USA) diluted in PBST (table G2). The spiked sera were tested with the β2GPI ELISA and recovery was calculated as the ratio of recovered to added β2GPI concentrations (expressed as percentages).

Table G2: Recovery of β2GPI added to a serum sample with an estimated low level of β2GPI.

	β2GPI (µg/mL)			Recovery (%)
	Added	Measured	Recovered	
Serum 1	0	54.6		
	50	102.6	48.0	96.0%
	100	152.9	98.3	98.3%
	200	252.7	198.1	99.1%
Serum 2	0	88.5		
	50	134.9	46.4	92.8%
	100	194.4	105.9	105.9%
	200	292.4	203.9	102.0%

G.23 Parallelism

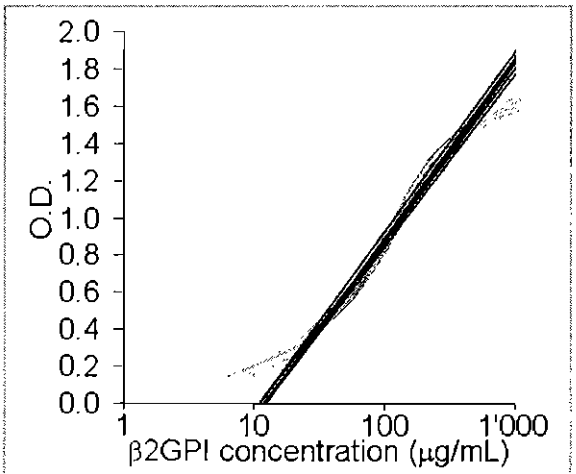

Figure G13: Several standard curves for the β2GPI assay. Darker lines are trendlines corresponding to the straight middle portions of the standard curves (average R^2: 0.9942).

Parallelism was checked by plotting a number of standard curves and by comparing their straight middle portion. As can be seen in figure G13, these straight middle portions were parallel.

G.24 Comments on the β2GPI ELISA protocol optimization

Variability associated with each step of an ELISA protocol often has various sources, making assay interpretation difficult. We have encountered noteworthy difficulties with the β2GPI assay, particularly because of the intrinsic physical properties of both the measured β2GPI molecule and the plastic support used for its quantification. The first part of our work was therefore focused on the optimization and standardisation of the β2GPI ELISA protocol in order to minimise technical errors that could have affected the assay's sensitivity and specificity.

To optimise the assay, we first focused on minimising the effect of non-specific binding of β2GPI to the plastic plate surface. β2GPI has been repeatedly proven to have a high propensity to bind to negatively charged surfaces, including oxygenated solid phase surfaces (Matsuura *et al*, 1994). The Nunc MaxiSorp™ immunoplates that were used in this study typically receive a certain dose of γ-irradiation in order to form charged groups on the polystyrene surface. It was thus not a surprise to observe significant binding of β2GPI to these plates. In order to prevent such binding, we tested two options: the use of a blocking substance and the saturation of the whole coating surface by the primary coating antibody.

We tested five blocking substances, three of them, gelatine (Merck and Sigma), glycine (Sigma), and agar (Merck and Sigma), did not significantly or consistently suppress non-specific binding of β2GPI to the plastic surface, even at a concentration as high as 1%. By contrast, two of them, carrageen (Sigma) and casein (Sigma), completely blocked not only non-specific binding of β2GPI to the plate, but also the specific recognition of β2GPI by the coating antibody, even at a concentration below 0.01%.

We could not explain why the last two blocking agents impeded specific recognition of the β2GPI antigen by the detecting antibody. Either these blockers created a physical shield above the coating antibody, obstructing any further reagent binding or, if small enough, they inserted themselves between the coating antibody-β2GPI complexes and by steric hindrance prevented further binding by the detecting antibody. The latter is probably what happened with casein that presents a relatively small molecular weight. Modifying the assay protocol by adding simultaneously the blocking substance and the antigen to be detected (instead of a pre-treatment of coated plates with the sole blocking substance prior to the addition of the antigen) may have permitted competition for binding sites between the blocking substances and β2GPI, and avoided possible excessive coverage of these sites by the blocking substances. We unfortunately did not test such *modus operandi*. Another -unlikely- explanation for the excessive blocking behaviour of these substances could be that exogenous impurities (such as lipid or protein particles) introduced together with the agents could have interfered with the

β2GPI assay reagents. Whatever the reason, none of the protein-based blocking agents that we tested was found suitable for the β2GPI assay. Bovine serum albumin (BSA), the most widely used blocking agent in β2GPI ELISA, was not considered suitable either since some commercial preparations of BSA have been found to contain significant amounts of bovine β2GPI ([Roubey, 1994]; personal comment from R. Sim, MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford) which presents a remarkable homology with human β2GPI ([Steinkasserer *et al*, 1991; Matsuura *et al*, 1991]).

As a substitute for a protein-based blocking agent, we opted for the use of non-ionic detergent Tween®20 in all dilution and washing buffers in combination with the use of a saturating concentration for the coating antibody. In doing so, we followed the advice of Nunc A/S that recommends that blocking agents and detergents should be administered as alternatives ([Esser, 1997b]), and of Rubin *et al* ([1980]) that indicated that storage and washing of surfaces coated with adsorbed antigen in PBS-Tween®20 significantly reduced leaching of protein. The latter was fixed at 3.2 µg/mL following the estimation of the antibody binding rate to the plate, of the capacity of irrelevant antibodies to block non-specific binding of β2GPI to the plastic surface (even at a concentration as low as 0.4 µg/mL), and following a checkerboard titration of the different components of the β2GPI ELISA. The concentration of 3.2 µg/mL was also selected because it was close to the theoretical saturating concentration estimated by Nunc A/S for MaxiSorp™ immunoplates ([Esser, 1997d]).

Having circumvented the issue of non-specific binding of β2GPI to the plastic support, we then faced another unusual variability in the disparity between plate batches related to the different certification status of the Nunc MaxiSorp™ plates. We found a significant difference between the batches, with several non-certified batches (especially one out of five) giving unacceptable maximum intra and inter-assay coefficients of variation (CV) (as high as 23.4% and 36.1% respectively), while the two certified batches that we tested did not show such variations. Both certified and non-certified surfaces are supposed to be identical except that Nunc provides for the certified plates a quality certificate for the immobilization homogeneity of IgG. This certificate attests that a representative sample from each manufacturing lot has undergone a "binding capacity test", i.e. an ELISA-like test in Nunc control laboratories to ensure binding capabilities ([Esser, 1997a]). However, it does not state whether the plates received the same amount of γ-irradiation as the non-certified ones. We could not explain the marked difference between the batches (certified or not), but, although the plates came from the same manufacturer, they possibly displayed different charge densities following doses of radiation that differed from one batch to another. Polar groups formation on the polystyrene surface caused by γ-irradiation ([Onyiriuka *et al*, 1990]) is known to promote the binding of water-soluble molecules, possibly by helping to overcome the effect of

intermediate-distance repulsive interactions ([Deshpande, 1996]). Because the coating of the monoclonal antibody onto the plate was performed by (direct) passive adsorption (without covalent bonding), it is possible that the variation in the charge density between the batches induced significant differences in the binding capacity of the plates and thereby altered the stability of coating antibody adsorption. A consequence of that can be an increased availability of binding sites on the plastic surface to which β2GPI, if present, would avidly bind thanks to its high avidity for negatively charged surfaces. On the other hand, the variation in polar charge density can affect the binding capacity of β2GPI to the plastic surface, especially on un-/sub-saturated plates. It can for instance allow better passive adsorption of β2GPI on the plastic surface, which renders the β2GPI assay even more susceptible to poor specificity. Plastics used for immunoplates are generally not of reagent grade and strict control of their chemical composition is probably not in the hands of the plate manufacturers, which adds uncontrollable variables at the level of the solid support. Thus, as suggested by numerous investigators ([Kricka *et al*, 1980; Shekarchi *et al*, 1984]), batches or sub-batches of plates intended for use in immunoassays should be screened for variability in protein adsorption. In the case of the β2GPI assay, although uncertified batches are most probably as reliable as certified ones, the lack of reproducibility in measurements for β2GPI level with one out of five uncertified batches renders them unsuitable for β2GPI quantification. We therefore decided to rely solely on certified plates. These certified plates gave consistent results throughout the study with an overall inter-assay CV (calculated with more than 70 measurements of a control serum sample) around 11.5%.

Another complication that could have affected the β2GPI assay was the potential interference between assay reagents and compounds present in the serum samples, as our protocol included no purification or extraction step prior to the actual quantification of β2GPI. β2GPI has a well-documented high propensity to bind to phospholipids ([Polz *et al*, 1979b; Schousboe, 1983b; Wurm, 1984]). We therefore particularly focused on the possible interfering action of these molecules. By artificially adding cardiolipin (CL) into serum dilutions, we found that, up to a concentration of 16.67 nM, CL had no effect on the β2GPI assay. Above that concentration, the only lowering effect that could be observed was caused solely by the ethanol diluent into which CL was diluted. As regards constituents such as factor H or complement receptor 1 with which β2GPI presents high structural homology, no cross-reaction was observed between these proteins and the Chemicon antibody (personal comment from J. Guerin, PhD).

Concerning the anti-β2GPI antibodies, we used a mouse monoclonal coating antibody from Chemicon International Inc. (Temecula, CA, USA) that was shown to recognise only β2GPI on Western blotting, to react with all β2GPI isoforms (personal comment from J. Guerin, PhD), and to recognise both free and lipid-bound β2GPI ([Mehdi *et al*, 1999]).

That antibody did not show any cross-reaction with proteins structurally related to β2GPI such as factor H, complement receptor-1, or C4b binding protein (personal comments from J. Guerin, PhD), with other non-related proteins or with any of the other β2GPI ELISA reagents potentially present in tested samples. The rabbit polyclonal detecting antibody from Dako A/S (Glostrup, Denmark) we used showed no significant binding to the coated plastic surface and was also found to be highly specific for β2GPI. To test whether the adsorption of the coating antibody to the polymer surface altered its affinity for the β2GPI antigen (thereby changing the reaction's kinetics), we inverted the order of use of the monoclonal and polyclonal antibodies. This test showed that, when used at the appropriate concentrations, both antibodies could recognise β2GPI as efficiently when bound to the plastic surface as in solution.

Thus, we concluded that any possible variability due to the potential blocking or interacting action of compounds present in the serum samples as well as all non-specific binding of β2GPI were annihilated when using certified Nunc A/S MaxiSorp™ immunoplates saturated with β2GPI-specific Chemicon antibody (3.2 µg/mL), no protein-based blocking agent but rather 0.05% of Tween®20 in all non-coating buffers, and screening dilutions of respectively 1 in 3,000 and 1 in 1,000 (0.6 µg/mL) for the serum samples and the detecting antibody, for a test volume of 100 µL.

The enzyme-linked immunosorbent assay (ELISA) format that was selected for this study was a sandwich (capture) direct protocol (figure 3.1). The β2GPI assay that we have been able to set up for the detection and quantification of β2GPI used Nunc MaxiSorp™ ELISA plates, non-labelled mouse monoclonal antibodies, and horseradish peroxidase-conjugated rabbit polyclonal anti-human β2GPI antibodies. It showed remarkably strong consistency and good reproducibility with intra- and inter-assay CVs that were of moderate amplitude.

Appendix H. Isoelectric focusing-immunoblotting materials and reagents

Reagent	Manufacturer	Catalogue#

Table H1: Isoelectric focusing materials and reagents

Clean Gel IEF (T=5%, C=3%)	Amersham Pharmacia Biotech (Uppsala, Sweden)	18-1035-32
2.2% Pharmalyte® 4-6.5	Amersham Pharmacia Biotech	17-0452-01
4.4% Pharmalyte® 5-8	Amersham Pharmacia Biotech	17-0453-01
3.1 M urea	BDH (Anala®)	102904W
1 M sodium hydroxide	BDH (Anala®)	102525P
0.05 M sulphuric acid	BDH (Anala®)	102760B

Table H2: Electric device

Power Supply	LKB Biochrom	2103

Table H3: Immunoblotting materials and reagents

TransBlot Transfer Medium 0.45 µm nitrocellulose membrane	Bio-Rad, Hercules (CA, USA)	162-0115
Chromatography paper 3mm	Whatman International Ltd (Maidstone, UK)	3030-672
Filter paper 41	Whatman International Ltd	1441-866
2 mM Trizma® Baze	Sigma	T1503
15 mM sodium chloride	BDH (Anala®)	102415K
Powder skimmed milk	Marvel (Merseyside, UK)	
Rabbit polyclonal anti-human β2GPI antibodies	Dako A/S	O 9152
Horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins	Dako A/S	P 0217
0.85 mM 3-amino-9-ethylcarbazole (20 mg tablets)	Sigma	A6926
Dimethyl sulphoxide	BDH	10323(2J)
3 mM acetic acid	BDH (Anala®)	10001
7 mM sodium acetate anhydrous	BDH (Anala®)	10236
Hydrogen peroxide	BDH (Anala®)	10128(4N)

Appendix I. DNA extraction (procedures)

Genomic DNA was extracted from peripheral blood leukocytes using standard methods either a salting out or a phenol/chloroform based procedure ([Dracopoli *et al*, 2003]). Both protocols involve selective lysis of erythrocytes, followed by selective lysis of DNA-containing leucocytes, removal of proteins and precipitation of nucleic acids. Samples of extracted DNA were stored at -20°C until use. Some of the DNA samples that were donated to us had been stored for a few months to several years (without significant DNA degradation).

Isopropanol/ethanol procedure

1. Add 3 mL of EDTA whole blood sample to 9 mL of erythrocyte lysis solution in a 15 mL polycarbonate tube.
2. Incubate at room temperature for 10 minutes, invert occasionally.
3. Centrifuge at 2,000 rpm for 10 minutes.
4. Pour off the supernatant (containing lysed erythrocytes) leaving 100-200 µL of residual liquid above the white pellet (containing unlysed leukocytes).
5. Vortex vigorously to resuspend the leukocyte pellet.
6. Add 3 mL of white cell lysis solution. Mix well to lyse cells fully - do not vortex.
7. Add 1 mL of protein precipitation solution. Vortex for 10 seconds.
8. Centrifuge at 3,000 rpm for 10 minutes to pellet proteins.
9. Pour supernatant (containing nucleic acids) into a fresh tube containing 3 mL of 100% isopropanol. Invert tube gently until DNA precipitates.
10. Centrifuge at 3,000 rpm for 5 minutes to pellet DNA.
11. Pour off supernatant.
12. Add 3 mL of 70% ethanol and invert tube gently.
13. Centrifuge at 3,000 rpm for 5 minutes.
14. Pour off supernatant and leave tube to drain fully on a paper tissue (10-15 minutes).
15. Add 200 µL of Tris-EDTA buffer to DNA pellet. Incubate at 65°C for 1 hour to fully dissolve DNA.
16. Estimate extraction yield and DNA purity by absorption spectroscopy, check DNA integrity by electrophoresis on agarose gel (appendix K) and store DNA at -20°C.

Phenol/chloroform procedure

1. Add 200 µL of each EDTA whole blood sample to 400 µL lysis buffer.
2. Incubate 1 hour/overnight at 37°C.
3. Add 450 µL phenol to each tube and mix by inversion. Centrifuge at 15,000 rpm for 5 minutes.
4. Make a chloroform iso-amyl alcohol mix.
5. Transfer aqueous (top) layer to a fresh tube containing 450µL chloroform iso-amyl alcohol. Place on shaker or invert for 5 minutes. Centrifuge at 15,000 rpm for 5 minutes to separate the layers.
6. Transfer aqueous (top) layer to a fresh tube containing 40 µL of sodium acetate pH 5.2. Add 800 µL 100% ethanol (-20°C). Mix thoroughly.
7. Precipitate nucleic acid overnight at -20°C.
8. Collect nucleic acid extract by centrifuging samples at 15,000 rpm at 0°C for 10 minutes.
9. Discard the supernatant. Blot on paper tissue. Try to remove as much ethanol as possible without disturbing the pellet. Allow the pellet to air dry.
10. Re-dissolve pellet in 15-50 µL nuclease free water. Allow 10 minutes to ensure entire pellet dissolution.
11. Estimate extraction yield and DNA purity by absorption spectroscopy, check DNA integrity by electrophoresis on agarose gel (appendix K) and store DNA at -20°C.

Appendix J. DNA extraction (reagents)

Final concentration	Reagent	Manufacturer	Catalogue#

Isopropanol/ethanol procedure

Table J1: Red blood cell lysis solution

155 mM	Ammonium chloride	BDH	100173D
10 mM	Potassium hydrogen carbonate	Lancaster synthesis (Lancashire, UK)	14110
1 mM	EDTA	BDH	421881L

Table J2: White cell lysis solution

25 mM	EDTA	BDH	421881L
2 %	Sodium dodecyl sulfate	BDH	301754L

Table J3: Other reagents

10 M	Ammonium acetate	BDH (Biochemical)	437453A
Pure	Isopropanol	BDH (GPR™)	296946H
Pure	Isopropanol	Sigma-Aldrich	I9516
70% v/v	Ethanol	BDH (Anala®)	1009712500

Table J4: Tris EDTA buffer (TEB)

10 mM	Tris-chloride pH 8.0	BDH	
1 mM	EDTA	BDH AnalaR®	421881L

Phenol/chloroform procedure

Table J5: Lysis solution

99 mM	Sodium chloride	BDH (Anala®)	102415K
50 mM	Tris pH 8.0	BDH (Anala®)	103156X
1 mM	EDTA pH 8.0	BDH	
0.50%	Sodium dodecyl sulfate	BDH	301754L
2 mg/mL	Proteinase K	Invitrogen™ Life Technologies Ltd, Paisley, Scotland, UK	25530-015

Table J6: Chloroform iso-amyl alcohol mix

50 volumes	Chloroform	Merck	UN1888
1 volume	Iso-amyl alcohol	Sigma-Aldrich	I9392

Table J7: Other reagents

Pure	Phenol	BDH (Anala®)	101884Y
3 M	Sodium acetate pH 5.2	BDH (Anala®)	102364Q
100% v/v	Ethanol	BDH (Anala®)	100971

Appendix K. Electrophoresis (reagents)

Final concentration	Reagent	Manufacturer	Catalogue#

Table K1: Electrophoresis gel

0.8 to 2.0%	Agarose	Pronadisa- CONDA (Madrid, Spain)	8016
1 x	Tris borate EDTA (TBE) buffer	Invitrogen™ Life Technologies Ltd (Paisley, Scotland, UK)	15581-028
0.5 µg/mL	Ethidium bromide	Sigma-Aldrich (Gillingham, Dorset, UK)	E8751

Table K2: Loading buffer (6x)

30% v/v	Glycerol	BDH AnalaR®	101184K
0.25%	Xylene cyanide	Sigma	X2751
0.25%	Bromophenol blue	BDH Indicators	200-17

Appendix L. PCR-RFLP (reagents)

Reagent	Manufacturer	Catalogue#
100 bp DNA ladder	Invitrogen™ Life Technologies	15628-019
100 mM dNTP set, PCR grade	GibcoBRL® Life Technologies (USA)	10297-018
Taq DNA polymerase, recombinant	GibcoBRL® Life Technologies	10342-053
Platinum® *Taq* DNA polymerase	GibcoBRL® Life Technologies	10966-018
Omniplate 96 25/pack	ThermoHybaid (Ashford, Midllesex, UK)	HBTR3MT
TD sealing tape 50/pack	ThermoHybaid	HBTDTAPE50
TD Silicone foam compression pad	ThermoHybaid	HBTDSFOAM
Primers	Sigma-Genosys	
Bst B I	New England BioLabs	R0519S
CviJ I	ChimerX (Milwaukee, WI, USA)	2125-01
Nsi I recombinant	New England BioLabs	R0127L
Rsa I recombinant	New England BioLabs	R0167L
*Tsp*509 I recombinant	New England BioLabs	R0576L

Appendix M. RNA extraction (procedure)

Total RNA was extracted from mouse liver tissues using either the single-step Tri-Reagent® (Molecular Research Center, Inc; Cincinnati; Ohio; USA) as prescribed by the manufacturer or the single-step guanidium thiocyanate-phenol-chloroform extraction procedure (slightly modified) from Chomczynski and Sacchi ([1987]). In order to prevent ribonuclease activity, only molecular biology grade reagents were used, all dilutions were made with RNase-free water, and all glassware and plastic materials that were used in this study were treated with DEPC or RNaseZAP™.

1. Disrupt (homogenise) the tissues (considering an approximate of 8 mg of RNA per g of liver tissue) in 0.5 ml of solution D (guanidium thiocyanate solution with sodium lauroylsarcosinate) with a glass-Teflon Potter-Elvejhem homogeneizer, a Pellet Pestle® (Kontes Glass Cº, Vineland, New Jersey; Sigma) with RNaseZAP™-treated pellet pestle polypropylene heads or by grinding tissues with sterile surgical blades on sterile glass slides.
2. Transfer the lysate/grinded tissues into a centrifuge propylene tube and add 3.5 ml of solution D (guanidium thiocyanate solution with sodium lauroylsarcosinate), mix well by vortexing.
3. Centrifuge at 10,000 rpm at 10°C for 10 minutes, and transfer the supernatant into a fresh centrifuge tube.
4. Add 0.1 ml of 2 M sodium acetate (pH 4.0) and mix.
5. Add 1 ml of water-saturated phenol and mix.
6. Add 0.5 ml of chloroform:isoamyl alcohol solution, and shake vigorously for 10 seconds.
7. Cool on ice for at least 15 minutes.
8. Centrifuge at 10,000 rpm at 4°C for 20 minutes, and transfer the supernatant into a fresh tube.
9. Precipitate RNA with an equal volume of isopropanol. Vortex and incubate at -20°C for at least one hour.
10. Centrifuge at 10,000 rpm at 4°C for 20 minutes, and remove the supernatant and resuspend the pellet in 1 ml of solution D (briefly heating to 68°C may aid dispersion of the pellet).
11. Precipitate RNA with one volume of isopropanol. Vortex and precipitate the RNA for at least one hour.
12. Centrifuge at 10,000 rpm at 4°C for 20 minutes, and carefully remove (and discard) the supernatant.
13. Resuspend/wash the pellet in 2 ml of absolute ethanol.
14. Centrifuge at 10,000 rpm at 4°C for 20 minutes, and carefully remove the supernatant. Dry briefly for 5-15 minutes.
15. Resuspend the pellet in 100 µl of deionised DEPC-treated water and store at -70°C.
16. Evaluate the extraction yield and the RNA purity by absorption spectroscopy, check RNA integrity by electrophoresis on agarose gel (appendix K) and store RNA at -20°C.

Appendix N. RNA extraction (reagents)

Final concentration	Reagent	Manufacturer	Catalogue#

Table N1: Guanidinium thiocyanate solution

4 M	Guanidinium thiocyanate	Fluka Riedel-de Haën®, Sigma-Aldrich	50985
25 mM	Sodium citrate, pH 7.0	BDH (Anala®)	10242
0.1 M	2-Mercaptoethanol	Sigma	M3148
0.5%	Sodium lauryl sarcosinate	Sigma	L5000

Sodium lauryl sarcosinate solution is prepared from a sarcosyl stock solution at 20% (w/v) and filtered with #1 Whatman filter paper or equivalent.
Heat GIT solution to 65°C to facilitate dissolving. 2-mercaptoethanol should be added just before use. The solution is light sensitive. Stable for a month in a dark bottle at room temperature.

Table N2: 2 M Sodium acetate solution pH 4.0

2 M	Sodium acetate, pH 4.0	BDH (Anala®)	102364Q

Mix very well and incubate for 2 hours (preferably overnight) at 37°C. Dispense into aliquots and autoclave for 30 min. Store at 4°C.

Table N3: Chloroform:Isoamyl alcohol solution

24 volumes	Chloroform	Sigma	C2432
1 volume	Iso-amyl alcohol	Sigma-Aldrich	I9392

The solution is light sensitive.

Table N4: Other reagents

Pure	Water-saturated phenol pH 4.5	BDH	43672 2V
Pure	Isopropanol	BDH (GPR™)	296946H
Pure	Isopropanol	Sigma-Aldrich	I9516
Pure	Ethanol	BDH (Anala®)	100971
0.2%	Diethyl pyrocarbonate (DEPC)	Sigma	D5758
Pure	RNaseZAP™	Sigma	R2020
Pure	TriReagent®	Molecular Research Center Inc. (Cincinnati, Ohio, USA)	TR118

Note: Solutions were made with DEPC-treated water and all materials used for RNA isolation or analysis (including electrophoresis tanks) were cleaned with detergent solutions, rinsed in water, dried with ethanol, filled with a solution of 3% H_2O_2 for 10 min in room temperature, and finally rinsed thoroughly with DEPC-treated water or cleaned with RNaseZAP™.

To treat water with DEPC: 1 mL of DEPC in 1 L of deionised water under constant shaking at room temperature (at least 1 hour) and autoclave.

Appendix O. Additional data

P value of χ^2-test for independence testing Hardy-Weinberg equilibrium (HWE) and expected heterozygosity (eH) given by $H = 1 - \Sigma (p_i^2)$, based on allele frequencies and assuming HWE.

Codon		88	247	306	316
Healthy	HWE P	0.8448	0.9998	0.9025	0.7552
	eH	0.1086	0.4208	0.0488	0.0794
Stroke	HWE P	0.9451	0.0968	0.8907	0.9109
	eH	0.0605	0.4228	0.0846	0.0765
ACS	HWE P	0.7059	0.0550	0.6885	0.8782
	eH	0.0848	0.3923	0.0852	0.0518
RFL	HWE P	0.9973	0.8851	0.9322	0.9756
	eH	0.0211	0.3803	0.1007	0.0618
Other T	HWE P	0.9968	0.5105	0.9869	0.9869
	eH	0.0253	0.3935	0.0500	0.0500

Tested individuals were healthy individuals, patients with stroke, with early onset acute coronary syndrome (ACS), recurrent foetal loss (RFL) or another thrombotic event (from a mixed bag of thrombotic disorders) (Other T).

Association between β2GPI genotype and disease (Pearson's χ^2-test P values and odds ratio calculated for heterozygotes and homozygous carriers relative to homozygous non-carriers).

Codon	Disease	Heterozygotes					Homozygous carriers				
		#	P value	OR	Lower	Upper	#	P value	OR	Lower	Upper
88	Stroke	431	0.1988	0.578	0.248	1.346	393	0.3919	n.a.		
	ACS	657	0.5227	0.844	0.502	1.420	596	0.1429			
	RFL	366	0.0700	0.188	0.025	1.411	334	0.5708			
	Other T	358	0.1177	0.228	0.030	1.716	326	0.6064			
247	Stroke	398	0.0971	1.455	0.933	2.269	237	0.1418	0.448	0.150	1.339
	ACS	601	0.0088	0.646	0.466	0.896	423	0.9353	1.022	0.604	1.731
	RFL	333	0.2684	0.690	0.357	1.335	216	0.6913	0.797	0.259	2.448
	Other T	328	0.6594	1.164	0.592	2.290	205	0.1994	0.283	0.036	2.198
306	Stroke	433	0.1387	1.845	0.812	4.193	407	n.a.			
	ACS	679	0.0470	1.859	1.000	3.457	631				
	RFL	367	0.1202	2.262	0.788	6.494	346				
	Other T	359	0.9724	1.027	0.227	4.645	341				
316	Stroke	427	0.9165	0.959	0.435	2.113	392	n.a.			
	ACS	671	0.1264	0.623	0.338	1.148	626				
	RFL	361	0.6554	0.755	0.219	2.601	332				
	Other T	353	0.4921	0.599	0.137	2.626	325				

#: number of subjects in the control and the experimental groups (as not all DNA samples were amplified for a given genetic marker, the total number of cases varies between the mutations examined); P value: 2-sided asymptotic significance of Pearson's χ^2-test; OR: odds ratio; Lower/Upper: limits of the 95 % confidence interval. ACS: early onset acute coronary syndrome; RFL: recurrent foetal loss; Other T: other thrombotic events (from a mixed bag of thrombotic disorders); n.a.: not applicable.

Association between β2GPI allele and disease (Pearson's χ^2-test P values and odds ratio calculated for each mutation).

Codon	Disease	#	P value	OR	Lower	Upper
88	Stroke	866	0.1216	0.527	0.232	1.201
	ACS	1318	0.2736	0.759	0.463	1.245
	RFL	736	0.0545	0.176	0.024	1.297
	Other T	720	0.0946	0.212	0.029	1.570
247	Stroke	862	0.9412	1.013	0.727	1.410
	ACS	1336	0.1810	0.850	0.670	1.079
	RFL	732	0.3652	0.796	0.486	1.305
	Other T	716	0.5631	0.856	0.505	1.451
306	Stroke	866	0.1449	1.806	0.807	4.039
	ACS	1358	0.0512	1.819	0.989	3.348
	RFL	734	0.1258	2.191	0.783	6.128
	Other T	718	0.9727	1.026	0.232	4.550
316	Stroke	854	0.9183	0.960	0.443	2.082
	ACS	1342	0.1331	0.633	0.347	1.155
	RFL	722	0.6622	0.763	0.226	2.573
	Other T	706	0.5012	0.609	0.142	2.618

#: number of subjects in the control and the experimental groups (as not all DNA samples were amplified for a given genetic marker, the total number of cases varies between the mutations examined); P value: 2-sided asymptotic significance of Pearson's χ^2-test; OR: odds ratio; Lower/Upper: limits of the 95 % confidence interval. ACS: early onset acute coronary syndrome; RFL: recurrent foetal loss; Other T: other thrombotic events (from a mixed bag of thrombotic disorders); ALL: data grouped from all the diseased patients; n.a.: not applicable.

IgG ACLA, IgM ACLA, and anti-β2GPI antibody prevalence and serum concentration (IU/mL) in patients with stroke according to the β2GPI genotype at codons 88, 247, 306 and 316.

Codon	88			247			306			316		
	SerSer	SerAsn	AsnAsn	ValVal	ValLeu	LeuLeu	CysCys	CysGly	GlyGly	TrpTrp	TrpSer	SerSer
IgG ACLA negative	95	5	0	42	55	3	91	10	0	93	8	0
IgG ACLA positive	7	0	0	2	4	1	7	0	0	6	1	0
P value		0.5546	n.a.		0.6320	0.1056		0.3821	n.a.		0.5557	n.a.
IgG ACLA level	5.90	4.49	n.a.	5.77	5.80	8.03	5.92	5.24	n.a.	5.90	5.34	n.a.
P value		0.5895			0.9752	0.5850		0.7179			0.7747	
IgM ACLA negative	89	5	0	40	50	4	88	7	0	86	9	0
IgM ACLA positive	9	0	0	1	8	0	7	2	0	9	0	0
P value		0.4781	n.a.		0.0529	0.7521		0.1298	n.a.		0.3340	n.a.
IgM ACLA level	3.43	1.51	n.a.	2.53	3.91	3.08	3.13	5.33	n.a.	3.45	1.97	n.a.
P value		0.3687			0.1540	0.6937		0.3596*			0.3606	
Anti-B2GPI negative	10	0	0	3	6	1	8	2	0	9	1	0
Anti-B2GPI positive	1	0	0	0	1	0	1	0	0	1	0	0
P value		n.a.	n.a.		0.4902	n.a.		0.6210	n.a.		0.7401	n.a.
Anti-B2GPI level	3.24	n.a.	n.a.	1.14	4.44	1.18	3.80	0.74	n.a.	3.50	0.73	n.a.
P value					0.4504	0.9316		0.5166			0.6650	

Prevalence and level were tested for heterozygotes and homozygous carriers versus homozygous non-carriers with respectively the Pearson χ^2 test and the student t-test. *Equal variances not assumed as P value of Levene's test for equality of variances equals 0.0464. As not all DNA samples were amplified for a given genetic marker, the total number of cases varies slightly between the mutations examined.

Mean serum β2GPI concentration (μg/mL) in patients with stroke according to the prevalence of IgG ACLA, IgM ACLA and anti-β2GPI antibodies, and β2GPI genotype at codons 88, 247, 306 and 316. P values from student t-tests comparing β2GPI level between individuals with and without antibodies.

Codon	88			247			306			316			Overall
	SerSer	SerAsn	AsnAsn	ValVal	ValLeu	LeuLeu	CysCys	CysGly	GlyGly	TrpTrp	TrpSer	SerSer	
IgG ACLA negative	168.9	158.1		165.3	169.0	153.1	174.0	109.5		170.8	129.7		168.6
IgG ACLA positive	187.0			156.3	207.4	167.1	187.0			198.6	117.6		187.0
P value	0.3288	n.a.	n.a.	0.8053	0.1104	0.0000	0.4292	n.a.	n.a.	0.1565	0.7341	n.a.	0.3244
IgM ACLA negative	172.8	158.0		170.4	172.0	156.6	175.4	120.2		175.9	128.4		172.3
IgM ACLA positive	158.2			69.9	169.3		178.0	88.9		158.2			158.2
P value	0.3775	n.a.	n.a.	0.0447	0.8772	n.a.	0.8786	0.5248	n.a.	0.2797	n.a.	n.a.	0.4004
Anti-β2GPI negative	168.2			127.5	188.7	167.1	188.0	88.9		173.8	117.6		168.2
Anti-β2GPI positive	154.6				154.6		154.6			154.6			154.6
P value	0.8381	n.a.	n.a.	n.a.	0.6271	n.a.	0.5508	n.a.	n.a.	0.7782	n.a.	n.a.	0.8381

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