OXIDATIVE STRESS AND VASCULAR DISEASE IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS AND ANTIPHOSPHOLIPID SYNDROME

by

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ABSTRACT

Systemic Lupus Erythematosus (SLE) and the Antiphospholipid Syndrome (APS) are related autoimmune diseases, characterised by a broad spectrum of clinical features associated with autoantibodies directed towards nuclear and phospholipid / protein antigens, respectively. Accelerated atherosclerosis has been recognized as a major feature of both conditions, however the mechanisms by which it occurs are not clear. Oxidative stress is an important factor in atherosclerosis and the presence of an increased oxidative state has been recognized in SLE and APS.

The overall aim of this thesis is to identify possible factors that contribute to this oxidative stress and to evaluate the mechanisms involved. Two different animal models were used to study the effects of anti-CL antibodies on nitric oxide (NO) synthesis. In a short term functional rat model (4 hours), a human anti-CL IgG monoclonal antibody (IS4) increased NO production through induction of iNOS, whilst in a long term mouse model (30 days), IS4 was associated with a pro-oxidant status with a decrease in paraoxonase activity (an anti-oxidant enzyme present in HDL) and nitric oxide levels, resulting in a decrease in total anti-oxidant capacity of plasma. Nitrotyrosine levels were increased suggesting a previous increased NO production, confirming the findings of the short-term model.

In a clinical study, serum from patients with APS, presented an inverse correlation between anti-phospholipid antibodies and paraoxonase activity, confirming the results reported in the animal models. In patients with SLE, anti-HDL antibodies were identified and inversely correlated with paraoxonase activity and with total anti-
oxidant capacity of plasma. These antibodies were shown to be a heterogeneous group, represented by antibodies cross-reacting with cardiolipin and antibodies directed against a different antigen, probably apolipoprotein A-I.

In another clinical setting, infertile women submitted to *in vitro* fertilization, positive for anti-cardiolipin antibodies but without the Antiphospholipid Antibody Syndrome, presented with similar pro-oxidant changes in their serum, suggesting that these can be a direct consequence of the antibodies rather than an independent characteristic of the syndrome.

In conclusion, anti-phospholipid antibodies may induce oxidative stress in patients with SLE and APS through a short-term up-regulation of nitric oxide synthesis, associated with an inhibition of the anti-oxidant activity of paraoxonase. In long-term, these effects combine to create a pro-oxidant environment, which can explain the enhance atherogenesis and thrombogenesis found in these patients.

**DECLARATION**

The work described in this thesis was carried out by the author, unless otherwise stated.
DEDICATION

To Liz, for making my life worth living...

To my parents and Madalena, of whom I am so proud...

To Adriano, for he will always be here...
ACKNOWLEDGEMENTS

To David Isenberg who was always present, knowing exactly when to support and when to let go....

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To Paul for all the support, both as a friend and as a mentor... for all this started with him during several long scientific sessions, walking along the Thames...

To Emma whose inner strength transforms the greatest sceptic into a proud believer...

To Maria who was always there in times of despair, and there were many...

To Liz Jury who showed up just in time...

To Ravi, Lesley, John, Selina, Kate, Anisur and all the staff from the lab and the department who helped me in every possible way...

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To Brian, who saved my life...

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>anti-CL</td>
<td>Anticardiolipin</td>
</tr>
<tr>
<td>anti-HDL</td>
<td>Anti-High Density Lipoprotein</td>
</tr>
<tr>
<td>anti-LDL</td>
<td>Anti-Low Density Lipoprotein</td>
</tr>
<tr>
<td>anti-PL</td>
<td>Antiphospholipid</td>
</tr>
<tr>
<td>anti-PS</td>
<td>Antiphosphatidylserine</td>
</tr>
<tr>
<td>anti-β2GP1</td>
<td>Anti-Beta-2-glycoprotein 1</td>
</tr>
<tr>
<td>APS</td>
<td>Antiphospholipid Antibody Syndrome</td>
</tr>
<tr>
<td>β2-GP1</td>
<td>Beta-2-glycoprotein 1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CL</td>
<td>Cardiolipin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ds</td>
<td>Double stranded</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>eNOS</td>
<td>Constitutive (endothelial) Nitric Oxide Synthetase</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen binding fragment of antibody</td>
</tr>
<tr>
<td>Fc</td>
<td>Crystallizable fragment of antibody</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FNG</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IMT</td>
<td>Intima-medial thickness</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric oxide synthetase</td>
</tr>
<tr>
<td>IVF</td>
<td><em>In vitro</em> fertilisation</td>
</tr>
<tr>
<td>LA</td>
<td>Lupus anticoagulant</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
</tr>
<tr>
<td>Lp(a)</td>
<td>Lipoprotein (a)</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthetase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidised Low Density Lipoprotein</td>
</tr>
<tr>
<td>PAI</td>
<td>Plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PAPS</td>
<td>Primary Antiphospholipid Antibody Syndrome</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipid</td>
</tr>
<tr>
<td>PON</td>
<td>Paraoxonase</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
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<tr>
<td>ss</td>
<td>Single stranded</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very Low Density Lipoprotein</td>
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Chapter 1:
INTRODUCTION

1.1 Systemic Lupus Erythematosus: a textbook of general medicine

Systemic lupus erythematosus (SLE) is a disease of unknown aetiology characterised by systemic inflammation and an increased production of a wide range of auto-antibodies directed against a multiplicity of nuclear, cytoplasmic and membrane antigens (Mason 1998).

It affects predominantly women during childbearing age but has been diagnosed at all ages, from childhood to the eighth and even the ninth decade of life. Lupus is found worldwide, but seems to be more common among black females. In the United Kingdom, its prevalence varies according to different ethnic origins between 36.2/100 000 for Caucasian females to 90.6 / 100 000 for Asians and 206/100 000 for Afro-Caribbeans (reviewed in Morrow 1999a).

The diversity of its clinical features and the broad range of its forms of presentation are responsible for an immense list of differential diagnoses from cancer to infections, and many other autoimmune systemic diseases.

In 1971 the American College of Rheumatology proposed a list of criteria for the classification of the disease (Levin 1984). These criteria, revised in 1982 (Tan 1982), and updated in 1997 (Hochberg 1997), were defined to provide clinicians and
researchers with objective means which would allow the identification of this entity amongst other immune conditions and the homogenisation of the populations in different studies, and not as a diagnostic tool. However, in practice this concept has been somewhat blurred.

1.1.1 Aetiopathogenesis

The aetiopathogenesis is multi-factorial. Evidence for genetic factors include increased prevalence of the disease in monozygotic (approx. 25%) when compared with dizygotic twins (approx. 3%), increased incidence in members of the same family (Block 1975), and correlations between the major histocompatibility complex (MHC) class II, disease and selected auto-antibodies (Reveille 1991, Arnett 1991). The hormonal system is clearly a susceptibility factor, as the ratio between female and male is 10:1 during the childbearing years but decreases to 2:1 after the menopause (Lahita 1997). Furthermore, several abnormalities in the metabolism of oestrogen and androgenic hormones have been reported (Lahita 1982). Environmental factors also seem to be a very important group in the pathogenesis of SLE and the effect of ultraviolet-B light in lupus flares has long been documented (Lehmann 1990). Other factors such as chemicals, drugs, viral and bacterial infections and psychological stress have also been implicated in the induction of the disease or its exacerbations (Hahn 1997a). All these factors interact with an abnormal immune response, characterised by a hyperactivity of B and T cells, and multiple abnormalities in immunoregulation (Hahn 1997a).
Dysfunction of apoptosis has been associated with autoimmune diseases and particularly with both SLE and APS. However, this phenomenon can be related to the pathogenesis of these conditions in different ways. Firstly, apoptosis of immune cells can induce T cell depletion, leading to immune dysregulation (reviewed in Ravirajan 1999). Secondly, apoptotic cells may present new antigens that have been kept away from the immune system until then, thus creating the conditions for the formation of auto-antibodies. This concept has been further developed in relation to the importance of a defective clearance of the apoptotic products in the generation of pathogenic auto-immunity (Bijl 2001).

Furthermore, mice with mutations of the Fas gene product or the Fas ligand, as well as mice over-expressing bcl-2 are used as animal models of SLE due to the development of a clinical condition very similar to human SLE (reviewed in Radway-Bright 1999).

The clinical diversity of SLE is matched by the multitude of abnormalities found in the sera of these patients. Circulating immune complexes are thought by some to be involved in the immunopathology of the disorder causing damage by depositing in several different tissues of the organism (Emlen 1996).

The complement system is involved in the inflammatory events present in SLE. It is fixed or consumed by immune complexes and localised in the tissues, particularly in the kidney. Different aspects of the importance of the complement include the release of vasoactive amines from mast cells (caused by C3a and C5a), and the increase of vascular permeability as a consequence of the kinin-like activity of C2 (Ruddy 1997).
One of the most important mechanisms relates to the fact that both classical (mainly through C4) and alternative (through C3) pathways prevent precipitation of immune complexes, thus protecting against vascular and organ damage in SLE. Zeitz et al showed that patients with SLE and deficiencies in early-reacting components of the complement were ineffective in making immune complexes soluble, when compared to non-deficient individuals (Zeitz 1981).

Congenital deficiencies of several complement proteins are also associated with a multitude of autoimmune diseases, and may be associated with SLE or SLE-like syndromes (Ruddy 1997). The most common are the deficiencies of proteins involved in the classical pathway, namely C1q, C2 and C4. Despite the similarity with SLE, these patients present several differences from the typical clinical picture of systemic lupus. There is an increased frequency of skin involvement, a decreased frequency of renal disease, with low or absent levels of anti-dsDNA antibodies (Ruddy 1997).

Complement proteins, and particularly C3 and C4, are the most important markers of disease activity in SLE. C4 levels fall prior to the deterioration of the clinical condition, and are usually followed by C3, C1q and C9. The accessibility of these markers is responsible for their wide use in clinical practice for monitoring disease activity (Morrow 1999a).

Research has focused on many different aspects of the disease, and several experimental models have been used, which provide a reasonable parallel with the condition in humans. The two most commonly used models are the New Zealand black and white hybrid (NZB/W) and the MRL/lpr mice. The NZB/W, has proved an excellent model of human lupus, with clinical symptoms becoming apparent from six months of
age (Kotzin 1987). MRL/lpr mice are a lupus-prone strain with a recessive lymphoproliferation gene (lpr) encoding a defective Fas molecule, leading to a defective Fas-mediated apoptosis. Massive lymphadenopathy and hyperplasia of all lymphoid organs are observed with elevated levels of several autoantibodies including antinuclear antibodies, anti-dsDNA antibodies, anti-Sm antibodies and rheumatoid factor. Death usually results from glomerulonephritis, which develops after two to three months of age (Wu 1994).

1.1.2 Auto-antibodies

Around fifty autoantibodies have been identified in the sera of patients with SLE (Harley 1988). The majority of these antibodies are directed against nuclear antigens (Rahman 1994). Of these, anti-dsDNA antibodies are the most important laboratory test for the diagnosis of lupus. They are found in up to 80-90% of the patients during the course of the disease (Cervera 1993; Morrow 1999a) and, unlike antibodies to ss-DNA, are very rarely found in any disease other than SLE (Koffler 1971). Their levels have been related to disease activity (Bootsma 1995), in many, though not all cases, and more specifically they seem to correlate with the degree of renal damage detected histologically (Okamura 1993). Furthermore, they have been shown to have a direct pathogenic effect, with the induction of glomerulonephritis in an experimental model (Ehrenstein 1995).

Other antibodies towards cell nucleus components include anti-SSA, anti-SSB, anti-RNP and anti-Sm with reported prevalence of 25, 19, 13 and 10% respectively, in a
large European study (Cervera 1993). Apart from specific auto-antibodies, which are directly associated with particular manifestations, such as anti-lymphocyte antibodies and lymphopenia or anti-platelet antibodies and thrombocytopenia, only anti-SSA has been consistently related to specific clinical features including the neonatal lupus syndrome, dermatitis, vasculitis and the Sjögren syndrome (Harley 1996).

Anti-cardiolipin (anti-CL) antibodies are present in 20-50% of patients with SLE and the Lupus anticoagulant in 15-35% (Gulko 1993; Morrow 1999a). These antibodies are now part of the classification criteria for SLE (Hochberg 1997) and their likely pathogenic role has been recognised in relation to thrombosis, obstetric morbidity, and more recently, to vascular disease in general and to atherosclerosis in particular (Levine 2002). However, a simplistic approach to the pathogenic role of different antibodies in the context of SLE might result in conflicting results for cross-reactivity has been shown not only amongst antibodies towards different phospholipids but also between anti-dsDNA and anti-cardiolipin antibodies (Diamond 1984). Understanding patterns of cross-reactivity amongst the total antibody population in the sera of patients with SLE becomes even more complex when antibodies against structures composed of multiple and variable antigens (e.g. lipoproteins), are considered.

1.1.3 Clinical manifestations, assessment and treatment

Systemic lupus can involve any organ or system in the body. Non-specific features like fatigue, fever or anorexia are almost universal at some point in the disease and are frequently responsible for delays in diagnosis, whilst searching for an infectious
disease or even a neoplasm. The most frequent organs involved are the skin, the joints, kidneys, lungs and brain. Haematological manifestations (usually cytopaenias) are also extremely common. Other clinical features concern the nervous and gastrointestinal systems, and less frequently, more specific organ involvement (e.g. eye, pancreas, adrenal glands) can be found (Cervera 1993).

Amongst the main clinical features of SLE, vasculitis and nephritis (with or without renal failure) are two of the major risk factors for atherosclerotic vascular disease.

Systemic vasculitis is associated with endothelial activation, leading to atherogenesis. There are several mechanisms that have been put forward to explain this association: firstly, the elevation of acute phase reactant proteins (e.g. CRP and fibrinogen), together with increased levels of pro-inflammatory cytokines, both known to be associated with vascular disease (Danesh, 2000). Secondly, the direct activation of endothelial cells, which is caused by the local inflammation. Thirdly, the higher predisposition for thrombosis, due to the turbulent blood flow and platelet stretch which takes place in the damaged vessel. Finally, endothelial stress caused by vasculitis leads to increased levels of oxidation, which in turn, enhances oxidative stress and endothelial and coagulation activation (Valente, 1997). In clinical practice, patients with SLE and vasculitis have a higher incidence of atherosclerosis, although the distinction between disease activity (as a consequence of inflammation), and vascular damage (as a result of atheroma formation) may not be clear (Morrow, 1999a).

Nephritis is also associated with vascular damage (Morrow, 1999a). Patients with renal disease in the context of SLE present increased levels of acute phase reactants
as a result of chronic or long-standing inflammation, which in turn is associated with atherosclerosis development (Danesh, 2000). Furthermore, glomerulonephritis, (even without renal failure), is associated with oxidative stress, a major factor in atheroma formation. Other aspects of renal involvement and atherosclerosis include hyperlipidaemia (particularly cholesterolaemia) associated with nephrotic syndrome, hypertension and the loss of proteins C and S, which might induce a pro-thrombotic state (Lahita, 1997).

In the context of such a complex clinical picture, distinction between disease activity, complications due to major organ failures, secondary effects related to drugs, co-morbidity and irreversible damage can be very difficult. Hence it is necessary to distinguish and define accurately, clinical activity and damage in order to create tools for assessing responses to treatment, prognosis, or simply to characterise the evolution of the disease. Since the late 1980s several reliable and validated global activity scores have been developed, (e.g. SLAM - Systemic Lupus Activity Measures, SLEDAI – Systemic Lupus Erythematosus Disease Activity Index) (Bombardier 1992; Liang 1988). The BILAG – British Isles Lupus Assessment Group) system offers a more comprehensive overview of activity in eight organs or systems. It was developed on the “intention to treat” principle (Hay 1993). In contrast, just one damage index, the SLICC/ACR (Systemic Lupus International Collaborating Clinics/American College of Rheumatology) damage index, has been described (Gladman 1996).

Treatment is based on a strategy of generalised immunosuppression, mainly with steroids and cytotoxic agents, (e.g. azathioprine, cyclophosphamide). In mild cases, with a predominance of skin and joint features, hydroxychloroquine is often prescribed.
Intravenous gammaglobulins and newer cytotoxics, (e.g. mychophenolate), together with a multitude of different approaches, including hormones, antibiotics and antioxidants have also been used, either as experimental treatments or in individual circumstances (Hahn 1997b).

Steroid treatment is still considered one of the corner stones of the management of SLE, despite the multiple side effects and deleterious consequences of long-term treatment.

Although there has been an increasing awareness of the pro-atherogenic potential of SLE itself, steroids are still perceived as the main culprit in the development of atherosclerosis-related vascular disease.

Steroids increase plasma levels of low-density lipoproteins (LDL), triglycerides and very low-density lipoproteins (VLDL) and are associated with a reduction of high-density lipoproteins (HDL) (Borba, 1997). These variations are associated with a higher rate of atheroma formation, hence the vascular risk associated with steroid usage. Furthermore, steroids can induce diabetes and hypertension, both conditions strongly associated with atherosclerosis.

Another important aspect relates to the possibility of increased oxidation as a consequence of steroid treatment. In fact, patients with SLE on higher steroid dosage (above 7.5mg / day) have been shown to have higher levels of plasma oxidation as measured by F2-isoprostanes (Ames, 1999).

Anti-malarials are the second most used drug in the management of SLE and represent the “other side of the coin” in relation to atherosclerosis. They are commonly used in the less serious forms of the disease, however their role has been extended with
the evidence of immunomodulatory and anti-oxidant properties of chloroquine and its derivates. Chloroquine diphosphate as been shown to revert the increased hepatic synthesis of lipoproteins induced by steroids and therefore is associated with a plasma reduction of the triglyceride and VLDL levels (Borba, 2001). Furthermore, hidroxychloroquine reduces thrombi formation induced by anticardiolipin antibodies in an experimental model (Edwards, 1997). In clinical studies, anti-malarial intake as been associated with a decrease in antiphospholipid titres, and anecdotal reports have suggested the correction of thrombocytopenia with chloroquine (Suarez, 1996). However, and despite cumulative evidence, the mechanism by which anti-malarials are effective is still not clear.

The prognosis of patients with SLE has improved considerably in the past four decades: in the 1950s, the survival rate was around 50% at 5 years (Hahn 1997b), whilst in the mid-1990s, for the same period of time, the survival rate was approximately 90% (Ward 1995). As a consequence of a more aggressive immunosuppression and renal care, using dialysis and transplant, the main cause of death has changed from renal insufficiency to superimposed infections (Cervera 1999; Urowitz 1976). In the last decade, more efficient antibiotic treatment has, in turn, reduced the number of infection-related deaths. At the beginning of the XXI century, the morbidity and mortality associated with SLE are now shifting towards the cardiovascular system and atherosclerosis.
1.2 Antiphospholipid Syndrome: old symptoms, new causes

Seventy-seven years, from 1906 till 1983, separate the first scientific report of an indirect test for the detection of antiphospholipid (anti-PL) antibodies (Wasserman 1906), and the recognition that this phenomenon could be directly related to a group of clinical features as important as stroke or venous thrombosis (Boey 1983).

The antiphospholipid syndrome represents the most recent piece to be assembled in the jigsaw puzzle that is the autoimmune diseases. Since the beginning of the 20th century, different pieces of this complex picture were collected: the identification of cardiolipin in 1941, the false positive serological test for syphilis in 1952, its association with recurrent foetal death, and the suggestion of the term “lupus anticoagulant” in 1977 (Feinstein 1972). Connections that now seem obvious were not so at the time, as most of these discoveries were made in patients with SLE, and were thus blurred in a complex background of symptoms and laboratory findings. In 1983, a definitive association between these biological markers and a group of clinical events was made (Hughes 1983) and, for the first time, these patients were considered as an individual sub-group with a condition which would finally be named in 1986, the Anticardiolipin Antibody Syndrome (Hughes 1986).

The antiphospholipid antibody syndrome, as it is now called is characterised by venous and arterial thromboses and recurrent foetal loss, in the presence of antiphospholipid antibodies, namely lupus anticoagulant, anticardiolipin antibodies, or
antibodies to other negatively charged phospholipids. Clinical and serological criteria were proposed for the classification of the syndrome in 1992, (Alarcon-Segovia 1992), and revised in 1999 (Wilson 1999).

Knowledge of the epidemiology of APS is still in its infancy, mainly due to the scattered distribution of patients with thrombosis through many specialties and the lack of uniformity in the measurement of anti-PL antibodies. Antiphospholipid antibodies are found among young healthy controls with a prevalence of 1-5% (Petri 2000), but reports of the prevalence of anti-PL vary widely. Fields et al reported the presence of IgG anti-CL in 12% of a cohort of 300 healthy elderly (mean age of 70 years) and in 2% of a group of 543 younger adults (Fields 1989), whilst others reported the presence of IgG anti-CL in 1.79% of a population of 1449 healthy pregnant women (without any pregnancy related morbidity) (Harris 1991).

The antiphospholipid syndrome can be found in patients with no clinical or laboratory evidence of any other definable condition (primary or PAPS), or associated with another disease (secondary APS), the most frequent being its association with SLE (Alarcon-Segovia 1989).

The clinical features are dominated by recurrent thromboses taking place in both the arterial and venous circulation, and affecting vessels of any size (Morrow 1999b). Histological findings are consistent with thrombosis, and there is no evidence of associated vascular inflammation. Concomitant vasculitis has been shown in the context of secondary APS, when associated with SLE or other systemic vasculitides (Lie 1996).
Another important aspect of the clinical picture are the obstetric manifestations, characterised mainly by foetal loss, intra-uterine growth retardation, stillbirth, and pre-eclampsia (Lockshin 1998) (see section 1.2.5 Obstetric complications and infertility).

At the extreme end of the clinical spectrum is a condition in which the thrombotic events predominantly involve small blood vessels or a combination of small and large vessel involvement inducing a systemic vascular response that is, in most cases, life threatening – the so-called catastrophic antiphospholipid syndrome (Asherson 2000).

Recently, attention has been drawn to small vessel disease and the similarity found between APS and accelerated atherosclerosis (Shoenfeld 1998). This new way of approaching the disease might give a different perspective to the mechanisms involved, together with suggestions for new therapeutic approaches.

1.2.1 Aetiopathogenesis

The aetiopathogenesis of the syndrome is unknown but, like most of the autoimmune syndromes, evidence so far suggests a multi-factorial origin. Several studies pointed to a genetic contribution based on the fact, for example, that there is an increased incidence of antiphospholipid antibodies in families (Radway-Bright 2000), and that some particular HLA antigens have been associated with the disease. In the UK, DR4 and DR53 antigens were found increased in a population of 13 patients with PAPS (Asherson 1992), whilst DQB1 was the strongest HLA association in a study with 20 patients based in the USA (Arnett 1991). An increased prevalence of DR7 was also
found in Italian women with recurrent pregnancy loss and anti-CL antibodies (Trabace 1991).

Several hypotheses have been proposed to explain the mechanisms by which anti-PL antibodies might promote thrombosis. One possibility relies on the fact that anti-PL antibodies activate endothelial cells with the consequent up-regulation of expression of adhesion molecules and cytokines (Meroni 2000). Others suggested that anti-PL antibodies might induce an oxidant-mediated injury of the vascular endothelium either via an increased up-take of oxidised low-density lipoprotein (oxLDL) by macrophages or through a direct pro-oxidant effect of anti-CL antibodies (Ames 1994). A popular, but perhaps simplistic hypothesis, is based on the importance of β2-GPI as a natural anticoagulant, hence the pro-thrombotic tendency found in consequence of antibody activity towards that protein (Arnout 1998). Even though a variety of hypotheses have been proposed to explain how anti-PL antibodies might cause thrombosis, and some support has been obtained with the use of experimental models, definitive evidence in humans is scarce and is based mainly on epidemiological associations.

1.2.2 Antiphospholipid and anti-β2-GPI antibodies

Antiphospholipid antibodies are the hallmark of the phospholipid syndrome. They are a family of immunoglobulins (IgG, IgM, IgA) with varying affinities for anionic phospholipids (e.g. cardiolipin, phosphatidylglycerol, phosphatidylinositol and phosphatidylserine), plasma phospholipid-binding proteins (e.g. β2-glycoprotein 1, prothrombin and protein C) or complexes of these proteins and phospholipids (Roubey
Cardiolipin (CL) is the most common antigen used in solid phase assays, and antibodies to it are usually detected in a standardised enzyme-linked immunosorbent assay (ELISA) (Harris 1987). Cross-reactivity between different phospholipids is frequent and negatively charged phospholipids, other than cardiolipin, may be substituted for cardiolipin in the ELISA assay (Radway-Bright 2000).

Other diseases, particularly infections, have also been suggested as possible factors for induction of both anti-PL antibodies and eventually APS. In fact, anti-PL production has been associated with malignancies (Duhrs en 1987), haematological conditions (Harris 1985), and a wide range of viral (Bloom 1986), and bacterial infections (Santiago 1989). However, these antibodies differ from the ones found in patients with SLE or APS. For example, in the context of autoimmune diseases, anti-CL antibodies are usually present in higher titre and are more commonly IgG (favouring IgG2 and IgG4 subclasses). They also have higher avidity, and generally require the presence of β2-GP1 as a co-factor, hence being designated β2-GP1 dependent (Hunt 1992; Levy 1990; Sammaritano 1990). It is of interest that these antibodies are often associated with in vitro anticoagulant activity whilst infection-associated anti-PL are not (Triplett 1998).

In contrast, infection-associated anti-PL antibodies are predominantly IgM with low affinity and their binding is independent of the presence of β2-GP1. An exception to this rule has been reported recently, with the identification of β2-GP1 dependent anti-PL antibodies in the serum of patients following parvovirus infection and the suggestion that this might in fact occur in other infectious diseases (Loizou 1997). More relevant
however, is the fact that auto-immune-related anti-PL antibodies are associated with thrombotic events whilst infection-related anti-PL antibodies are not (Morrow 1999b).

As with anti-dsDNA antibodies in patients with SLE, both the isotype and the titre of anti-PL antibodies seem to be relevant to the development of the clinical features of the disease. A strong association between high levels of IgG anti-PL, but not IgM anti-PL, and the incidence of thrombosis, foetal loss and thrombocytopenia has been shown (Alarcon-Segovia 1989). Harris et al have also shown that patients with very high IgG anti-PL levels were more likely to present with repeated thrombosis or foetal loss, than those with low to moderate levels (Harris 1986). However, in a minority of patients with APS, these features can occur in the presence of IgM or IgA anti-PL (Petri 1997).

Particular clinical features have also been associated with IgM (thrombocytopenia) (Khamashta 1998) and IgA isotypes (skin lesions and vasculitis) (Burden 1996; Tajima 1998).

1.2.2.1 Lupus anticoagulant

In 1952 Conley and Hartmann described an association between a circulating anticoagulant and SLE (Hartmann 1952). The term “Lupus Anticoagulant” (LA), still persists even though it is inaccurate; the same phenomenon can be found in other diseases (namely APS) and the anticoagulant activity is only present in vitro (Triplett 1998). It is, in fact, nothing more than a functional assay for anti-PL antibodies. It detects their ability to inhibit phospholipid-dependent reactions in the clotting cascade (Brandt 1991; Pierangeli 1997). Most of the antibodies identified by this method have
prothrombin or $\beta_2$-GP1 as their main target antigens, and like anti-CL antibodies, they are also strongly associated with thrombosis (Triplett 1998).

1.2.2.2 $\beta_2$-GP1 and anti-$\beta_2$-GP1 antibodies

$\beta_2$-GP1 has been recognised as a major player in several pathophysiological mechanisms known to be present in patients with SLE and APS. Structures or systems with which it has relevant interactions include plasma lipoproteins, endothelial cells, monocytes, macrophages and the coagulation cascade (Matsuura 2000). $\beta_2$-GP1 possesses five complement control protein domains, one of which is cryptic and can become exposed when the molecule binds to anionic phospholipids (PL) (Koike 1998). It can also bind negatively charged macromolecules other than PL, including heparin and platelets (Asherson, 1997).

In the beginning of the 1990s this plasma protein was identified as a cofactor for the binding of anti-PL antibodies to anionic phospholipids, as detected in solid phase ELISA (Hunt 1992; Matsuura 1992). Galli et al, first reported that affinity-purified anti-CL could bind directly to the cofactor coated plates (Galli 1990). Another possibility raised at the time was that the target antigen could be the PL-$\beta_2$-GP1 complex, a cryptic / neoepitope generated by the interaction of these two components (McNeil 1990).

Arvieux et al, using a $\beta_2$-GP1 ELISA reported a good correlation between anti-CL and anti-$\beta_2$-GP1 antibodies (Arvieux 1991). Purified anti-CL antibodies were also shown to bind $\beta_2$-GP1 coated plates in the absence of PL, but not $\beta_2$-GP1 in the fluid phase (Keeling 1992). An important hint to help understand these mechanisms was provided by Matsuura et al, when they showed that anti-CL antibodies would bind to
β2-GP1 when coated on oxidised plates (Matsuura 1994). These findings support the view that a conformational epitope is generated when anionic PL or any other pro-oxidant factor and β2-GP1 interact, and some anti-β2-GP1 antibodies recognise this neoepitope. Studies addressing the exact binding location of anti-PL to β2-GP1 have provided different results. Domains I, the inter domain region of I-II, III and IV have all been suggested as potential candidates (Blank 1999; Iverson 1998; Matsuura 1995).

Evidence of the immunogenic potential of β2-GP1 was first provided by Rauch et al (Rauch 1992), who reported that β2-GP1 when injected with CL, acted as a co-immunogen, required for the production of anti-CL antibodies in Balb/c mice. Furthermore, β2-GP1 administration, with or without CL, induced the production of anti-β2-GP1 antibodies. In contrast, mice immunised with CL alone did not produce anti-CL antibodies. Immunisation of NIH/Swiss mice and NZW Rabbits with β2-GP1 also resulted in anti-β2-GP1 and anti-PL antibody production without cross-reaction (Gharavi 1992). Another group, using lipid-free adjuvants to avoid lipid contamination of the β2-GP1, confirmed these findings (Pierangeli 1993). More recently, it was shown that immunisation of mice with a PL-binding viral peptide, which has a structural homology with the PL-binding region of β2-GP1, induced β2-GP1-dependant anti-PL antibodies (Gharavi 1999).

The clinical significance of anti-β2-GP1 antibodies was first addressed by Viard et al, who reported that anti-β2-GP1 antibodies were associated with thrombosis in patients with SLE (Viard 1992). Interestingly, IgG anti-β2-GP1 antibodies were also shown to be associated more strongly with manifestations of APS than anti-CL (Amengual 1996) in another SLE cohort. Balestrieri et al, found an association of IgG
anti-β2-GP1 antibodies with recurrent fetal loss, and of IgM anti-β2-GP1 antibodies with a history of thrombosis and thrombocytopenia (Balestrieri 1995). Anti-β2-GP1 antibodies were also found to be associated with recurrent foetal loss in patients without an underlying disease (Ogasawara 1996).

Two studies have addressed the value of anti-β2-GP1 antibodies as a marker for the clinical features of APS. In both, the positive predictive value of anti-β2-GP1 antibodies for thrombosis was found to be higher than anti-CL antibodies (Roubey 1996; Tsutsumi 1996). However, anti-β2-GP1 antibodies, despite being highly specific were not sensitive for a history of thrombosis (Khamashta 1998).

1.2.3 Experimental models

The full range of clinical manifestations seen in the human syndrome has proved difficult to reproduce in any one experimental model of APS.

Experimental animal research on APS has used several strains of lupus-prone mice on the grounds that this disease is frequently associated with APS. Two strains have been more frequently used: MRL/lpr and (NZW X BXSB) F1. However, anti-PL antibodies have been reported in association with other strains of mice, notably the NOD, usually regarded as a model for insulin -dependant diabetes mellitus (Radway-Bright 1999).

MRL/lpr strains have high titres of anti-CL, present by two months of age in females and by three months in males, which are maximal at five months with a decline at about six months (Gharavi 1989). The anti-CL activity of autoantibodies from this
strain was not found to be \( \beta_2 \)-GPI dependant (Hashimoto 1992), but monoclonal anti-CL antibodies derived from MRL/lpr mice have anticoagulant activity (Ichikawa 1988).

Thrombotic lesions in central nervous system vessels of the MRL/lpr strain have been reported (Smith 1990), but this was not confirmed by other studies and the dominant pathology was generally described as an arteritis. In 1993, cognitive impairment and neurological deficits, without evidence of cerebral infarction, were also reported (Hess 1993). However, the overall neuropathological findings in MRL/lpr mice have limited overlap with those seen in human disease.

NZBW F1 mice develop a very high incidence of right-sided thrombotic coronary vascular disease and myocardial infarction, particularly in males (Hang 1981). Hashimoto et al reported that NZBW F1 male mice produced anti-CL antibodies and the titre increased with age. These anti-CL antibodies were mainly IgG and more importantly, their binding activity to CL was \( \beta_2 \)-GPI -dependent (Hashimoto 1992).

Unfortunately, these models present only a few of the clinical features which characterises human APS, (e.g. in the NZBW F1 strain, arterial occlusion only develops in male mice, even though anti-CL antibodies have also been detected in females).

Two main methods have been used to induce symptoms of APS in naive mice: passive infusion of autoantibodies into a healthy animal, or active immunisation with an antibody (anti-PL) or with an autoantigen (\( \beta_2 \)-GPI).

Healthy strain ICR mice infused with IgG anti-CL antibodies derived from a patient with PAPS presented with lower fecundity, smaller litter sizes and smaller placentae, indicating the importance of anti-CL in the induction of foetal loss (Blank...
1991). The same picture emerges in nearly all the studies in which anti-CL antibodies were infused into naive mice (Suzuki 1996).

Rather than focusing on pregnancy outcome in experimental APS models, Pierangeli and co-workers have established an *in vivo* model for thrombosis using healthy strain, CD-1 mice (Pierangeli 1994). However, this model uses a "pinch" injury as a stimulus for thrombus formation, which does not represent a common physiological pathway for the development of clots. When polyclonal IgG and IgM anti-CL antibodies were injected, 72 hours prior to the "pinch", the thrombi formed were significantly larger, and clots persisted for significantly longer periods, compared with controls.

Active induction of autoimmunity with an autoantibody is suggested to work by inducing disease via the idiotypic network (Bakimer 1995). Successful APS induction, including anti-PL antibody production, has been reported (Cohen 1994). Clinical features of induced APS in these studies were poor outcomes of pregnancies relating to decreased embryo and placental weights, increased foetal resorption and low pregnancy rates. Other shared features included prolonged activated partial thromboplastin time (pANTI-PTT) and thrombocytopenia (Cohen 1994).

Experimental APS in female BALB/c mice also manifests itself neurologically (Ziporen 1997). The mice performed less accurately compared to controls in placing reflex, postural reflex and grip tests, pointing to affected CNS function in the cortex level and brain stem. Spatial behaviour, motor co-ordination and learning ability were also impaired. A more recent study tried to determine the natural course of APS: four mice were injected with β2-GP1-induced anti-PL. After ten months, three of the four mice showed progressive neurological symptoms. Vacuolization and degeneration of
spinal cord neurones, as well as thrombosis in small vessels was observed. This study is
the first description of neurological involvement and thrombosis in mice with \( \beta_2 \)-GPI-
induced APS (Garcia 1997).

Mice with severe combined immune deficiency (SCID) have also been used to
demonstrate the pathogenic role of anti-PL antibodies. Levy et al, injected peripheral
blood mononuclear cells (PBMC) from a patient with APS into SCID mice. Two of the
five mice injected developed anti-PL activity which increased with time, in parallel with
the increase in human IgG concentrations in their sera, up to a peak 8 weeks after
injection. After three months, the mice which had anti-PL antibodies developed renal
injury (deposits in the subepithelial and intramembranous areas of the glomeruli, and

Induction of anti-PL and anti-\( \beta_2 \)-GP1 antibodies was also recorded in larger
mammals such as the New Zealand white (NZW) rabbits (Kouts 1995; Pierangeli 1993).
In an attempt to demonstrate a stasis model of venous thrombosis, IgG anti-CL was
administered to NZW rabbits, which had been submitted to a ligation of the external
jugular vein. Thrombi were induced, however there was a need for an "artificial second
hit" to induce clot formation, in that particular location (reviewed in Radway-Bright
1999).

1.2.4 Coagulation activation and thrombosis

The antiphospholipid antibody syndrome is a thrombophilic disorder in which
patients may develop both arterial and venous occlusions. Any size of vessel may be
affected. Recurrent deep venous thrombosis of the legs is the most common feature on the venous side, but other territories, including axillary, renal, hepatic or even the inferior vena cava can be involved (Asherson 1997). Arterial occlusions leading to transient ischaemic attacks, strokes, myocardial infarction, renal and mesenteric thrombosis amongst others have also been reported (Asherson 1997). Long known thrombotic syndromes have found a new cause: APS is now considered one of the most frequent causes for Budd-Chiari syndrome (Pelletier 1994), and Addison’s disease may be caused by thrombosis of the adrenal veins (Asherson 1991).

Other manifestations include valvular heart disease, livedo reticularis, thrombocytopenia, migraine, epilepsy, chorea, and transverse myelitis (Khamashta 1998). Some of these symptoms have been suggested solely on the grounds of clinical experience in major centres, and though a clear statistical relation with anti-PL antibodies has been reported in some cases, the mechanisms responsible for such clinical features remain unclear.

Although a comprehensive understanding of how anti-PL antibodies might induce the clinical features is still lacking, much evidence has been collected about coagulation activation in the context of APS. Antiphospholipid antibodies have been shown to inhibit the anticoagulant activity of $\beta_2$-GP1 (Arnout 1998), impair fibrinolysis, reduce protein C activation, decrease levels of free protein S, decrease antithrombin III activation and inhibit the factor XII/prekallikrein-mediated fibrinolytic activity (Roubey 1998).

Other reported mechanisms, directly or indirectly related to coagulation activation, include complement activation (Khamashta 1998), platelet activation and
aggregation (Joseph 1998), increase of tissue factor expression (Dobado-Berrios 1999), and decrease function of annexin V (Levy 1998).

However, coagulation activation and endothelial cell studies *in vitro* do not accurately reproduce the complex relationships that take place in the human circulation. Furthermore, the necessity for aggressive anti-coagulation once the syndrome is diagnosed, hampers the conclusions, which might otherwise have resulted from subsequent studies in these patients.

### 1.2.5 Obstetric complications and infertility

Recurrent miscarriages are one of the most consistent features of APS. Losses can occur at any stage of pregnancy, although anti-PL-associated foetal losses are much more frequent in the second and third trimester (Branch 1994). The prevalence of anti-PL antibodies, either anti-CL or LA, in normal pregnant women is less than 2% and fewer than 0.2% have high titres (Harris 1991). In contrast, persistently positive titres of anti-PL antibodies have been found in 15% of women with a history of three or more miscarriages (Rai 1995), and patients with APS, if not treated, have a pregnancy loss rate of up to 80-90% (Branch 1989). The presence of anti-PL antibodies represents an important predictor of poor foetal outcome (Lima 1995): a patient with SLE whose LA test is positive, or with moderate titres of anti-CL antibodies has a 30% risk of miscarriage during the first pregnancy and 70% subsequently (Lockshin 1987). Foetal growth retardation and pre-eclampsia are the other most common features of obstetric morbidity related to APS (Branch 1992; Polzin 1991).
The mechanisms of pregnancy loss in APS are not fully understood. The antibodies do not cross the placenta implying that local pathogenic mechanisms are likely to be responsible (Levy 1998). Fetal death would then result from placental insufficiency, and thrombosis would be the logical explanation for this event. However, not all placentae examined have shown areas of thrombosis (Out 1991), and therefore other mechanisms must exist. Furthermore, even though thrombosis is frequently observed in placentae from patients with APS, approximately half of the women with fetal deaths have placental thrombosis (Levy 1998). Thrombosis may then be related to damage of the decidual/placental interface, regardless of the presence of anti-PL.

Complement activation has also been associated with the pregnancy-related morbidity in APS. Activated complement fragments are known to activate inflammatory and endothelial cells as well as to induce a prothrombotic phenotype (Wetsel 1995). Interestingly, Holers et al have shown that blockage of C3 activation prevents fetal loss and growth restriction in a murine model of APS (Holers, 2002). More recently, Girardi et al put forward the hypothesis that the complement component C5 and its cleavage product C5a, are the key mediators of fetal injury (Girardi, 2003), reinforcing the concept that complement activation is a necessary in vivo intermediary step for the clinically relevant pregnancy-related deleterious effects of APS.

Lesions described in placentae from patients with APS include fibrinoid necrosis, acute atherosis (eosinophilic spiral vessel wall with mural foamy cells) and thickened vessels retaining muscular and elastic fibers (Salafia 1997). It is interesting to note that such pathological findings are strikingly similar to those found in atherosclerotic vessels.
Several mechanisms have been put forward to explain the pathogenesis of anti-PL in this context: blockage of prostacycline and thromboxane production (Peaceman 1995), blockage of placental gonadotrophin secretion (Di Simone 1997), interference with Annexin V function (Sammaritano 1992), and displacement from its placental sites (Rand 1997). Regardless of multiple possible mechanisms to account for the pathogenicity of anti-PL in pregnancy, the histological findings of the placentae of patients with APS are of low specificity as well as sensitivity, suggesting that, rather than a rare or peculiar mechanism, obstetric morbidity is probably due to the combination of several factors commonly found in other pathologies, enhanced by the presence of the autoantibodies.

More recently infertility has been suggested as a possible manifestation of APS. Taylor et al found an increased incidence of anti-CL in infertile women when compared to pregnant controls (17% versus 6%) (Taylor 1989). Another study looked at a complete panel of IgG, IgM and IgA antibodies towards six different phospholipids in 45 women with unexplained infertility. The incidence of anti-PL antibodies in general was 42% compared to 7% in healthy, non-pregnant controls (Roussev 1996), but most of the positives were of antibodies towards different phospholipids and the methods were not standardised. In a third study, investigators assayed stored sera from women with unexplained infertility and compared them to a group of women with infertility of known cause. A greater proportion of women with unexplained infertility was positive for anti-CL antibodies, but the sera were selected from a bank enriched with patients who had previously demonstrated positive autoimmune test results (Aoki 1995). Interestingly, in a blinded study to control for interassay variation, the prevalence of
anti-PL antibodies in women with unexplained infertility was no different from fertile controls (Hatasaka 1997).

The reported prevalence of anti-PL in women attending an IVF clinic has varied widely, the most consistent finding being a relatively high prevalence, compared to normal parous women (Kutteh 1997), normal nulliparous women (Geva 1995) or oocyte donors (Schenk 1996). The cause of this high prevalence is unclear. Studies have found a higher prevalence of antibodies in women with tubal blockage or endometriosis (Nip 1995), while others have found no association between antibodies and a particular infertility diagnosis (Kowalik 1997). In any case, in most studies it is unclear whether the antibody testing was performed before or after IVF treatment.

In respect to this issue, two studies showed that the high prevalence of anti-PL is not caused/exacerbated by IVF treatment. Birdsall et al found no elevation of anti-PL in the serum of women who had previously undergone IVF, compared to those who had not (Birdsall 1996). Fisch et al found no differences in anti-PL antibodies titres at three different time points during IVF treatment (Fisch 1995). There was an elevation of the anti-PL titres among the women undergoing IVF over the normal control women, but the authors could not confirm that previous IVF treatment caused higher anti-PL production. In contrast, Geva et al found that IVF patients with three or more previously failed cycles had a higher rate of anti-PL than women who had conceived within three IVF attempts (22.0% vs. 2.5%) (Geva 1995). Most studies agree however that there is no relationship between the presence of the autoantibodies and IVF outcome (Branch 1998).
The concept of antiphospholipid-mediated infertility is popular and hypothetically attractive. However the data are conflicting and the possibility that IVF can be a cause for the induction of anti-PL antibodies raises interesting questions and opens important avenues regarding management and even the pathogenesis of this condition.

1.3 Atherogenesis and vascular disease

Atherosclerosis literally means hardening of the arteries. It is the most important vascular disorder, and the main cause of death in the western world (Woolf 1999). In general terms, it can be defined as a disorder in which there are widely prevalent, focal lesions, confined mainly to the elastic and muscular arteries.

Patient characteristics such as age, race, gender and geographical variation are related to disease progression and interact with an ever-growing list of risk factors. Hyperlipidaemia, hypertension, smoking, overweight, dietary factors and diabetes mellitus are classical risk factors. Recently, homocysteinemia and chronic inflammation have been found to enhance atheroma progression and are now considered high risk factors (Poulter 1999).

Thrombosis is the most important clinical feature associated with atherosclerosis and both coronary artery disease (CAD) and stroke have been shown to be equally good clinical markers of the disease (Poulter 1999).

Even though dyslipidaemia is still considered the primary insult in the initiation of atherogenesis, and the basic feature of the arterial lesion remains the accumulation of
clusters of “foam cells”, a much broader concept of the pathogenesis of atherosclerosis has led to the acceptance of oxidation and inflammation as key factors in several stages of the disease (Ross 1993). Several authors have, in fact, suggested that atherosclerosis should be thought of as an immune disease such is the preponderance of the components of the immune system involved in the formation of atheroma (Ross 1999).

An important aspect of the pathophysiology of atherosclerosis relates to the atherosclerotic plaque itself and its importance in the development of the clinical features of the disease.

In this condition, arteries are diffusely involved with confluent atherosclerotic plaques. The composition, vulnerability, and thrombogenicity of individual plaques vary greatly without any obvious relation to the well-known risk factors for clinical disease (Burke 1997).

The plaque is composed of two major parts, the atheromatous core and the fibrous cap. The core is rich in extracellular lipids, (mainly cholesterol and its esters), and hypocellular. The macrophage foam cells are present at the periphery of the core (Guyton 1989). The formation of the core is mainly due to necrosis and apoptosis of foam cells, induced by oxLDL, but lipoproteins trapped in the extracellular space without first being taken up by the macrophages may also contribute to core formation and enlargement (Guyton 1994).

Calcification of coronary plaques increases with age and the overall plaque burden, but not with the degree of luminal obstruction (Mintz 1997). Interestingly, culprit lesions responsible for unstable angina and/or myocardial infarction are less calcified, indicating that calcium might stabilise plaques against disruption and
thrombosis (Mintz 1997). In fact, only a small subset of mature uncomplicated plaques is unstable and rupture-prone. Overall, there are three major determinants for the vulnerability of the plaque and its risk for rupture: 1- size and consistency of the atheromatous core, 2- thickness of the fibrous cap, and 3- inflammation and repair within the cap.

Disrupted fibrous caps are usually heavily infiltrated by macrophage foam cells, and recent observations have revealed that such rupture-related macrophages are activated, indicating ongoing inflammation at the side of plaque disruption (van der Wal 1994). Of the multiple factors that might be associated with inflammation and subsequent rupture of the plaque, oxidative stress is particularly important for it induces both endothelial and macrophage activation. This leads to coagulation activation and release of cytokines by mononuclear cells, which will further enhance plaque inflammation.

Another interesting aspect of the importance of oxidative stress in the development of vascular disease relates to type 2 diabetes mellitus and insulin resistance. In fact, early manifestations of vascular disease are present often before hyperglycemia becomes evident. Steinberg et al have shown that endothelial dysfunction is associated with insulin resistance (Steinberg 1996). This was confirmed by Balletshofer et al who confirmed this as a possible mechanism for the induction of vascular damage (Balletshofer 2000). Furthermore, Petrie has shown that NO production is also associated with insulin resistance, suggesting that oxidation and oxidative stress could be the mechanism by which insulin resistance and atheroma formation relate (Petrie 1996).
1.3.1 Oxidative stress: ageing of the blood, ageing of the vessel

Oxidative stress can be considered to occur when the flux of partially reduced forms of oxygen is greater than the ability of the biological system to cope with their production. Under conditions of stress, biological molecules are exposed to pro-oxidant species resulting in irreversible oxidation reactions. Such reactions result in chemical modification of these molecules, which can lead to cellular dysfunction.

In the beginning of the 1990s, a new concept in atherogenesis was put forward, in which oxidative stress in general, and oxidation of LDL in particular, were the driving forces for atheroma formation (Ross 1993). This hypothesis has been widely tested and confirmed, however knowledge of the full extent of oxidation as a pathological mechanism is still far from being completely understood (Witztum 1994).

Two major factors have emerged: the endothelium-derived relaxing factor, now identified as nitric oxide (Palmer 1987), and lipid peroxidation, the most common process of lipid oxidation, also present in lipid-containing structures such as cell membranes or lipoproteins. The multiple interactions between these two substances determine the balance between endothelial function and atherosclerosis.

Nitric oxide is synthesised in animals through sequential oxidation of L-arginine. It is produced by three different nitric oxide synthases (NOS), two of which are constitutively expressed, predominantly in neurons (nNOS) and endothelial tissue (eNOS) (Knowles 1994). An inducible form (iNOS) can be expressed in immune and
endothelial cells and in many other tissues, in response to infection, inflammation or immune activation (Xie 1994).

NO is an important mediator of the microbicidal and tumoricidal actions of macrophages (Nathan 1991), and endothelial NO production is fundamental in the maintenance of endothelial function (Christopherson 1997). Being small and hydrophobic, NO can pass through membranes but its diffusion is limited to only several cell diameters, as it has a half-life of a few seconds (Lancaster 1994).

Nitric oxide is not a strong oxidant and is not capable of beginning the peroxidation chain reaction in unsaturated fatty acids, but in combination with other agents it can modulate lipid oxidation (Darley-Usmar 1992).

Lipid peroxidation has been suggested to be one of the inducers of atherogenesis (Steinberg 1989). It has been demonstrated that NO is a potent inhibitor of LDL oxidation (Hogg 1995), and can protect endothelial cells against the toxic effects of oxLDL (Struck 1995). Therefore NO, by preventing lipid peroxidation, may have a protective role in atherosclerosis. In fact, compromised nitric oxide formation is an early event in atherosclerosis and the disease can be ameliorated by the restoration of nitric oxide synthesis (Maxwell 1998).

Further evidence of the deleterious effects of lipid peroxidation in atherogenesis has been reported in mice, where oxidative stress and lipid peroxidation has been associated with aortic fatty streak formation (Liao 1994), and with the expression of the CD36 scavenger receptor and the cellular uptake of oxLDL by macrophages (Fuhrman 2002). An interesting aspect of the work presented by Fuhrman et al focussed on PON
activity, and they reported a protective role from this enzyme against the effects of oxidative stress in the activation of the immune cells.

Activation of vascular NOS is not the only phenomenon associated with inflammatory or immune conditions, and the global activation of macrophages and endothelial cells results in the production of other active substances, amongst them, superoxide.

Elevation of endothelial superoxide generation is also thought to be an initial component of the atherosclerotic process (Ohara 1993). NO and superoxide react rapidly in biological systems, hence superoxide elevation would result in a decrease in nitric oxide levels (Darley-Usmar 1996). The reduction of the available NO would consequently reduce the suppression of lipid peroxidation. The peroxynitrite formed from the reaction between NO and superoxide would add to the overall oxidative stress (Radi 1991). In addition, peroxynitrite can increase iNOS expression, establishing a vicious circle (Cooke 2002).

The mechanisms by which peroxynitrite compromises cell functionality are diverse: DNA fragmentation leading to a futile repair cycle in macrophages and smooth muscle cells, cellular energy depletion and cell death (Szabo 1996). Moreover, peroxynitrite inhibits mitochondrial electron transport and respiration, with a negative effect on cellular energy production (Radi 1994), and deactivates enzymes involved in energy metabolism (Castro 1994). Peroxynitrite has been shown to oxidise LDL (White 1994) and nitration of tyrosine residues in proteins, (a “marker” of the previous presence of peroxynitrite), has been detected in human atherosclerosis (Beckmann 1994).
The oxidation hypothesis of atherosclerosis, rather than definitively explaining the mechanisms of vascular dysfunction, has generated more questions, unravelling new pathways and interactions, which will provide much controversy for years to come.

1.3.2 Plasma lipoproteins, paraoxonase and β₂-GP1.

Increased plasma levels of very-low density lipoprotein (VLDL), low-density lipoprotein (LDL) and cholesterol along with a reduction in high-density lipoprotein (HDL) are the “classical” features of atherosclerosis (Libby 1998). However, it has been now established that lipids, by themselves, are incapable of inducing atherosclerosis. In fact, “lipid-related atherosclerosis” is dependent on the action of oxygen free radicals, which act as intermediates between these molecules and the target structures: endothelium and monocytes (Ross 1993).

1.3.2.1 Low-density lipoprotein and oxidised low-density lipoprotein

Goldstein et al in 1979, first proposed that modification of LDL was a pre-requisite for their uptake by macrophages (Goldstein 1979). They showed that acetylation led to enhanced macrophage uptake by a novel receptor, termed the “acetyl LDL” receptor. Other similarly chemically modified forms of LDL such as malondialdehyde-conjugated LDL (MDA-LDL) were recognised by the same receptor (Fogelman 1980). Subsequently, the modification induced was shown to be due to oxidation (Steinbrecher 1984). In important parallel studies, it was shown that LDL was cytotoxic to endothelial cells and smooth muscle cells and that the cytotoxicity depended
on oxidation of LDL (Morel 1984). Many studies have now documented that oxLDL, produced by a variety of different techniques, is more easily incorporated into macrophages, and can lead to cholesteryl ester accumulation and foam cell formation (Steinberg 1990). oxLDL is also chemotactic for circulating monocytes, and inhibits the motility of tissue macrophages (Steinberg 1989). It is cytotoxic (Morel 1984), and can induce the expression of monocyte chemotactic protein-1 and adhesion molecules of neighboring cells (Berliner 1990). It is immunogenic and can elicit autoantibody formation (Salonen 1992). Finally, it can activate coagulation pathways (Schuff-Werner 1989), and alter arterial vasomotor properties (Bossaller 1987).

However, oxLDL is not a single homogeneous entity. There are many oxidation products that form from both the peroxidation and fragmentation of the lipid components of LDL, and the modification and oxidative degradation of apoprotein B (Witztum 1991). In fact, different forms of LDL oxidation may be related to different pathophysiological mechanisms and eventually to different clinical aspects of vascular disease. Recently, Holvoet et al reported an association between both oxLDL and malondialdehyde-modified LDL (MM-LDL) and CAD. Similar data have previously been reported by others, however, this study has shown a dichotomy in this association whereby oxLDL was related to stable coronary artery disease whilst MM-LDL was associated with acute coronary syndromes and atherosclerotic plaque instability (Holvoet 1998).

Oxidation of LDL can be affected by multiple factors both intrinsic and extrinsic to the LDL complex itself: the main intrinsic factors are the composition of fatty acids, the LDL content of antioxidants and the phospholipase A2 activity, whilst the most
relevant extrinsic factors are the plasma concentrations of free oxygen radicals, the concentration and activity of plasma anti-oxidant enzymes and the levels of HDL (Witztum 1991).

1.3.2.2 High-density lipoprotein

HDL is a heterogeneous lipoprotein produced in the liver and small intestine. It consists primarily of phospholipids and different proteins (70%), with small amounts of triglycerides. It accounts for approximately 25% of the cholesterol in the blood. It has been sub-classified into four classes according to size and lipid content. Of these, HDL$_2$ and HDL$_3$ predominate in human plasma (Pownall 1999). Apolipoprotein A-I (Apo A-I) is the specific apoprotein of HDL. It exists predominantly in HDL$_2$ whilst in HDL$_3$ there is a balance between Apo A-I and apolipoprotein A-II (Apo A-II) (Atmeh 1983).

An association of low levels of HDL with an increased incidence of cardiovascular events, (the Framingham heart study demonstrated that low levels of HDL are the most potent lipid predictor of CAD) (Castelli 1986), implies an important anti-atherogenic role of this lipoprotein. In fact, HDL has been associated with a decrease in the expression of adhesion molecules by endothelial cells, with an improvement in plaque stability and a reduction in plaque susceptibility to rupture. Furthermore, HDL is associated with anti-oxidant enzymes responsible for preventing LDL undergoing oxidation (Libby 1998).

Endothelial dysfunction is the ultimate step leading to atherosclerosis and thrombosis, and a decrease in the bioavailability of nitric oxide (NO), causing an inhibition of the endothelium-dependent relaxation, is the most prominent feature of this
dysfunction (Schoen 1999). In 1993, Matsuda et al, reported that HDL reversed the decrease in endothelium-dependent relaxation caused by oxLDL in rabbit aorta (Matsuda 1993). These findings were confirmed later by Watts et al, who showed an association between high HDL levels and normal endothelial response to acetylcholine in humans (Watts 1996). The mechanisms by which HDL fulfils its protective role are not completely understood. However, the fact that the pro-atherogenic effect of oxLDL is mainly due to the generation of superoxide and not to the impaired activation of endothelial nitric oxide syntetase (eNOS) (Cominacini 2001), suggests that HDL might carry out its role through its anti-oxidant properties, associated with Apo A-I and paraoxonase.

In cultured endothelial cells, HDL complex attenuates the expression of vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule (ICAM-1) and E-selectin (Cockerill 1995), through a direct inhibition of nuclear factor-kB (NF-κB) (Xia 1999), and counteracts endothelial cell apoptosis induced by oxLDL.

Prostacyclins are powerful vasodilators produced by endothelial cells, which increase the potency of the effects of NO, and inhibit platelet aggregation, hence being considered an important factor in preventing thrombotic events. Incubation of HDL with endothelial cells has shown an increase in prostacyclin synthesis (Fleisher 1982), whilst in plasma HDL has been found to prolong the half-life of prostacyclin (Yui 1988). Another important aspect of HDL is its effect on thromboxane A₂ (TXA₂), a potent vasoconstrictor and platelet activator. HDL₂ subfraction inhibits TXA₂ formation in a dose-dependent fashion. Interestingly, HDL₃ stimulates TXA₂ in cultured endothelial
cells, but the prostacyclin/TXA₂ ratio is still favourable to prostacyclin, unless very high concentrations of HDL are present (Oravec 1998).

HDL has also been recognised as a regulator of platelet function and of the coagulation and fibrinolytic pathways. It inhibits the synthesis of platelet-activating factor (PAF) in a way which is independent of the HDL PAF-acetyl hydrolase enzyme (Sugatani 1996). Plasma levels of HDL have been inversely associated with von Willebrand factor (Blann 1995), and directly with tissue factor pathway inhibitor (Zitoun 1996). HDL has also been associated with protein C and S activation (Griffin 1999), and with the correction of the imbalance between tissue plasminogen activator and plasminogen activator inhibitor-1 induced by glycated LDL (Ren 2000).

Finally, HDL plays a fundamental role in capturing cholesterol from endothelial cells and transporting it back to the liver: the reverse cholesterol transport. Apo A-I and particularly the lecithin:cholesterol acyltransferase (LCAT) are recognised to have an important part in this process (Fielding 2000). The relevance of LCAT in atherosclerosis has been reinforced recently by Vohl et al who reported that this enzyme could also prevent the accumulation of oxidised lipids in LDL (Vohl 1999). However, the presence of MM-LDL would inhibit plasma LCAT activity, thus impairing HDL metabolism and reverse cholesterol transport (Bielicki 1999).

Although it is now possible to conclude that HDL improves endothelial function, fundamental questions regarding the relationship between HDL and endothelial cells remain unanswered. Nevertheless, HDL seems to have a predominantly regulatory role, (as opposed to a more structural one carried out by other lipoproteins), in the maintenance of the balance between endothelial structures and plasma.
This regulatory role is highly dependent, not only on the function of the different HDL components, but also on the structure of the overall complex. This has been highlighted by an important study presented by Van Lenten et al, which showed a loss of protective effect of HDL against LDL oxidation in aortic wall cell cultures (Van Lenten 1995). A change in HDL structure characterised by an increase in serum amyloid A and a decrease in Apo A-I in the acute phase HDL, resulted in a decrease of PON activity in more than 70%. A significant increase in ceruloplasmin was also found in HDL collected from patients two days after having been submitted to cardiac surgery. Again, parallel to an increase in the complex content of ceruloplasmin and the correspondent decrease in Apo A-I, paraoxonase (PON) activity was reduced by 75% (Van Lenten 1995). The authors concluded that absorbance of acute phase proteins into the HDL complex can dislodge Apo A-I and induce dramatic changes in the enzymatic function of paraoxonase, the main anti-oxidant mechanism associated with HDL.

1.3.2.3 Apolipoprotein A-I

Apolipoprotein A-I is an integral component of both spheroid circulating HDL particles and the discoid nascent HDL particles. It is a 243-residue protein, which contains a globular N-terminal domain and a lipid-binding C-terminal domain (Segrest 1994). Apo A-I is an important factor in reverse cholesterol transport by activating the enzyme LCAT, which is necessary for the esterification of the cholesterol molecules of HDL, and leads to the conversion of discoid to spheroid (circulating) HDL (Banka 1991). This process is the main step of a continuous mechanism by which cholesterol is taken from the endothelial cells back to the liver to be excreted in the bile, and is
considered by some authors as the "classical" anti-atherogenic mechanism, associated to HDL.

Apolipoprotein A-I can also block contact-mediated activation of monocytes by T-lymphocytes, inhibiting the production of IL-1β and TNFα (Hyka 2001). This study shows that Apo A-I has anti-inflammatory activity and is able to block one of the most significant activation pathways, not only in atherosclerosis, but also in autoimmune diseases such as rheumatoid arthritis and SLE.

Another emerging aspect of the influence of Apo A-I concerns the antioxidant capacity of HDL, which resides mostly in the activity of paraoxonase (PON). Sorenson et al have showed that PON binding to the HDL particle is dependent on Apo A-I which represents a major factor for the stabilisation of the enzymatic activity (Sorenson 1999). These results were confirmed a year later by Oda et al, who reported that cysteine substitutions in the N-terminal region of Apo A-I significantly increased paraoxonase activity, whilst the same type of mutations in the C-terminal did not change the antioxidant capacity of the enzyme (Oda 2001). This observation suggests that Apo A-I, through its N-terminal region, is an important factor in the co-assembly of the enzyme onto HDL, leading to an optimal PON activity. Furthermore, Apo A-I can increase the formation of phosphatidylcholine core aldehydes, (formed after HDL oxidation by peroxynitrite), which will in turn be hydrolysed to lysophosphatidylcholine by PON (Ahmed 2001). These data suggest that HDL can counteract its own oxidation through a system involving both APO A-I and PON.
1.3.2.4 Paraoxonase

Paraoxonase is a 45KDa "A"-esterase (it hydrolyses organophosphates) named after paraoxon, the substrate commonly used to measure enzyme activity. It requires calcium for both activity and stability. It is inhibited by sulphhydryl reagents, and this inhibition is reversed by cysteine (Sorenson 1995).

PON is part of a multigene family that includes PON1, PON2 and PON3. All three PON genes are located on the long arm of chromosome 7. PON1 (or simply PON) codes for a 355-amino-acid protein (Humbert 1993), and is recognised as the main phenotype, with which anti-oxidant properties have been associated. No mature protein products have been identified in association with PON2 (Mackness 1999), however a 40kDa protein associated with the PON3 gene has recently been cloned and also prevented the formation of mildly oxidised LDL in a similar fashion to PON1 (Reddy 2001).

PON activity is modulated by different polymorphisms. Genetic studies have shown that different activities, associated with different polymorphisms, are controlled by two alleles operating at a single autosomal locus, determining low- and high-activity PON alloenzymes (Playfer 1976). The molecular basis of the PON polymorphism has been shown to be an aminoacid substitution at position 192, with arginine being associated with the high activity and glutamine with the lower activity enzymatic form (Adkins 1993). More recently, another polymorphism at position 55, (leucine to methionine substitution), not originally believed to affect PON activity, has been reported to modulate enzyme activity independently of the 192 polymorphism (Mackness 1997a).
PON activity is present in newborn and premature infants at about half the levels found in adults, and the adult level is reached one year after birth (Ecobichon 1973). After the fourth decade of life, PON activity seems to reduce in association with the ageing process (Senti 2001). Although PON is associated with HDL, and there is an obvious difference between the overall circulating HDL concentration in men and women, no gender differences in PON activity have been reported.

Dietary modifications may influence the enzymatic activity, and the response seems to be modulated by the genetic variance of PON (Rantala 2002).

In fact, food with anti-oxidant properties (e.g. green vegetables, several types of beans and vitamins C, E and D) are associated with an increase in PON activity, probably due to the fact that they may prevent plasma oxidation, which may in itself reduce the enzyme antioxidant capacity (Rantala 2002).

Paraoxonase is synthesised by the liver, and has been found in liver, kidney, lung and brain tissue in rats (Rodrigo 2001). In humans, a search of GenBank for PON sequences has found sequence tags in lung, brain, liver, pancreas and placenta (Mackness 1996). Paraoxonase has also been found in human aortas, both in endothelial and in smooth muscle cells, associated with atheroma progression. The concentration of PON expression was proportional to the extension of the atherosclerotic lesions, suggesting a potential protective response to the oxidative stress present at those locations (Mackness 1997b).

Most studies use a functional assay to establish PON activity, by measuring the capacity of the enzyme to metabolise paraoxon (Eckerson 1983). Indirect quantification of the enzyme became possible after the development of ELISA assays for paraoxonase
(Blatter Garin 1994). However the relationship between quantity and activity of the enzyme, and the correlation between organophosphates- and anti-oxidant-related activities are still matter of debate (Durrington 2001). An important addition to this discussion was provided by Kujiraoka et al, who produced a new “sandwich-ELISA” using a combination of two anti-PON monoclonal antibodies and purified paraoxonase. The authors reported a correlation between serum PON concentration and activity after correction for the presence of the two polymorphisms (Kujiraoka 2000).

The association between PON and atherosclerosis was first hinted at following a report showing a lower enzyme activity in subjects with insulin-dependent diabetes mellitus and familial hypercholesterolemia (Mackness 1991a).

In 1995, Abbott et al demonstrated that lower PON activity levels found in diabetic patients were not a consequence of a reduction in the concentration of PON, but rather that they were caused by a lower PON-specific activity (Abbott 1995). Since then, several epidemiological studies have shown an association between PON polymorphisms and activity with different clinical manifestations of the atherosclerotic disease (Pfohl 1999).

A further contribution to the role of PON as a protective mechanism against atherosclerosis was provided by studies in experimental models. In 1998, Shih et al reported the production of a PON-knockout mouse achieved by targeted disruption of exon 1 of the PON gene. These mice were characterised by an increased susceptibility to atherosclerosis when compared to wild-type littermates (Shih 1998). These results were reinforced by Tward et al, who reported a significant reduction in atherosclerotic lesions in PON transgenic mice, when compared with control animals (Tward 2002).
PON anti-oxidant activity derives from its capacity to minimise the accumulation of phosphatidylcholine oxidation products by hydrolysing the phosphatidylcholine isoprostanes and core aldehydes to lysophosphatidylcholine (Ahmed 2002) in HDL and LDL. This may account for the protective role of HDL in preventing LDL oxidation and thus protecting against the induction of inflammatory and pro-atherogenic responses of endothelial cells, when activated by oxidative radicals (Watson 1995).

Indirect but important information has also been provided by therapeutic studies, which showed an increase in PON activity induced by fibrates and statins, suggesting that this could be another mechanism by which these drugs exert their anti-atherosclerotic action (Balogh 2001).

The importance of PON in atherogenesis and lipoprotein metabolism has now been widely accepted. However, its specific role in atherogenesis and/or thrombosis of each particular disease with vascular involvement will still have to be clarified. No doubt different mechanisms will be present in different diseases, however acute-phase reagents, oxygen radicals and auto-antibodies, all part of inflammatory and immune conditions, are premium candidates for interaction with HDL, thereby jeopardising PON’s protective role.

1.3.2.5 Beta 2-Glycoprotein 1

β₂-GP1 has multiple functions, from regulation of coagulation activation to antioxidant, in relation to the different locations in which it can be found. It has a molecular mass of 50kDa, and is present in plasma at an approximate concentration of 200μg/ml. Approximately 30-40% of plasma β₂-GP1 is associated with different
lipoproteins, namely chylomicron, VLDL and HDL, while approximately 10% is in an apparently free form (McNally 1995a). Its plasma levels are independent of age or gender (Bancsi 1992). It binds negatively charged phospholipids, (hence its association with cellular membranes), lipoproteins, platelets and plasma and viral proteins.

In vitro, $\beta_2$-GPI inhibits thrombin formation at two different levels: contact activation and prothrombinase activity (Nimpf 1987). Even though excessive formation of thrombin and an increased risk of thrombosis are in theory associated with a deficiency in $\beta_2$-GPI, this in itself, is an unlikely risk factor for the development of thrombosis (Bancsi 1992), which is probably due to other haemostatic regulatory factors coming into play. $\beta_2$-GPI also seems to have some anti-oxidant activity and has been described as part of a system which operates principally in abnormal conditions of oxidative stress and inflammation (Harper 1998). The possible anti-atherosclerotic effect of $\beta_2$-GPI via macrophage oxLDL interaction may be another example of its multiple roles (Hasunuma 1997).

Patients with primary hyperlipidaemia have been shown to have significantly higher $\beta_2$-GPI levels, compared to controls, as well as a different distribution between the lipoprotein fractions, suggesting a direct relationship between plasma lipoprotein and $\beta_2$-GPI levels (McNally 1994). Lin et al showed that $\beta_2$-GPI present in a macrophage culture system inhibits LDL oxidation and electrophoretic mobility. Furthermore, it decreased cellular accumulation of cholesterol via a reduction in cholesterol influx and an increase in cholesterol efflux (Lin 2001).

Adding to its interactions in plasma, $\beta_2$-GPI has an important relationship with the endothelium. Localisation of this molecule has been demonstrated in human
atherosclerotic plaques and CD-4 positive lymphocytes densely infiltrated the areas in which \( \beta_2\)-GP1 was expressed (George 1999a). The importance of this location is highlighted by Del Papa et al, who showed that the binding of certain autoantibodies to endothelial cells and platelets depends on the presence of \( \beta_2\)-GP1 (Del Papa 1995). In 1997, Hasunuma et al reported an important interaction between \( \beta_2\)-GP1 and oxLDL uptake by macrophages, which is a fundamental step in early atherosclerosis. This group has shown that uptake of \( \text{Cu}^{2+}\)-mediated oxLDL by macrophages was inhibited in the presence of exogenous \( \beta_2\)-GP1. This finding is particularly relevant in patients with APS, due to the presence of anti-\( \beta_2\)-GP1 antibodies.

\( \beta_2\)-GP1 has been the subject of many studies in the last decade, in part due to its recognised role in SLE and APS (see sections 1.2.2.2 and 1.4.4.2 of this chapter).

### 1.3.3 Atherosclerosis and the immune system

The cellular component of the atherosclerotic process is present from the earlier stages of the disease and reflects a much more comprehensive systemic response. Plasma lipids and particularly oxLDL activate endothelial cells, inducing the production of the stimulators of leucopoiesis, macrophage, granulocyte-macrophage and granulocyte colony-stimulating factors, which will increase the maturation and release of monocytes from the bone marrow and induce monocyte and T lymphocyte infiltration in the intima of the artery wall (Anderson 1995).

Cells in atherosclerotic lesions express the immune mediator CD40 and its ligand, CD40L (Mach 1997). CD40 ligation is required for T-cell priming, and leads to
an enhanced production of Th1 cytokines (IL-2 and INFγ), which induce macrophage
activation and production of IL-12, that, in turn, promotes INFγ production by activated
T-cells, creating in this way a positive loop (Lee 1999).

Furthermore, INFγ induces the expression of the scavenger receptor in
macrophages increasing the oxLDL uptake, and inhibits the adenosine triphosphate-
binding cassette transporter-1, a key structure in the mechanism of cholesterol efflux,
thus contributing with a “double hit” to the formation of “foam cells” (Panousis 2000).

Several authors have questioned whether macrophages were the dominant
cellular element in the process or whether they were merely effectors, controlled by
activated lymphocytes. Atherosclerotic changes in pig and human arteries implanted in
immunodeficient mice (without lymphocytes), were induced by INFγ, suggesting that
macrophages rather than lymphocytes are the driving force in atherogenesis (Tellides
2000). These results confirmed the findings of Fyfe et al, who showed the development
of typical atherosclerotic fatty streaks in different types of immunodeficient mice. The
interesting findings, however, were that SCID and athymic “nude” mice had the same
extension of atherosclerotic lesions as controls, whilst MHC class I deficient mice had
an increased rate of atheroma progression. These data suggest that cellular immune
response and particularly T-suppressor cells may in fact act to suppress lesion
development (Fyfe 1994). Such a possibility could be consistent with the observation
that mice treated with cyclosporin A exhibit increased atherosclerotic progression
(Emeson 1993), since cyclosporin exerts a preferential immunosuppressive effect on T
suppressor cells (Sakaguchi 1989).
Another important aspect of the relevance of monocytes in the first steps of the atherosclerotic process regards the production of tissue factor (TF). In fact, its role as an important activator of the coagulation cascade has lead to the hypothesis that its importance was not limited to the induction of atheroma, but also that its presence could contribute to a higher chance of rupture of the atherosclerotic plaque. Several authors have reported not only an induction of the expression of TF on monocytes by oxLDL and cholesterol in vitro (Wada 1994), but also a reduction of the expression of the tissue factor pathway inhibitor, which would tip the balance even further (Petit 1999). Interestingly, one of the few studies made in patients’ sera, showed a decrease in monocyte tissue factor response in patients with hyperlipidemia (Rugeri 1999). However, the interpretation of this result should take into account that these were circulating cells and not monocytes adherent to the endothelial wall, as the ones analysed in in vitro studies. The extremely short half-life of most cytokines, which are responsible for monocyte activation, may account for the limitation of the activation process to the surroundings of the atherosclerotic plaque, preventing in this way a systemic activation of coagulation.

Another aspect of the systemic character of atherosclerosis is clearly illustrated by the elevation of inflammatory parameters in the serum of these patients. However, it is unclear whether the pattern and magnitude of this increase vary with the site and extent of the disease. Different acute-phase reactants and even the white-cell count have been associated with cardiovascular risk (Kanda 1996). Of these, C-reactive protein (CRP) seems to be the most relevant, and this ability has been reinforced by the
discovery of its capacity to bind oxLDL and increase its uptake by different cells (Chang 2002).

The elevation of several other inflammatory markers has also been reported: serum amyloid A, IL-6 and TGF-β, neopterin and calcitonin (Erren 1999). Interestingly, these markers were not independent predictors suggesting that they might be associated with the disease but are a consequence of previous events and do not provide discriminatory power above established risk factors.

The concept that acute phase proteins may be involved in the atherosclerotic process, either by slowly contributing to its progression or by inducing sudden disturbances, which may trigger acute events like thrombosis, has been suggested by clinical observation and epidemiological studies. Bacterial infections seem to be associated with an increased risk of atherosclerosis (Valtonen 1993), and deaths from cardiovascular disease increase during and after influenza epidemics (Tillett 1983). Case-control studies have suggested that 4% of bacteraemic patients will develop acute myocardial infarction within a month of the onset of infection and that 10% of all strokes may be associated with preceding bacteraemic infections (Valtonen 1993).

Several mechanisms have been put forward to explain this link. A systemic inflammatory response would increase circulating clotting factors including fibrinogen. Alternatively, endothelium function might be altered following exposure to pro-inflammatory cytokines, or it may be that oxidative stress, induced by infections, can hamper the protective mechanisms of the vascular wall leading to endothelial dysfunction (Bhagat 1996). An interesting hypothesis has been summarised by Vallance et al, whereby the activation of iNOS by infective or inflammatory states would down-
regulate eNOS, and that after recovery of the acute insult, the endothelium would remain dysfunctional for a long period, with an increased predisposition towards vasospasm and thrombosis (Vallance 1997).

Plasma levels of CRP have predictive value for individuals admitted to hospital with acute coronary ischemia (Biasucci, 1999). The role of CRP is difficult to assess because acute ischemia may itself trigger an inflammatory response, however, epidemiological data supporting the role of CRP as a marker of vascular risk are consistent across different study populations (Danesh, 2000). In a prospective case control study, Ridker et al have shown that CRP was the most significant predictor of the risk of cardiovascular events amongst 12 different variables, including inflammation markers, plasma lipids and lipoproteins and homocysteine (Ridker 2000).

To support the clinical data, CRP has been found in atheromatous plaques and correlate with endothelial dysfunction. Furthermore, CRP increases the expression of adhesion molecules, in this way promoting coagulation activation (Ridker, 2001).

The analysis of the endothelial cell response to different pathophysiological studies has lead to the study of nuclear factor-κB. This transcription factor has been shown to modulate the cellular response to both immune and inflammatory stimuli, inducing the expression of adhesion molecules, cytokines and nitric oxide synthetase (Collins 1993). A common intracellular link between the oxidation and immune pathways would not only explain the confirmed association of the two systems (Collins 1993), but would also suggest targets for therapeutic approaches of atherosclerosis-related conditions.
1.4 Is atherogenesis present in patients with SLE and APS?

The concept that atherosclerosis could be an important issue in patients with SLE was first hinted at by Urowitz in 1976 when he described a bimodal pattern of mortality: in the first peak, infection and renal involvement represented major causes of death, occurring early in the disease, whilst atherosclerosis-related complications were the major cause of mortality in the second, later peak (Urowitz 1976). At the time, steroid-related complications, together with nephrotic syndrome and chronic inflammation, seemed the obvious causes, and for the following two decades vascular morbidity was put down mainly as an unavoidable drawback of steroid use.

This concept was modified in 1997 by Manzi et al who reported that patients with SLE had a x50 higher risk of developing cardiovascular disease and stroke, even after the effect of the traditional risk factors defined in the Framingham study were removed (Manzi 1997). Added to this was an overall clinical impression, that some patients with SLE and vascular disease did not have renal disease, had less obvious inflammation than patients with other chronic diseases, and had always been on very low steroid doses or even none at all. Since then, several reports have addressed atherogenesis in patients with lupus. However, whilst atherosclerotic complications are well described, very few studies have investigated the prevalence of objectively documented atherosclerosis in patients with SLE.
In patients with APS, the information is even more limited and little objective data has been produced. In fact, the clinical characteristics of APS raise a methodological problem for the study of atherosclerosis, since most of the studies analyse the prevalence of atheroma-related complications (e.g. cardio- and cerebro-vascular disease), as an indirect method of assessing vascular disease. In patients with APS, stroke and myocardial infarction are direct features of the syndrome and therefore how can they be distinguished from atherosclerosis-related events? Furthermore, recurrent thrombosis, regardless of the cause, might accelerate the atherosclerotic process (Chambless 1997), which leads us to two major dilemmas: firstly, accelerated atherogenesis in patients with APS might be simply due to the fact that they had previous thrombotic events, and secondly patients with non-APS-related thrombosis should always be included as a control group.

1.4.1 *In vitro* studies in SLE and APS

Most of the *in vitro* assays so far have targeted the cellular components, which constitute the atherosclerotic plaque, namely monocytes and endothelial cells and focus on the first steps of atheroma formation.

Anionic phospholipid-containing vesicles are pro-coagulant structures present in plasma as a result of lipid metabolism and oxidation/redox system interactions. Monocytes clear plasma from these vesicles, avoiding undesirable activation of the coagulation cascade. $\beta_2$GP1 plays an important part in this process by binding to the anionic vesicles and facilitating their incorporation into macrophages through a receptor-
mediated pathway (Thiagarajan 1999), hence the presence of anti-β2-GP1 or anti-PL antibodies, as in APS syndrome, might hamper the protective action of β2-GP1.

Macrophages are also responsible for the normal clearance of oxLDL, which is another aspect of the contribution of these cells to the overall control of oxidation in plasma. Increased levels of oxLDL can induce the production of anti-oxLDL antibodies, recognised now as important participants of the atherosclerotic process (Salonen 1992). Anti-oxLDL-oxLDL immunocomplexes are incorporated by macrophages at a higher rate, leading to the production of "foam-cells". β2-GP1 is important in preventing an increased uptake of these immunocomplexes by the macrophage (via the scavenger receptor), thus preventing the trigger of the atherosclerotic process. Again, the presence of anti-PL or anti-β2-GP1 antibodies can block the protective action of β2-GP1 (Hasunuma 1997).

Monocyte adherence to human umbilical vein cells can be induced by anti-CL antibodies purified from APS patients (Simantov 1995). This effect, which constitutes the next step in atheroma formation is mediated by activation of endothelial cells, resulting in the production of the adhesion molecules ICAM-1, VCAM-1 and E-selectin (Del Papa 1995). Binding of monocytes to the stressed endothelium is, in turn, reinforced by the production of monocyte chemoattractant protein-1 (MCP-1). This protein facilitates trafficking of activated monocytes to endothelium and crossing of the endothelial barrier (Cho 2002). Furthermore, MCP-1 can induce the production of tissue factor, integrin, pro-inflammatory cytokines and arachidonic acid by monocytes (Schecter 1997).
Another aspect of *in vitro* activation of endothelial cells by anti-PL antibodies involves the fact that β2-GPI acts as a "co-factor" for the binding of anti-CL antibodies to endothelial cells (Del Papa 1995).

Anti-β2-GP1 antibodies can induce adhesion molecule expression and IL-6 secretion from both HUVEC and human brain micro-vascular endothelial cells, suggesting a common pattern of reactivity with both micro- and macro-vessel related endothelial cells, in accordance with the spectrum of clinical manifestations (Raschi 2000).

Binding of anti-β2-GP1 monoclonal antibodies to endothelial cells in the vessels of placental villi and to membranes of trophoblast cells has also been reported, in relation to potential pathogenic mechanisms for explaining the obstetric manifestations of APS (La Rosa 1994).

Similar to anti-CL antibodies in patients with APS, anti-endothelial cell antibodies, isolated from sera of a patient with SLE were also found to induce the production of E-selectin, ICAM-1 and IL-6 in a dose dependent manner. The authors also showed an activation of NF-κB, in relation to a decrease in the levels of I-κB, in an inverse fashion in relation to antibody concentration (Yazici 2001). None of the monoclonal antibodies used in this study had activity towards dsDNA, CL or β2-GP1.

These *in vitro* experiments have lead to novel treatment options, specifically directed at this particular mechanism. Thus, in 1999, Blank *et al* reported the inhibition of endothelial activation by anti-β2-GP1 monoclonal antibodies, using peptides (selected from a phage display library), which reacted specifically to the antibodies considered. Endothelial cell activation was assessed through measurement of E-selectin, VCAM-1
and ICAM-1 expression. These peptides, when administered to mice previously injected with the respective antibody, prevented the development of clinical manifestations of APS (Blank 1999). Recently, Meroni et al., reported that statins reduced the activation of endothelial cells by anti-β2-GP1 antibodies. Both fluvastatin and simvastatin reduced, in a concentration-dependent manner, the adhesion of U937 cells to HUVECs and the expression of E-selectin and ICAM-1. Furthermore, the authors showed that these effects were mediated by inhibition of NF-κβ translocation and could be reversed by mevalonate, suggesting a link between the inhibition of the enzyme HMG-CoA reductase and the activity of the drug (Meroni 2001).

### 1.4.2 Experimental models

Experimental models are one of the main options for the study of atherosclerosis in general and APS in particular. However, experimental models for atherosclerosis in SLE do not exist and most of the data are extrapolated from mixed (SLE/APS) models, where most of the thrombotic or atherosclerotic-related events are associated with the presence of anti-PL antibodies.

Cardiolipin and anti-CL antibodies, β2-GP1 and oxLDL are the most common substances used to trigger atherosclerotic progression in animal models. Two pro-atherogenic mouse models, LDL-receptor knockout and Apolipoprotein E deficient mice, have been used as "background" models of atherosclerosis on top of which, changes can be induced by active or passive immunisation.
In 1997, George et al described acceleration of atherosclerotic progression in LDL-receptor knockout mice after immunisation with anti-CL antibodies. Atherosclerosis, quantified by calculating the area of the fatty streak lesions on the aorta of the mice was increased in the anti-CL immunised group when compared with irrelevant IgG immunised controls (George 1997a). In a similar experiment, the same type of mice were divided into three groups according to different administered antigens: β2-GP1, ovalbumin or non-immunised. Within each group, mice were fed either a normal chow or high cholesterol pro-atherogenic diet. All the animals immunised with β2-GP1 developed antibodies against that protein. Aortic fatty streak lesions were larger in the mice immunised with β2-GP1 and submitted to a normal chow diet, when compared to ovalbumin and non-immunised groups. Interestingly, there were no significant differences amongst the groups fed with a high-cholesterol pro-atherogenic diet, regardless of the immunisation protocol (George 1998).

In both studies, increased atherogenesis is achieved in an animal lacking LDL-receptors, in an attempt to overcome the natural resistance to atherosclerosis shown by mice. However, interference with this receptor might introduce a different variable not found in the human form of the disease, particularly when the importance of LDL uptake has already been emphasised by several groups using different methodological approaches. Furthermore, the fact that there were no differences between animals when fed with a high-cholesterol diet suggesting that the effect of β2-GP1 immunisation, if indeed there is one, could easily be overcome.

In 2000, the same group reported an increase in atherosclerotic lesions in mice transplanted with lymphocytes and splenocytes from β2-GP1 immunised mice, when
compared with mice transplanted with cells collected from controls. Interestingly, when T-lymphocytes were depleted from the splenocytes, these failed to induce the same lesions (George 2000). This study highlights the importance of T cells in atherosclerosis together with the suggestion that β2-GPI may be a relevant antigen in the process. However, both facts have already been reported in the context of general atherosclerosis, hence the reported results are not specifically relevant to APS.

Immunisation with β2-GPI was also used in apolipoprotein E-deficient mice, fed a high-cholesterol diet, with similar results. Atherosclerotic-related lesions were larger in immunised animals when compared with controls, as has previously been reported with other models (Afek 1999; George 1999b).

Another study reported an exacerbation of the clinical manifestations of an experimental model of APS induced by oxLDL. Mice previously immunised with anti-CL antibodies were subsequently infused with oxidised and native LDL. The oxLDL group exhibited a significantly more severe form of the disease in comparison to the native LDL group (George 1997b). This study highlights the role of oxLDL in the pathogenesis of APS and corroborates similar findings reported by other investigators in in vitro and epidemiological studies (Hayem 2001; Witztum 1991).

The complexities of the oxidative balance in living organisms has frustrated a more comprehensive understanding of the interactions between oxidants and antioxidants in in vitro systems. Currently, only a few studies have addressed this issue in experimental models, and they are predominantly descriptive, reporting oxidation-related characteristics of known autoimmune models. Venkatraman et al reported a weak defensive system against oxidative stress in MRL/lpr mice. These mice have a
lower mRNA expression of catalase, glutathione peroxidase and superoxide dismutase, which corresponds to lower antioxidant activities of these enzymes (Venkatraman 1994).

An interesting study was performed in MRL/lpr mice by Weinberg et al, which showed increased expression of iNOS with corresponding NO activity. Furthermore, oral administration of N-monomethyl-L-arginine (NMMA), a NOS inhibitor prevented the development of glomerulonephritis and reduced the intensity of inflammatory arthritis in this lupus model (Weinberg 1994). This study was complemented by Oates et al, who reported the same therapeutic effect of NMMA, following the onset of the clinical manifestations in the same mouse model (Oates 1997).

These three studies suggest the existence of a significant imbalance in the control of oxidation in SLE. An increase in NO production, as suggested by the later studies, would not be compensated for by the impaired performance of the antioxidant mechanisms (as suggested by the former), resulting in increased oxidative stress.

1.4.3 Oxidative stress in SLE and APS

Oxidative stress is now recognised as one of the major factors contributing to vascular disease in general and atherosclerosis in particular. Free radicals activate immune and endothelial cells and induce “oxidative damage” in isolated or membrane-incorporated lipids and lipoproteins (Ames 1994).

Between 1992 and 1994, together with the first animal studies, a few reports were published, describing changes in the oxidative status of patients with SLE. Serum
lipid peroxide levels were increased and superoxide dismutase activity decreased in a cohort of 83 patients with SLE when compared with 29 healthy controls. Both parameters correlated with ANA and dsDNA titres, C3 levels and disease activity, with a significant difference between lipid peroxide levels and dismutase activity being found between active/not treated and inactive/controlled patients (Jiang 1992). Unfortunately, a multifactorial analysis was not performed and it was not established if either of the two parameters was an independent predictor, hence the authors could not draw any conclusions about the significance of a decrease of the activity of superoxide dismutase on serum lipid peroxide levels.

Based on the capacity of eicosapentaenoic and docosahexaenoic acids to modulate oxidant stress and their suppressive actions on the production of IL-1, IL-2 and TNFα, Mohan et al performed a comprehensive analysis of the oxidative status of patients with SLE before and after oral supplementation of these two essential fatty acids. They reported a decrease of the levels of lipid peroxides, along with an increase in NO levels, and superoxide dismutase and glutathione peroxidase activities, after treatment (Mohan 1997), which also induced significant clinical improvements. These results reinforced the concept that oxidative stress is present in patients with SLE and suggested that its manipulation could be a therapeutic option in this condition.

However, low NO levels in plasma of SLE patients, were not confirmed in subsequent studies. In fact, NO production has been repeatedly reported as elevated in patients with SLE, mimicking previous studies performed in other autoimmune diseases (Wanchu 2000). Belmont et al found higher levels of plasma NO in patients with SLE when compared to controls. Skin biopsies performed in these patients, also showed
increased expression of both iNOS and eNOS in endothelial cells and keratinocytes (Belmont 1997). Similar results were described by Wanchu et al, which reported an increase in NO levels in the plasma of a group of 26 patients with SLE when compared with healthy controls (Wanchu 1998). A different method to assess NO levels, but with similar results, was presented by Rolla et al, who measured the NO content in exhaled air of patients with SLE when compared to controls. An increased NO level was found in the SLE group and it correlated with disease activity (Rolla 1997). However, this method does not allow discrimination between a systemic or respiratory origin for the increased nitric oxide.

Definitive data came from two prospective studies looking at NO levels and disease activity: Gonzalez-Crespo et al showed an increase in NO levels and urinary nitrate/creatinine ratio in 50 patients with active SLE when compared to inactive patients or healthy controls (Gonzalez-Crespo 1998) and Gilkeson et al reported increased plasma levels of NO and nitrotyrosine in a cohort of 26 patients with SLE. In the first study, the authors could not find a correlation between both serum and urinary NO levels and disease activity as measured by SLEDAI, however NO determinations may have been influenced by the high rates of infection (24%), found in this study. In the second study, NO levels correlated with disease activity, particularly in patients with nephritis (Gilkeson 1999). Nitrotyrosine, as a marker for overproduction of NO was also found to be elevated in patients with SLE in a third study, and its levels also correlated with disease activity (Oates 1999).

In 1999, Ames et al showed an increase in the levels of F2-isoprostanes (a marker for lipid peroxidation) in patients with SLE. Interestingly, steroid dosage
correlated with F2-isoprostane levels in a "u-shaped" pattern, with higher levels of lipid peroxidation being found in patients not taking steroids or on prednisolone dosages above 7.5mg/day (Ames 1999). A possible explanation would be that low oxidative stress was present in patients with good disease control (requiring only low dose steroids), as opposed to the non-medicated ones. On a high-dose regimen, the proatherogenic effects of steroids, (along with a more active disease), would predominate, leading to an enhanced lipid peroxidation.

Unfortunately, none of the previous studies considered anti-PL antibodies or associated APS in the analysis of oxidation markers. In 1997, luliano et al analysed the levels of F2-isoprostanes in a cohort of 30 patients with SLE compared with a control group. Not surprisingly, lipid peroxidation was increased in the SLE group, but when anti-PL antibodies were considered, a strong correlation was found between antibody titres and F2-isoprostane levels (luliano 1997). Furthermore, patients with SLE and without anti-PL antibodies had similar levels to the controls, and all the patients positive for anti-PL had F2-isoprostane levels in the same range, regardless of the existence of previous thrombotic events. These results suggest that oxidative stress was related to the presence of anti-PL antibodies and not to the syndrome itself. However a definitive conclusion could not be reached due to the small number of patients studied. Furthermore, the design of the study (cross-sectional), did not allow any conclusions to be drawn regarding a causal effect of one of the variables on the other.

Different results were reported by Porta et al, who could not find a significant difference in plasma levels of NO in a population of patients with SLE, with or without anti-CL antibodies, and healthy controls (Porta 1997). The small number of patients
(n=13), was further divided into several groups: anti-CL positive, anti-CL negative, active SLE and PAPS (only 3), which may account for the lack of statistical significance.

In 1998, Ames et al published the first study looking at oxidative stress in patients with PAPS. F2-isoprostanes and urinary NO were assessed in 10 patients with PAPS and 10 healthy controls. Despite the small number of patients, a significant difference was found between plasma levels of F2-isoprostanes in the two populations, with higher values being present in the PAPS group. Furthermore, a positive correlation was found between the lipid peroxidation marker and anti-CL titres, confirming the findings reported previously, in patients with SLE (Ames 1998). Interestingly, anti-CL antibody titres inversely correlated with urinary NO levels. These results suggest a decrease in NO production in relation to the presence of anti-CL antibodies in patients with PAPS, contrasting with the results in patients with SLE. Again, a causal relationship between the immune response and lipid peroxidation could not be determined. Furthermore, a definitive conclusion about whether the low levels of NO might be due to a decrease in production or an increase in consumption could not be reached. However, the fact that lipid peroxidation was found to be increased, suggests a previous excess of pro-oxidant radicals (mainly peroxynitrite).

The importance of oxidative stress in PAPS was reinforced by Ames et al, in 2000, with a reported decrease in elevated plasma oxidative markers (F2-isoprostanes, urinary NO and thromboxane B₂) in a group of patients (n=14) with PAPS, treated with probucol (a cholesterol lowering agent with anti-oxidant properties). These findings were independent of cholesterol levels and did not occur in a control population.
Furthermore, all the tested markers returned to pre-treatment levels three weeks after stopping treatment (Ames 2000a). Throughout the study, NO levels were always significantly lower in patients with PAPS when compared to the control population and correlated in an inverse fashion with anti-CL antibody titres, confirming the previous observation made by the authors. This pilot study supported the presence of oxidative-sensitive mechanisms in PAPS, and a potential role for antioxidant treatment in this disease.

In 2000, Lambert et al reported a study in which several biological markers for atherosclerosis were measured. Seventy-one patients with different autoimmune diseases, positive for anti-CL antibodies, were compared with a control group matched for age and gender. Paraoxonase activity was found to be dramatically decreased in these patients, but the enzymatic activity according to the different disorders was not assessed and no correlation was detected between the clinical and biological variables and the enzymatic activity (Lambert 2000). A reduction of PON activity could account for the increased lipid peroxidation previously reported, however the authors offered no explanation for their findings.

1.4.4 Lipids, associated plasma proteins, autoantibodies and vascular disease in patients with SLE and APS

Dyslipidaemia, in its multiple forms, is another major risk factor for atherosclerosis (Kannel 1987a). In patients with SLE, multiple deviations from the normal plasma lipid pattern have been reported, either as a complication of treatment,
secondary to nephrotic syndrome or renal failure, or associated with different features of the disease (Manzi 2000).

1.4.4.1 Plasma lipids and lipoproteins

In a cohort of 100 patients, total cholesterol, LDL and TG were found to be significantly elevated in those patients with SLE who had renal disease or were on high dose of steroids. A decrease in total HDL was also found in patients on high-dose steroids (>30mg prednisolone/day) (Leong 1994). Interestingly, the authors did not find any lipid abnormalities in active lupus patients, provided they did not have renal involvement. Similar findings were reported by Borba and Bonfa, who described high levels of VLDL and TG plus low levels of HDL in 36 women with SLE when compared with controls, but none of the patients had been treated with steroids or chloroquine in the previous 3 months, nor had renal disease (Borba 1997). Furthermore, there was a direct correlation between disease activity (as measured by the SLEDAI score), and the lipid abnormalities found, suggesting that these changes were a feature of the disease itself. More recently, the same authors also showed that chylomicron metabolism was disrupted with decreased lipolysis and remnant removal from the plasma (Borba 2000).

Total cholesterol was increased in 75% of 134 patients with SLE in a Canadian cohort. Old age at onset, increased cumulative dose of steroids and no antimalarial therapy were significant predictors of the result (Bruce 1999). In 2001, Formiga et al confirmed these results, reporting an increase in total cholesterol, triglycerides, apolipoprotein A-I and B, and HDL$_3$ subclass when compared to HDL$_2$. Corticosteroid therapy and proteinuria were the best predictors. In addition, there was an abnormal
distribution of the HDL subclasses, with a predominance of HDL3 over HDL2 (Formiga 2001). The latter has a predominance of Apo A-I whilst the former has an equal distribution of both A-I and A-II, hence the relative content of Apo A-I is reduced if a shift towards HDL3 is present (Atmeh 1983). Differences in the content of Apo A-I might be important in the maintenance of the anti-oxidant properties of HDL, which are mainly attributed to paraoxonase.

None of the previous studies however, considered the presence of anti-CL antibodies in the populations studied. The possibility that anti-CL antibodies could be relevant in this context was first addressed by MacGregor et al in 1992, who found an increased risk of vascular disease in SLE patients with raised levels of triglycerides, particularly in the presence of elevated anti-CL antibodies (MacGregor 1992). In this study, the abnormal lipid profile was associated with high doses of steroids.

Another interesting report of 75 patients with SLE suggested that anti-CL might play a role in determining plasma levels of lipoproteins (Lahita 1993). In this study, Lahita et al compared 57 patients positive for anti-CL antibodies with 18 negative for this autoantibody and reported low levels of total cholesterol, HDL and Apo A-I in the anti-CL antibody negative patients. It is noteworthy that the overall SLE population studied had lower levels of cholesterol when compared to a control population, contrary to other reports. Whether this was due to different treatment regimens, different degrees of disease activity, renal involvement or even different epidemiological characteristics, was not possible to assess.

Lipoprotein (a) is a LDL-like molecule, from which it differs mainly in the addition of a large glycoprotein: apolipoprotein (a). Epidemiological studies have
identified elevated levels of Lp(a) as an independent risk factor for cardio- or cerebrovascular diseases (Nagayama 1994). Lp(a) has been reported to be elevated in patients with SLE, with or without secondary APS (Borba 1994), and in PAPS (Yamazaki 1994). Some of these studies claim an association between Lp(a) levels and arterial thrombosis. This finding was not confirmed by Atsumi et al (Atsumi 1998). In their study, APS patients had higher plasma Lp(a) levels than controls, regardless of sub-grouping for arterial or venous thrombosis and recurrent miscarriages. In addition, Lp(a) levels were associated with an increase in the levels of D-dimer and PAI, suggestive of a relative fibrinolysis impairment. None of these studies found any association between Lp(a) levels and anti-CL titres, suggesting that the possible role played by Lp(a) in APS is probably independent of these antibodies.

In patients with primary APS, no significant changes have ever been reported in levels of total cholesterol, HDL, and LDL, suggesting that other factors might be involved in the increased tendency to thrombosis (Ames 2000a).

1.4.4.2 Plasma proteins

Fibrinogen (FNG), the most abundant coagulation-related protein, is an independent and powerful predictor of cerebrovascular and cardiovascular disease (Kannel 1987b). In patients with SLE, plasma FNG increases throughout follow up, regardless of disease activity and may represent an independent risk factor for the development of vascular disease in this context (Ames 2000b).

High levels of plasma homocysteine, a non-essential aminoacid, are strongly associated with an increased risk of atherosclerotic events (Clarke 1991). Suggested
mechanisms include a direct toxic effect on endothelial cells (Harper 1970), and the induction of a vascular endothelial cell activator (Rodgers 1986). In patients with SLE, plasma homocysteine is also an independent risk factor for thrombotic events (Petri 1996), and in PAPS it correlates with intima-media thickness (IMT) of carotid arteries (Ames 2002).

A first report showing an increased level of total β2-GP1 in patients with SLE (Cohnen 1970) was complemented by Ichikawa et al, who showed that β2-GP1 levels were relatively higher in SLE patients with hyperlipidaemia when compared with healthy controls (Ichikawa 1992). McNally et al reported that hyperlipidemia rather than lupus itself was the factor associated with an increased level of total β2-GP1 (McNally 1994). The results of the original study by Cohnen in 1970 may have been due to a secondary hyperlipidaemic state, caused by lupus nephritis and/or prednisolone treatment, which were more recently shown to increase β2-GP1 levels in SLE patients. The possibility that high levels of β2-GP1 could act as a second risk factor for thrombosis, along with the hyperlipidaemia, is contradicted by the fact that β2-GP1 levels did not correlate with thrombotic events in patients with SLE or APS. This observation suggests that the elevated levels might be an attempt to counteract the effect of the lipids (Ichikawa 1992).

Total β2-GP1 levels in patients with SLE, positive for anti-PL antibodies are significantly increased when compared with anti-PL negative patients, and normal control groups. However, there were no significant differences in the free β2-GP1 levels between normal controls and patients with lupus, even when the presence of anti-PL antibodies was considered (McNally 1995a). The difference found is therefore likely to
be due to an increase in β₂-GP1 together with other plasma constituents such as lipoproteins, and immune complexes. Increased total β₂-GP1 levels were also associated with a clinical history of thrombosis and/or foetal loss in the patients studied, suggesting that β₂-GP1 might play a role in the pathogenic mechanism of thrombosis when associated with anti-PL antibodies (McNally 1995b). All the patients in this study had a normal plasma lipid profile therefore excluding the possibility that elevated levels of β₂-GP1 were due to a hyperlipidaemic state.

Reports of β₂-GP1 antigen levels in patients with aPS have produced conflicting results with both normal, elevated and decreased levels (Galli 1992; Ichikawa 1992; McNally 1995b). In women with recurrent miscarriage, however, β₂-GP1 concentrations seem to be consistently normal (Rai 1995). Whether functional activity of β₂-GP1 is compromised by anti-PL antibodies without any demonstrable change in plasma levels, is still a matter for debate.

Immunocomplexes containing β₂-GP1 were also found in another cohort of patients with SLE. In this study, the presence of such particles was associated with higher levels of lipoprotein (a), thrombocytopenia and venous thrombosis (George 1999c). This study however, includes patients with secondary APS amongst the SLE cohort, hence it is not clear whether these associations are related with APS or SLE.

1.4.4.3 Autoantibodies

In 1992, Kabakov et al reported that sera from patients with SLE stimulated the accumulation of cholesterol in cultured smooth muscle cells from human aorta. The authors suggested that the binding of LDL to DNA-anti-dsDNA immunocomplexes
would increase the incorporation of the lipoprotein into the smooth muscle cells (Kabakov 1992).

A few anecdotal reports have suggested associations between cerebral vasculopathy and some extractable nuclear antigens (anti-Ro/SSA and anti-La/SSB), however nuclear magnetic resonance (NMR) identified the lesions as most likely to be due to vasculitis (Provost 1991). A more interesting result was presented by Mecocci et al, who showed increased levels of anti-histone antibodies, (but not anti-dsDNA antibodies), in a population with vascular dementia, when compared to normal controls (Mecocci 1993). Another study carried out in patients with atherosclerotic ischemic cardiomyopathy failed to show an increased frequency of ANA when compared with a control population (Fiorito 1992).

Recently, Grainger et al reported an increase in the levels of a nucleolar pattern of ANA in a population with CAD when compared with a control group. The antigen was not identified, though the most common extractable antigens were excluded (Grainger 2002).

One of the most interesting findings regarding the pathogenicity of anti-dsDNA antibodies was presented by Mason et al, who described the formation of thrombi in the kidneys of SCID mice injected with hybridoma cells producing a human IgG anti-dsDNA antibody. These thrombi stained positively for IgG and fibrin. The authors suggested that thrombi formation could have been secondary to the deposition of immunocomplexes, however concomitant signs of nephritis, previously described in this model, were not as evident (Mason 2001).
In contrast with anti-dsDNA antibodies, anti-PL antibodies have been reported to be the major culprits in the activation of several pro-atherosclerotic pathways. Antiphospholipid antibodies have been linked with clinical features of vascular disease, as reported by Leung et al, who showed that heart valve lesions and myocardial dysfunction were associated with the presence of anti-CL antibodies in patients with SLE (Leung 1990). More recently, anti-PL and to a lesser degree anti-β2-GP1 antibodies were found to have good predictive value for the extent of atherosclerotic occlusion of the coronaries in a cohort of 80 patients submitted to coronary angiography (Shoenfeld 1998). Cognitive dysfunction (as a possible marker for cerebrovascular disease) has also been associated with anti-PL antibodies. Menon et al found a significantly poorer cognitive function in patients with SLE who had sustained high levels of IgG anti-CL antibodies over a 2-3 year period, when compared with patients negative for anti-PL (Menon 1999). In contrast, sustained high anti-dsDNA antibodies or low C3 levels were not associated with cognitive dysfunction. Patients with higher anti-CL antibody levels also had abnormal MRI results with general atrophy and diffuse white matter changes, but without focal changes, suggesting that cognitive impairment was due to small vessel disease rather than major cerebral thrombotic events.

Different coagulation-related factors have also been directly related with anti-PL antibodies. Total IgG isolated from sera of patients with SLE and APS and incubated with endothelial cells, increased the production of von Willebrand factor antigen, when compared to IgG collected from normal healthy controls (Lindsey 1993). Ferro et al reported increased levels of von Willebrand factor, TNF-α, tissue plasminogen activator
and human prothrombin fragments (F1+2) in anti-CL antibody-positive patients with SLE when compared with patients lacking anti-CL antibodies (Ferro 1997).

Tissue factor (TF) expression has also been associated with anti-CL antibodies. Cuadrado et al showed an increase in TF expression in monocytes of patients with PAPS, when compared to patients positive for anti-CL antibodies but without the syndrome, patients with non-APS related thrombosis and healthy controls. Interestingly, tissue factor expression was only increased in patients positive for IgG anti-CL antibodies and not in patients with IgM anti-CL or LA. These data would suggest that TF expression might be a discriminating factor between pathogenic and non-pathogenic anti-CL antibodies (Cuadrado 1997).

Outside the lupus setting, anti-CL antibodies appear to be an independent risk factor for atherosclerotic vascular disease (Glueck 1999) and a 15% prevalence of anti-CL antibodies has been shown in middle aged patients who underwent surgery for peripheral vascular disease (Nityanand 1995). Anticardiolipin antibodies were found to be predictive of future myocardial infarction in a different cohorts of middle-aged men (Vaarala 1995; Wu 1997). However, inspite of these being prospective studies, the occurrence of infarctions might not be due, simply, to atherosclerosis, but might be a thrombotic event in the context of a possible antiphospholipid syndrome.

In 2002, Ames et al reported a direct correlation with increased IMT in PAPS (Ames 2002), suggesting that anti-CL antibodies could also have a direct influence in different pro-atherogenic mechanisms.

A confounding matter in the association between anti-PL and atherosclerosis concerns the cross-reactivity of these antibodies with oxLDL. In 1993 Vaarala et al
described a cross-reaction between antibodies to oxLDL and cardiolipin. Autoantibodies towards oxLDL were found in 80% of the 61 patients with SLE studied. Of the total cohort, 46% had raised levels of anti-CL antibodies, and cross-reactivity was found in 76% of the patients positive for both. This study suggests that in the context of SLE (and APS), antibodies against oxLDL might be a cross-reaction with anti-CL antibodies, thus providing a possible link for atherogenesis in these diseases (Vaarala 1993). Further evidence was provided by Mizutani et al, who reported a significant positive correlation between anti-CL and anti-oxLDL antibodies in (NZW x BXSB) F1 mice, suggesting that the same cross-reactivity phenomenon could be occurring (Mizutani 1995).

This cross-reaction however should not be unexpected, for cardiolipin is present in lipoproteins, and LDL contains more than 50% of the total plasma cardiolipin (Deguchi 2000), hence anti-CL antibodies binding to oxLDL might not constitute a cross-reaction but rather the normal binding of these antibodies to their natural antigen, as it is present in the LDL complex. In fact, the same pattern was not seen when anti-β2-GP1 antibodies were considered. Tinahones et al showed no cross-reaction between anti-β2-GP1 and anti-oxLDL antibodies in a cohort of 93 patients with APS, even when high concentrations of oxLDL were used in the inhibition studies (Tinahones 1998). These data were confirmed by Romero et al who reported that the association between anti-β2-GP1 antibodies and arterial disease, in a cohort of 118 patients with SLE, was not mimicked by anti-oxLDL, suggesting that these were different antibody groups. In addition, re-examining the data according to the presence or absence of APS did not show any difference in relation to a possible association between anti-β2-GP1 and anti-oxLDL antibodies (Romero 1998).
Several studies have subsequently addressed the issue of autoantibodies directed against different forms of phospholipids and their crossreactivity patterns with oxLDL. For example, an interesting study was provided by Wu et al, who reported a crossreactivity pattern between antibodies towards CL, oxLDL and lysophosphatidylcholine (Wu 1999). Again, this result is not surprising as the latter is part of the LDL complex. However, lysophosphatidylcholine is one of the active components of oxLDL and specific antibodies against this antigen have been shown to contribute significantly to the antigenicity of oxLDL (Wu 1998). These results suggest that this specific crossreaction within the LDL complex could in fact be responsible for the atherogenic potential previously reported with these antibodies. It is however reasonable, to consider whether a pure anti-oxLDL antibody family exists. If so, it might be present in the normal population, outside the context of autoimmune diseases, but this issue has not been addressed adequately.

Nonetheless, antibody production against a specific antigen, in this case oxLDL, perhaps triggering a subsequent pathologic mechanism can only take place after the antigen has been provided. Therefore, a more relevant question would be: why do patients with APS have such high levels of oxLDL?

Other lipoproteins and related components have been reported as possible targets for autoantibodies in patients with SLE or APS. Merrill et al, in 1995, showed the immunogenecity of Apo A-I using a mouse complementary DNA lambda phage expression library, and reported the presence of anti-Apo A-I antibodies in 5 out of 30 patients with SLE and/or APS (Merrill 1995). These data were confirmed by Dinu et al who demonstrated a high prevalence of antibodies to Apo A-I in a cohort of 175 patients.
with SLE and suggested that these autoantibodies had a high affinity towards mature HDL (Dinu 1998).

HDL is known for its protective role in atherogenesis, particularly through prevention of LDL oxidation (Watson 1995). Hence studying the interactions between autoantibodies and the HDL complex could lead to the understanding of the basic mechanisms, which trigger LDL oxidation and atherogenesis in SLE and APS.

1.4.5 Evidence for atherosclerosis in clinical practice

In the beginning of the 1970s initial observations, in the form of anecdotal reports, described patients with SLE, mostly young women with disease duration of more than 6 years, who had suffered myocardial infarctions (MI) (Meller 1975). Complementing the observations of Urowitz et al (Urowitz 1976), several other groups documented CAD-related deaths in their cohorts (Dubois 1974; Rosner 1982), although the mortality rates were significantly different. Autopsy studies confirmed the histological presence of CAD, with a greater than 50% narrowing of at least one of the coronary arteries in 36 patients with SLE (Bulkley 1975), and more than 75% narrowing in 12 other young female patients when compared with a matched control group (Haider 1981). Had APS been known at that time, then other mechanisms for these events might have been suggested. However the presence of atherosclerotic lesions, regardless of the possibility of anti-CL-related thrombotic episodes, was striking.

The risk for CAD in a SLE population was first compared with an age matched general population in Sweden in 1989, and found to be 9-fold higher (Jonsson 1989).
The increasing importance of atherosclerosis-related morbidity and mortality in the general population has boosted the awareness of it, in the context of autoimmune diseases. Urowitz et al identified CAD manifested as angina or MI in 8.9% of more than 500 patients with SLE (Gladman 1987). The prevalence of cardiovascular events including MI, sudden cardiac death or angina was reported as 8.3% in 229 patients of the Baltimore Lupus Cohort and comprised 30% of all lupus deaths (Petri 1992). In this study, the main risk factors for atherosclerotic events included age, higher mean serum cholesterol, hypertension or use of antihypertensive medication. Interestingly, steroid use was not significantly associated with these events although the duration of prednisone use did predict for CAD. This study was the first to suggest that factors other than apart from the traditional risk factors, might be involved in the premature onset of atherosclerosis in patients with SLE. Comparison between risk factors in SLE populations and the data reported in the Framingham study provide the most relevant epidemiological evidence that SLE is itself a risk factor for atherosclerosis. Confirming previous observations by Manzi et al (Manzi 1997), Esdaile et al reported an 8.3-fold and a 6.7-fold increase risk for MI and stroke respectively in patients with SLE after controlling for the Framingham risk factors: gender, age, systolic and diastolic BP, smoking, diabetes, cholesterol and left ventricular hypertrophy (Esdaile 1998). However, none of these studies considered anti-PL antibodies as a possible factor in assessment of the risk.

Early atheromatous disease has also been addressed, and in one study, abnormal thallium perfusion, suggestive of myocardial ischemia, was reported in 25% of a group of women with lupus (Hosenpud 1984), and in another cohort, dual-isotope myocardial
perfusion abnormalities were found in 40% of a total of 60 female patients with SLE (Bruce 2000).

The need for objective quantification of atherosclerosis in patients has led to the standardisation of ultrasonographic techniques for measuring intima media thickness (IMT). Using this method, Roman et al in 1997 reported the presence of focal plaques in 42% of patients with SLE and APS, however possible causal associations were not addressed (Roman 1997). More recently, the same group has reported a higher prevalence of carotid atherosclerosis and left ventricular hypertrophy in patients with SLE when compared to controls (Roman 2001). Similar results were reported by Manzi et al, who found that 40% of their SLE population had focal plaques which were predicted by age, higher systolic blood pressure, a previous coronary event, prolonged treatment with steroids and higher levels of LDL (Manzi 1999). In this study, anti-CL antibodies bore no relation to IMT.

More recently, Svenungsson et al compared IMT in patients with SLE, with and without cardiovascular manifestations with normal controls. All the groups were corrected for traditional risk factors. IMT was greater and focal plaques were more frequent in SLE patients with history of CAD when compared with SLE and population controls. Interestingly, there were no differences between the SLE group without CAD and the control population, suggesting that the increased risk of atherosclerosis could be related to previous events rather than being an inherent feature of the disease. Furthermore, a greater IMT was present in the patients with higher titres of anti-CL and anti-oxLDL antibodies (Svenungsson 2001), suggesting that APS could be involved.
In 1992, a comparison was made between a group of 19 patients with arterial manifestations of APS and a population of 1078 patients with classical atherosclerotic disease admitted to a peripheral vascular surgery unit. Patients with APS were found to be younger, non-smoking, with a higher percentage of upper extremity involvement and had a higher incidence of early graft failure (Shortell 1992). Apart from highlighting some basic epidemiological characteristics of the APS population, it raised the possibility of the presence of an ongoing, possibly endothelium-independent factor in this disease, which could account for the increased incidence of graft failure and disease perpetuation. The study methodology did not allow further investigation of the possible role of anti-CL antibodies, LA or any other specific features of the syndrome. Several case reports have subsequently shown similar clinical features between PAPS and "normal" atherosclerotic disease (Hughson 1995; Mandreoli 1993). An objective assessment of atherosclerotic involvement in patients with PAPS was reported in 2002 (Ames 2002). IMT was increased in patients with PAPS in comparison with normal controls. Furthermore, elevated IgG anti-CL, homocysteine and fibrinogen were identified as independent predictors of IMT in the carotid arteries of these patients. Even though this study provided the first objective demonstration of atherosclerosis in APS, the lack of a non-APS-related thrombotic group still raises the question of whether the lesions described in the vascular bed are related solely to previous thrombotic events or if they are indeed a feature of the syndrome.
1.5 Aims of this thesis

General aim:

To determine the pathogenic role of antiphospholipid antibodies in the induction of oxidative stress in patients with systemic lupus erythematosus and antiphospholipid syndrome.

1- To determine if a human IgG anti-CL monoclonal antibody can induce NO production by endothelial cells in an ex vivo model, and to characterise the role of iNOS and eNOS in this context.

2- To confirm the induction of iNOS by an anti-CL antibody in a short-term animal model.

3- To determine whether anti-PL monoclonal antibodies (anti-CL and anti-β2-GP1, murine and human), can influence the anti-oxidant activity of PON and establish its consequences in the induction of a pro-oxidant state in a long-term mouse model.

4- To identify the production of autoantibodies against HDL and Apo A-I in patients with SLE and PAPS and to establish whether these antibodies are mutually cross-reactive, and also bind to cardiolipin.
5- To investigate the association between anti-CL, anti-β2-GP1 and anti-HDL antibodies and oxidative stress in a cohort of patients with SLE and PAPS.

6- To confirm the association of anti-PL antibodies and oxidative stress in a group of infertile women positive for anti-PL antibodies induced by IVF treatment, who do not have APS or any other autoimmune disease.
Chapter 2:
METHODS

2.1 Immunology-related methods

2.1.1 ELISA

2.1.1.1 Anti-Human IgG antibodies

Polystyrene 96-well plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with 2.5μg/ml of goat anti-human IgG (Sigma) diluted in PBS (pH 7.2). After incubation for 16h at 4°C, the plates were washed three times with PBS and blocked with 1% (w/v) BSA (Sigma) in PBS for 1h at 37°C. Serum samples were diluted (1:200) in PBS containing 0.05% Tween 20 (Sigma), and incubated on the plate for 1h at 37°C. Following three washes, bound antibodies were detected by incubation for 1h at 37°C with a 1:1000 dilution of affinity purified goat anti-human IgG conjugated to alkaline phosphatase conjugate (Sigma) and following washing, developed with the substrate p-nitrophenol phosphate (Sigma). Optical density was measured at 405nm with a reference filter at 490nm. The sample concentrations were calculated by reference to the linear portion of a standard curve of purified human IgG (Sigma) run on every plate. Inter/intra plate coefficients of variation were less than 10%.
2.1.1.2 Anticardiolipin antibodies

IgG and IgM anti-CL were measured by an ELISA technique using microtitre plates (Polysorp, Nunc, Life Technologies, Paisley, UK) coated with cardiolipin (bovine heart, Sigma-Aldrich Ltd, Poole, UK). Briefly, half of the plates were coated overnight at 4°C with 50μg/ml cardiolipin in 70% ethanol. Blocking was performed using PBS for 1 hour at room temperature. Plates were then washed once using PBS. Samples were diluted 1:100. 100μl of positive control was added to duplicate wells for 1 hour at room temperature. After washing three times 100μl of alkaline phosphatase conjugated anti-human IgG (1:1000 in the blocking agent) was added for 1 hour. 100μl p-nitrophenyl phosphate (Product 104/105, Sigma-Aldrich, Poole, UK) in BIC buffer (pH 9.8) was added and incubated at 37°C for colour development and the absorbance read at 405nm after 1 hour. Assays were standardised with sera calibrated against the appropriate International Reference Material (Harris 1987), and the results were reported as antiphospholipid (PL) units. Inter/intra plate coefficients of variation were less than 10%.

2.1.1.3 Anti-dsDNA antibodies

ELISA plates (Maxisorp, Nunc, Roskilde, Denmark) were half coated with 5μg/ml of calf thymus DNA (Sigma) in citrate buffer (0.15M sodium chloride and 0.015M sodium citrate, pH 8.0) and half with buffer alone for 2hrs at 37°C. After washing in PBS, the plates were blocked with 2% casein (Sigma) in PBS for 1 hour at 37°C. Serum samples were titrated from 1:100 in PBS/Tween 20 and incubated on both
DNA coated and uncoated sides of the plate for 1 hour at 37°C. Bound antibodies were detected as described for anti-cardiolipin antibodies. Serum from a patient known to have anti-dsDNA antibodies was used as a positive control and results were expressed in OD as a percentage of the positive control for each plate. Inter/intra plate coefficients of variation were less than 10%.

2.1.1.4 Anti-β2-GP1 antibodies

IgG anti-β2-GP1 was measured by ELISA, using a commercial kit (Diastat anti-β2-GP1, Axis-Shield Diagnostics, Dundee, UK) based on a method previously developed at the Middlesex Hospital (McNally 1995c). IgM anti-β2-GP1 were assayed by an adaptation of the IgG kit components to accommodate the detection of IgM antibodies using a μ-specific conjugated secondary antibody. Both assays were standardised using serum with a known high concentration of antibody and units were expressed in mg/dl. The cut-off point for the upper limit of normal had been previously determined as the geometric mean + 95% confidence interval of values obtained from a population of 30 healthy adults. Inter/intra plate coefficients of variation were less than 10%.

2.1.1.5 Anti-prothrombin antibodies

IgG and IgM anti-prothrombin were analysed by ELISA using γ-irradiated microtitre plates (Maxisorp, Nunc, Life Technologies, Paisley, UK) coated overnight at 4°C with 10μg/ml human prothrombin (Enzyme Research Laboratories, Swansea, UK) in PBS (0.01M phosphate, 0.145M Nanti-CL, pH 7.2). Blocking was performed using
PBS containing 0.1% Tween 20 and 1% BSA (A7030, Sigma-Aldrich, Poole, UK) for 1 hour at room temperature. Plates were then washed once using PBS containing 0.1% Tween 20. A standard curve was prepared from reference plasma with a known high anti-PT activity. Test samples were diluted 1:50 in PBS containing 0.1% Tween 20 and 1% BSA. 100μl of standard or test was added to duplicate wells for 1 hour at room temperature. After washing three times 100μl of alkaline phosphatase conjugated anti-human IgG or IgM was added for 1 hour. 100μl p-nitrophenyl phosphate (Product 104/105, Sigma-Aldrich, Poole, UK) in BIC buffer (pH 9.8) was added and incubated at 37°C for colour development, which was stopped using 3M NaOH and the absorbance read at 405nm. All assays were validated by the inclusion of internal quality control samples of known activity and the units were expressed in OD as a percentage of the standard control. The cut-off point for the upper limit of normal was previously determined as the geometric mean + 95% confidence interval of values obtained in 30 healthy adults. Inter/intra plate coefficients of variation were less than 10%.

2.1.1.6 Anti-HDL antibodies

IgG anti-HDL antibodies were measured by ELISA using γ-irradiated microtitre plates (Polysorb, Nunc, Life Technologies, Paisley, UK) half-coated overnight at 4°C with 20μg/ml human HDL in 70% ethanol. HDL was isolated from healthy subjects as previously described (Mackness 1992). Blocking was performed using PBS containing 1% BSA (A7030, Sigma-Aldrich, Poole, UK) for 1 hour at room temperature. Plates were then washed once using PBS. Samples were diluted 1:100 in PBS containing 1%
BSA. 100µl of a positive control was added to duplicate wells for 1 hour at room temperature. After washing three times 100µl of alkaline phosphatase conjugated anti-human IgG (1:1000 in the blocking agent) was added for 1 hour. 100µl p-nitrophenyl phosphate (Product 104/105, Sigma-Aldrich, Poole, UK) in BIC buffer (pH 9.8) was added and incubated at 37°C for colour development and the absorbance read at 405nm after 1 hour. All assays were validated by the inclusion of internal quality control samples of known activity. The results were expressed in a percentage of the positive control present in each plate. Inter/intra plate coefficients of variation were less than 10%.

2.1.1.7 Anti-Apolipoprotein A-I antibodies

Ninety-six well plates (Polysorp) were half-coated (background OD was discounted from the wells with the respective samples) overnight, at 4°C with 10µg/ml human apolipoprotein A-I (Sigma-Aldrich) in PBS. Blocking was performed using PBS containing 2% BSA (Sigma-Aldrich) for 1 hour at 37°C. Serum samples (1:100 dilution in PBS containing 2% BSA) and hybridoma cells culture supernatant containing human IgG anti-CL monoclonals (neat) were loaded in duplicate together with a positive control (kindly supplied by Dr. Joan Merrill, Oklahoma, USA) and the plates incubated for 1 hour 37°C. After washing, 100µl of alkaline phosphatase conjugated anti-human IgG (1:1000 in the blocking agent) was added for 1 hour. 100µl p-nitrophenyl phosphate (Sigma-Aldrich) in 5 ml of BIC buffer (pH 9.8) was added and incubated at 37°C for colour development and the absorbance read at 405nm after 1 hour. All assays were
validated by the inclusion of internal quality control samples of known activity. The
results were expressed in a percentage of the positive control present in each plate after
subtraction from the background in the uncoated half of the plate. Inter/intra plate
coefficients of variation were less than 10%.

2.1.1.8 Anti-phosphatidylserine antibodies (this assay was performed
by Dr. E. Radway Bright)

Half of the wells of 96-well ELISA plates (Polysorp, Nunc, Life Technologies,
Paisley, UK) (50ul/well) were coated with phosphatidylserine (PS) (Sigma, Poole,
Dorset, UK), 50ug/ml in ethanol whilst the other half (control) with ethanol alone, and
air-dried overnight at 4°C. The plates were blocked with 100ul/well 10% foetal calf
serum (Sigma, Poole, Dorset, UK) in phosphate-buffered saline (10% FCS / PBS) for
one hour at 37°C and washed three times with PBS. The serum diluted 1:100 in 10%
FCS / PBS was added in duplicate (50ul/well) to both PL-coated and uncoated halves of
the plates. After incubation for 1.5 hours at 37°C and three washes with PBS, alkaline
phosphatase conjugated goat anti-human IgG or IgM (Sigma) was added at 1:1000
dilution (50ul/well), for one hour at 37°C. Colour was developed with 50ul/well alkaline
phosphatase substrate tablets (Sigma) dissolved in bicarbonate (BIC) buffer [pH9.6] to
give a 1mg/ml solution supplemented with 2mM MgCl2, after three washes with PBS
and two with BIC. Plates were incubated at 37°C for one hour and optical density (OD)
was measured at 405nm (with reference 490nm) using an ELISA reader. Background
values were obtained from wells containing no antigen (control wells), and their
absorbances subtracted from all sample readings (test wells). Results were expressed as a percentage of the positive control: OD (sample) / OD (positive control) x 100. Values >3 standard deviations (SD) above the mean of fifty healthy controls were deemed to be positive.

2.1.1.9 Nitrotyrosine

Nitrotyrosine was measured by ELISA, using a commercial kit (Hbt nitrotyrosine, HyCult biotechnology bv, Uden, Netherlands), and in accordance with the instructions of the manufacturer. Standard curves were performed in each plate, using samples with known nitrotyrosine concentrations included in the kit.

2.1.2 Western blotting

2.1.2.1 iNOS and eNOS in mouse heart homogenates

Immunoblot assays were performed according to a method previously described (Laemmli 1970), with some modifications. In brief, homogenates from mouse heart were obtained after sonication, and proteins were separated under reducing conditions on 7% polyacrylamide gels. Proteins were then transferred to polyvinylidene difluoride membranes (PVDF, Millipore) and quenched with blocking buffer, containing 5% non-fat milk in 0.1% PBS Tween-20 for 1 h at room temperature. After one wash, membranes were incubated overnight at 4°C with primary antibody (rabbit anti-eNOS or rabbit anti-iNOS, Santa Cruz Biotechnology, USA), 1:1000 in blocking buffer. After
several consecutive washes, the membranes were incubated with HRP-conjugated secondary antibody (donkey anti-rabbit, Santa Cruz Biotech) in blocking buffer for 1h. Assays were performed using the enhanced chemiluminescence (ECL) detection system (Pharmacia Biotech).

2.2 Cell culture-related methods

2.2.1 Hybridoma production

All manipulations were carried out in a sterile tissue culture hood, and all media and equipment used were kept sterile and not used for any other purpose.

2.2.1.1 Maintenance of CB-F7 cells in culture

CB-F7 cells are a mouse-human heteromyeloma cell line. It is HAT (hypoxanthine, aminopterin, thymidine) sensitive, ouabain resistant, non-secreting cell line, characterised by rapid cell growth (doubling time about 16 hours) (Grunow 1988).

CB-F7 cells were grown in Growth Medium (GM) in 80cm² or 175cm² Falcon tissues culture flasks (Beckton-Dickinson, New Jersey, USA). The cells were maintained in continuous culture by changing the GM twice a week as follows: a tissue culture flask containing 25ml of healthy CB-F7 cells was removed from the tissue culture incubator (37°C, 5% CO₂) and gently shaken to detach any cells adhering to the base of the flask. Nine tenths of the old GM was aspirated, replenished with fresh GM (1:10 dilution) and

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returned to the incubator. For the second feed of the week, flasks were gently shaken and
2.5ml of cells were transferred to fresh flasks and made up to 25ml with fresh GM. To
avoid long passages in vitro, fresh batches of cryoconserved CB-F7 cells were thawed
and put into culture every eight to twelve weeks.

2.2.1.2 Separation of peripheral blood lymphocytes from whole blood

In one 50ml Falcon tube (Becton-Dickinson), 25ml diluted heparinised blood
(1:1 with RPMI 1640 medium) was slowly layered onto the surface of 25ml
Lymphoprep (density 1.077 ± 0.001g/ml, Nycomed, Oslo, Norway) and centrifuged at
2000rpm for 25 minutes at 21°C. Lymphocytes were harvested from the interface
between the Lymphoprep and plasma / RPMI and made up to 50ml in RPMI. The cells
were washed three times in RPMI (wash = centrifugation at 2000rpm for 15 minutes at
21°C, pour off supernatant and resuspend the pellet in 50ml RPMI). After the final
wash, the cell pellet was resuspended in 10ml RPMI in preparation for counting, using a
haemocytometer.

To count the cells and determine viability, 20μl of the cell suspension was mixed
with 20μl of a 1:1 mixture of ethidium bromide and acridine orange. The cells were then
placed in a modified Neubauer counting chamber (Gallenkamp, Loughborough, UK)
and allowed to settle for one minute prior to counting. The cells were counted under
ultraviolet light where live cells were viewed green and dead cells were orange. All the
green cells in the triple hatched area were counted. The total number of cells was
calculated using the formula:
cells counted x dilution \((1/2) \times 10^4 \times 10\text{ml}\) (volume of cell suspension)

2.2.1.3 Freezing, storage and thawing of lymphocytes and CB-F7 cells

All excess lymphocytes and CB-F7 cells, surplus to the requirements for fusion, were transferred to 50ml Falcon tubes, and washed once at 2000rpm for 10 minutes at 21°C. The supernatant was decanted off, and the pellet resuspended in pre-cooled 10-20ml Freezing Medium at a concentration of \(1 \times 10^6\) cells/ml. This suspension was quickly aliquotted into 1ml freezing vials and frozen slowly at \(-70°C\). The labelled vials were kept in liquid nitrogen, for prolonged storage.

Lymphocytes: Frozen vials of cells were transferred from liquid nitrogen to a 37°C water bath. When only a small clump of ice was visible, the contents were transferred into a Falcon tube, diluted in 10ml RPMI and washed at 2000rpm for 10 minutes at 21°C. The supernatant was decanted off and the pellet of lymphocytes was resuspended in 25ml RPMI in preparation for counting, prior to fusion.

CB-F7 cells: Frozen vials of cells were transferred from liquid nitrogen to a 37°C water bath. When only a small clump of ice was visible, the contents were transferred into a Falcon tube, diluted in 50ml pre-warmed (to 37°C) Growth Medium and washed at 2000rpm for 10 minutes at 21°C. The supernatant was decanted off and the pellet of cell was resuspended in 30ml Growth Medium and plated into a 6-well tissue culture plate (Becton-Dickinson). Half the Growth Medium volume was changed after 24 hours, and the cells were transferred into 50cm² or 175cm² tissues culture flasks when they were flourishing.
2.2.1.4 Generation of hybridoma cells by fusion of lymphocytes with CB-F7

Sensitivity of CB-F7 to HAT was checked 24hrs prior to fusion by culturing one flask of cells in HAT Medium and determining viability. No cells were alive at the end of this procedure, as shown by acridine orange / ethidium bromide staining. The remaining flasks of CB-F7 were transferred into 50ml Falcon tubes, washed three times (wash = centrifugation at 2000rpm for 15 minutes at 21°C, pour off supernatant and resuspend the pellet in 50ml RPMI), finally resuspended in 10ml RPMI and counted.

Lymphocytes in RPMI and CB-F7 cells in RPMI were mixed together at a ratio of 1:2 (lymphocytes : CB-F7), made up to 50ml with RPMI and centrifuged at 2000rpm for 15 minutes at 21°C. The RPMI was decanted off, and the tube was tapped gently to loosen the pellet of lymphocytes and CB-F7. One millilitre (for every 5 x 10⁶ lymphocytes) of pre-warmed PEG 1500 (Polyethylene glycol 1500; Boehringer Mannheim, Germany) was added dropwise to the pellet, over a period of one minute. The tube was swirled gently for 90 seconds before the contents were very slowly diluted with pre-warmed RPM, over a period of 10 minutes. The volume was made up to 50ml with RPMI and centrifuged at 1500rpm for 15 minutes at 21°C. The RPMI was decanted off, and the loosened pellet was resuspended in 1ml pre-warmed Post Fusion Medium for every 1 x 10⁶ lymphocytes.

The cells were seeded (100 µl/well) into 96-well tissue culture plates. The peripheral wells of the 96-well plates were not used for culture because of the increased
risk of drying out and infection. RPMI containing a few drops of 10000 IU/ml Penicillin / 10mg/ml Streptomycin solution was placed in these wells instead. From this point onwards, all reference to the tissue culture plates refers to the 60 wells in the middle of the plate. After 24 hours in the incubator (37°C, 5% CO₂), 100 μl/well HAT Medium (with 2 x HAT) was added into the wells. Growth was usually observed in the first 7-10 days of culture, and the HAT Medium was changed once or twice a week (yellow culture medium was used as an indicator of cell growth). In the first two to three weeks, the number of wells with initial cell growth (containing clusters of more than 50 viable cells) were marked and observed daily. The culture was maintained in HAT Medium for a maximum of eight weeks, during which wells containing growing cells (clones) were screened for immunoglobulin secretion.

2.2.1.5 Detection of human IgG molecules in hybridoma supernatant

Half of a 96-well ELISA plate (Maxisorp, Nunc, Denmark) was coated with 5μg/ml goat-anti-human IgG [F(ab')₂ fragment] (Sigma, I-9885) dissolved in BIC buffer, while the control half was coated with BIC buffer alone. The plate was incubated overnight at 4°C. PBS containing 2% Bovine Serum Albumin (BSA) was added to each well, after the plate was washed twice with PBS. This was to block non-specific binding of immunoglobulin to the plastic. The plate was incubated for one hour at 37°C then washed twice with PBS containing 0.1% Tween 20 (Sigma) (PBS-Tween). Supernatant from the clones (hybridomas) was loaded directly onto the ELISA plate, such that for each well in the test half there was a well in the control half containing the same
supernatant. The plate was incubated for one hour at 37°C. After washing three times in PBS-Tween, bound antibodies were detected by incubation for one hour at 37°C with a 1:1000 dilution of affinity purified goat anti-human IgG conjugated to alkaline phosphatase (Sigma, A-3150). The conjugate was diluted in PBS-Tween.

Following five washes in PBS-Tween, then one with BIC buffer, the plate was developed with alkaline phosphatase substrate tablets dissolved in BIC buffer to give a 1mg/ml solution supplemented with 2mM MgCl₂. Plates were incubated at 37°C and optical density (OD) was measured at 405nm (with reference 490nm) using an ELISA reader, after 30-60 minutes or when a yellow colour developed. For each clone supernatant, the result was calculated as the difference between the OD in the test well and that in the control well. This ensured that only conjugate bound directly to IgG in the cell supernatant, which had itself be captured by the goat anti-human IgG on the plate, would contribute to the result. Positive and negative controls were included on each plate. The positive control was derived from 10ml of serum from a patient with APS who had high titres of IgG. The negative control was derived from serum collected from a SCID mouse that tested negative for human and mouse IgG. Each serum sample was aliquoted for use in multiple experiments.

The transfer of the supernatants to the ELISA plates was conducted under sterile conditions.
2.2.1.6 Detection of human IgM molecules in hybridoma supernatant

This procedure was identical to that described in section 2.2.1.6, except that the plate was coated with 2μg/ml goat anti-human IgM (Sigma, I-2386) and the conjugate used was affinity purified goat anti-human IgM conjugated to alkaline phosphatase (Sigma, A-3275). The positive control was derived from 10ml of serum from a patient with APS who had high titres of IgM. The negative control was derived from serum collected from a SCID mouse that tested negative for human and mouse IgM.

Each serum sample was aliquoted for use in multiple experiments.

2.2.1.7 Detection of human anti-CL and anti-dsDNA antibodies (IgG and IgM) in hybridoma supernatant

This procedure was identical to that described in sections 2.1.1.2 and 2.1.1.3, except that supernatant was loaded directly onto the ELISA plate, such that for each well in the test half there was a well in the control half containing the same supernatant. The transfer of the supernatants to the ELISA plates was conducted under sterile conditions.
2.3 Tissue staining-related methods

2.3.1 Haematoxylin and eosin histological stain

Formalin-fixed, paraffin-embedded kidney sections from the SCID mice were stained with haematoxylin and eosin. These were then examined by a histopathologist, (Dr. Meryl Griffiths, Histopathology Department – UCL Hospitals), for morphological evidence of thrombosis or any other abnormalities.

2.3.2 Immunohistochemistry (iNOS and eNOS)

Paraffin-embedded sections of kidney blood vessels were de-waxed and antigen retrieval achieved by boiling the sections for 180 seconds in Tris-EDTA (pH 9.0). Following flushing in tap water, slides were rinsed in 0.05% Tween 20 in Tris buffered saline (TBS/Tween). Endogenous peroxidase activity was blocked for 30 minutes using Dako Peroxidase block (Dako, Ely, UK). The sections were rinsed in TBS/Tween and primary antibody was applied for 60 minutes at room temperature (iNOS and eNOS were diluted 1/100 in TBS). The sections were rinsed in TBS/Tween and EnVision reagent (Dako) was applied for 60 minutes. The sections were rinsed in TBS/Tween and EnVision DAB (Dako) was applied for 10 minutes. The slides were counterstained with haematoxylin before being dehydrated and mounted.
2.4 Biochemistry-related methods

2.4.1 Paraoxonase

Serum PON activity was measured as described by Eckerson with some modifications (Eckerson 1983). Paraoxon substrate (Sigma, UK) (1.0mM), freshly prepared in 300μl of 50mM glycine buffer containing 1mM calcium chloride (pH 10.5) was incubated at 37°C with 5μl of serum, for 15 min in 96 well plates (Polysorp, Nunc, Life Technologies, Paisley, UK). p-Nitrophenol formation was monitored at 412nm and activity expressed as nmol p-nitrophenol per ml serum per min. All samples were tested in triplicate, and each set of experiments was repeated twice to check for reproducibility. Inter/intra plate coefficients of variation were less than 10%.

The cut-off point for the limits of normal were previously determined as the mean ± 3 SD of values obtained in a population of 35 healthy adults.

2.4.2 Total anti-oxidant capacity of plasma

Peroxynitrite (ONOO⁻) quenching: 100μl of phosphate buffer (50 mM; pH 7.4) containing Pholasin® (1.7 μg/ml) were pipetted into a microcuvettes and plasma or buffer for control (5 μl) added. The reaction was initiated by adding SIN-1 (2 μl of 2 mg/ml in water) and the light emission measured continuously at 5 min intervals until the maximum reading was obtained. Antioxidant capacity was expressed as the time at which maximum light was emitted. Lower values reflected a decreased TAC of plasma (peroxynitrite related).
Superoxide anion (O$_2^-$) quenching: 100µl of phosphate buffer (50 mM; pH 7.4) containing Pholasin® (0.5 µg/ml) and adjuvant K (50 µl/ml) were pipetted into a microcuvette and plasma or buffer for control (5 µl) was added. Next, 5µl of xanthine (50 mM) was added and the reaction initiated by the addition of 20 µl of xanthine oxidase (0.5 U/ml phosphate buffer). The signal was recorded over a period of 5 min on a 121 LKB recorder. Antioxidant capacity was expressed as the counts (mV) at maximum light emission. Higher values reflected a reduced TAC of plasma (superoxide related). All samples were tested in triplicate, and each set of experiments was repeated twice to check for reproducibility. Inter/intra plate coefficients of variation were less than 10%.

2.4.3 Total serum nitric oxide

NO was determined using the Griess reaction, as previously reported (Giovannoni 1997). Briefly, serum was diluted one in four with PBS, and 200µl of this solution was ultra-filtered by centrifugation at 10000g for 1 hour, using 10000kD molecular weight filters (Ultrafree-MC, Millipore). Only clear and colourless filtrates were tested. The assay was performed in a standard flat-bottomed 96-well polystyrene microtitre plate, containing 50µl/well of standard or sample. The assay was blanked against PBS. 50 µl of nitrate reductase and β-NADPH were added to each well giving final concentrations of 300U/l and 25µmol/l, respectively. The plate was incubated at room temperature for 3h. Excess β-NADPH was consumed by addition of 50µl of PBS containing L-glutamic dehydrogenase, α-ketoglutaric acid, and NH$_4$Cl, (final
concentrations 500U/l, 4mmol/l and 100mmol/l, respectively), followed by a 10 min incubation at 37°C. NO concentration was then measured by the addition of 50µl each of Griess reagents 1 and 2, and after 10 min incubation at room temperature the absorbance was read at 540nm. All samples were tested in triplicate, and each set of experiments was repeated twice to check for reproducibility. Inter/intra plate coefficients of variation were less than 10%.

2.4.4 Total cholesterol, HDL and HDL sub-fractions 2 and 3

Plasma total cholesterol and HDL cholesterol were determined by the CHOD-PAP method with the use of an enzymatic test reagent (Infinity™ cholesterol test reagent, Sigma Diagnostics, Poole, UK). HDL was first isolated by selective precipitation of LDL from plasma using heparin-Mn²⁺. Specifically, 1ml of plasma was treated with 75µl of heparin, briefly vortexed and allowed to incubate at room temperature for 2 minutes. Following the addition of 100µl of MnCl₂, samples were again vortexed and incubated at 4°C for a further 30 minutes. Samples were then centrifuged at 12,000 x g for 5 minutes to pellet precipitated LDL particles and the supernatant collected for HDL cholesterol measurements. Aliquots of plasma or HDL containing supernatant (10µl) were then mixed with 1ml of enzyme assay reagent, the resultant mixture incubated at room temperature for 20 minutes and then assayed spectrophotometrically at 500nm. Triplicate determinations were made and the average value taken. HDL sub-fraction 2 and 3 were obtained according to the method described
by Gidez at al (Gidez 1982). Briefly, HDL$_3$ was isolated by precipitation after adding 50 μl dextran sulphate (14.3 mg/ml in saline) to 500 μl of total HDL prepared as described above. After incubation at room temperature for 30 min, samples were centrifuged at 12000x g for 2 min. Supernatants (HDL$_3$ containing) were removed and values were obtained by spectrophotometry at 500nm, were multiplied by 1.225 to correct for additions. HDL$_2$ was obtained by subtracting HDL$_3$ to total HDL.

2.5 Experimental protocols

2.5.1 Induction of NOS expression by a human monoclonal IgG anti-CL antibody in an ex vivo and in an experimental model

2.5.1.1. Hybridoma cells, antibodies, drugs and LPS content

Hybridoma cells producing IS4 were cultured in RPMI 1640 medium containing 1% L-glutamine, 1% sodium pyruvate, 2% MEM non-essential amino-acids, 1% penicillin/streptomycin, 0.2% gentamycin (all from Gibco, UK) and 10% FCS (Sigma, UK). IS4 is a human monoclonal anti-cardiolipin IgG$_3$ antibody, which binds to cardiolipin and β$_2$-GP1 but does not have LA activity. It was derived from patients with anti-phospholipid syndrome (a kind gift from Dr. Pojen Chen, UCLA, CA, USA) (Zhu
Antibodies were purified in an LPS free environment (Cymbus Biotechnology limited). Human non-specific IgG (Sigma, UK) was used as control antibody and diluted in PBS, in sterile conditions.

Phenylephrine hydrochloride, acetylcholine chloride, L-NAME, arginine, sodium nitroprusside, lipopolysaccharide, polymixin and cardiolipin were obtained from Sigma (Poole, Dorset, UK). 1400W was obtained from Alexis Biochemical (Nottingham, UK). Dulbecco’s modified Eagle’s medium came from GibcoBRL (Paisley, UK). Preparation of all stock solutions and their subsequent dilution were made using sterile normal saline, with the exception of polymixin and cardiolipin, which were dissolved in DMEM.

LPS content in both antibody solutions was measured by a kinetic chromogenic assay (BioWhittaker Inc, Belgium). LPS in the IS4 solution was 0.7739 EU/ml and in the control IgG solution was 0.6406 EU/ml.

2.5.1.2 Ex-vivo aorta bath model

Male Sprague Dawley rats (200-250g) were stunned and killed by cervical dislocation. The thoracic aorta was dissected out, cleaned of connective tissue and cut into four rings (3-4mm wide). Rings were incubated in 0.5ml of serum free Dulbecco’s modified Eagle’s medium (DMEM) in a 24 well plate for 4 hours (at 37°C, 95% O₂/5% CO₂). Initially 20ul of phosphate buffered saline (PBS), 20ul of IS4 (final concentration 0.4ug/ml), 20ul of non-specific IgG (0.4ug/ml) or 20ul of lipopolysaccharide (Salmonella typhi; 500ng/ml) were added to each well before incubation (n=8). In order
to confirm the antibody-specificity of the results, an inhibition assay was performed by pre-incubating IS4 and non-specific IgG with cardiolipin (100 fold excess) in the media, before the addition of the aortic rings (n=6). To exclude lipopolysaccharide contamination of the antibodies a set of experiments were performed in which rings were incubated with IS4 or IgG in the presence or absence of polymixin (10μg/ml) (n=7).

Following incubation aortic rings were suspended in 25ml organ baths containing Krebs-bicarbonate buffer (composition (mM): Na^+ 143; K^+ 5.9; Ca^{2+} 2.5; Mg^{2+} 1.2; Cl^- 128; HCO_3^- 25; HPO_4^{2-} 1.2; SO_4^{2-} 1.2; D-glucose 11), maintained at 37°C and gassed with 95% O_2/5% CO_2. A resting tension of 1g was applied to each tissue and changes in isometric tension measured using a force displacement transducer (FT03) connected through a MacLab to a computer. The tissues were allowed to equilibrate for 60 minutes prior to experimentation. During this time the tissues were washed three times and the tension readjusted to baseline.

Initial maximal contraction was obtained with the addition of KCL (4.8x 10^{-2}M). After washing for 30 minutes, a phenylephrine (PE) concentration-response curve was produced (10^{-9} – 10^{-5}M). Tissues were then washed for another 30 minutes and sub-maximally (70-85% of maximum) contraction with PE was again achieved. Once stabilised (5min), the vessels were relaxed with acetylcholine (Ach; 10^{-6}M) to assess endothelial integrity; no rings needed to be discarded during these studies. In the first set of experiments the vessel rings were then washed for 30 minutes and incubated with nitro-L-arginine methyl ester (L-NAME; 3x10^{-4}M) for a further 30 minutes in order to
non-selectively inhibit all isoforms of nitric oxide synthase. A concentration-response curve to PE was then repeated.

In the second set of experiments, incorporating cardiolipin, at the end of the PE concentration-response curve the vessels were allowed to stabilise before D-arginine (3x10^{-4}M) was added to each bath. Following the completion of any response L-arginine (3x10^{-4}M) was added to the baths to see the maximal relaxation achievable by direct arginine supplementation. Vessels were then washed for 30 minutes before sub-maximal contraction with PE (70-85%) and a concentration-response curve to Ach (10^{-9} – 10^{-5}M) was obtained.

In the polymixin experiments the initial PE concentration-response curve was followed by the construction of a concentration-response curve to sodium nitroprusside (SNP; 10^{-9} – 10^{-5}M), to confirm that the effects seen were endothelial dependent. After washing for 30’, the rings were exposed to (10^{-5}M) of N-(3-(Aminomethyl)benzyl)acetamidine (1400W), (a specific inhibitor of iNOS), for another 30’, in order to selectively inhibit iNOS. Finally the PE response was repeated.

2.5.1.3 In vivo experimental model

(Surgical procedures were made by Raymond Stidwill, Intensive Care Department of UCL).

The experiments were performed on an anaesthetised, spontaneously breathing, male Sprague-Dawley rat model. The animals were given free access to food and water until the time of surgery. Anaesthesia was established with an intraperitoneal injection of thiobutabarbitone, 60mg/kg, and the animals were placed on a heated operating table to
maintain core temperature. Neck dissection was performed to allow placement of vascular lines using polythene tubing of 0.9mm external diameter stretched over a heat source to reduce the diameter of the ends. A right jugular venous line for infusion of fluid and drugs and a left carotid arterial line for continuous monitoring of blood pressure (pressure transducer MX860; Medex, Haslingdon, UK, and Monitor 78353A; Hewlett-Packard, Bracknell, UK) and intermittent sampling for blood gas analysis (165μl heparinised capillary tube samples; processed by ABLA; Radiometer, Copenhagen, Denmark) were placed. A tracheostomy (2.08mm external diameter polythene tubing) was sited and cut to a length approximating anatomic dead space to secure the airway and allow tracheal toilet. A 1ml bolus of 0.9% saline solution was given after placement of these lines followed by a continuous infusion of 4ml/h/250g of body weight. A midline laparotomy was performed to gain access to the abdominal vasculature and bladder. Doppler flow probes were placed on the left renal artery (1mm, J reflector [1RB]) and on the infrarenal abdominal aorta (2mm, J reflector with sliding gate [2SB]) and connected to a flow monitor (T206; monitor and probes from Transonics, Ithaca, NY). One cannula (1.57 mm outside diameter) was inserted surgically into the bladder, via the laparotomy, through an incision in the avascular area at its dome, to allow free drainage of urine. A sample of the saline solution was then put through the blood gas analyser and the electrode was calibrated to this value after temperature compensation (correlation factor x 1.04/°C). This procedure was repeated after each experiment to check for electrode drift. After instrumentation, the animals were left to stabilise until three consistent haemodynamic measurements at 5 min intervals were obtained. The animals were injected with IS4 (3mg/Kg; n=6), control
IgG (3 mg/Kg; n=6) and normal saline to a total volume of 400μl. Mean arterial blood pressure, aortic and renal artery flows and renal tissue oxygenation were recorded before administration of the antibodies and every 15 minutes thereafter, for 240 minutes. Blood for the measurement of oxidation markers was collected before the antibody administration and at the end of the experiment. Approval for this study was obtained from the Home Office (according to the Animals [Scientific Procedures] Act 1986).

2.5.1.4 Immune and biochemistry assays

Paraoxonase activity, total anti-oxidant capacity of plasma, total serum nitric oxide and immunohistochemistry (iNOS and eNOS) were performed as described in sections 2.3 and 2.4 of this chapter.

2.5.1.5 Data analysis

Responses were plotted graphically as means from each set of eight experiments with vertical bars representing standard error of the means. PE responses are expressed as active tension above the baseline tension and Ach, SNP and arginine responses as percentage reversal of the PE-induced tone. In studies with L-NAME and 1400W plots show PE response both before and after incubation with the inhibitors. All curves were compared by 2-way ANOVA using Prisma software. A p value of <0.05 was taken to indicate a statistically different difference. Further statistical analysis was performed using the Statistical Package for Social Sciences (SPSS), Inc (Chicago, USA). Non-
parametric (Kruskall-Wallis test) and parametric (t-student) were employed to compare differences between two groups. Overall comparisons were performed with one-way ANOVA with a post hoc Bonferroni correction.

2.5.2 In vitro inhibition of PON activity by IS4

2.5.2.1 Experimental protocol

Fifty microliters of sera from 5 healthy controls was incubated with 50\(\mu\)l of IS4 (anti-CL IgG) supernatant (10\(\mu\)g/ml), collected from IS4-producing hybridoma cells, for 1 hour at 37°C. As controls, irrelevant human IgG (Sigma-Aldrich, Poole, UK) at the same concentration, and PBS were used. Samples were then kept at room temperature and PON activity was measured every 10 minutes for half an hour and then every 30' for 4.5 hours, according to the method described in chapter 2.

In the second part of the experiment, IS4 supernatant, at 10\(\mu\)g/ml (maximum concentration obtained in supernate), was diluted (2, 4, 8 and 16 fold) and 50\(\mu\)l of the dilution was incubated, in triplicate, with 50\(\mu\)l of sera from a healthy subject. Paraoxonase activity was again measured every 30' for 4 hours.
2.5.3 Induction of oxidative stress in vivo by antiphospholipid antibodies

2.5.3.1 Mice and hybridoma cells

Female BALB/c SCID mice were obtained from Harlan UK (Bicester, UK) at 8 weeks of age. The mice were all housed in sterile conditions on vented racks. All procedures were carried out in accordance with the 'Animals Scientific Procedures Act 1986'.

A human IgG monoclonal antibody producing hybridoma (IS4), which binds to cardiolipin and β2-GP1, and a human IgG hybridoma cell line (TW), secreting human IgG of unknown specificity, which tested negative against cardiolipin and β2-GP1 (a kind gift from Thomas Winkler, Erlangen, Germany), were used. In addition to these human hybridoma derived antibodies, 12A1-A17.3 and 16A3-14.11 which are murine IgG1 anti-β2-GP1 and murine IgM anti-CL antibody producing cell lines, and 29J3-119 and 16B4-2 which are murine IgG1 and IgM antibodies that do not bind to β2-GP1 or cardiolipin were also used (Price 1996). CB-F7, the non-secreting mouse-human heteromyeloma fusion partner cell was also used as a negative control.

Cells were cultured in RPMI 1640 medium containing 1% L-glutamine, 1% sodium pyruvate, 2% MEM non-essential amino-acids, 1% penicillin/streptomycin, 0.2% gentamycin (all from Gibco, city, UK) and 10% FCS (Sigma, city, UK).
2.5.3.2 Experimental schedule

Mice were acclimatized for one week and then primed with 500μl i.p. of pristane (2,6,10,14-tetramethylpentadecane, Sigma), which activates macrophages to produce growth factors and create an optimal environment for hybridoma cell growth. Ten days later the mice were injected intraperitoneally with hybridoma cells (1x 10^6 cells in 500μl of RPMI 1640). Mice were injected with: IS4 (n=5), TW (n=4), 12A1-A17.3 (n=5), 29J3-119 (n=5), 16A3-14.11 (n=5), 16B4-2 (n=5), CB-F7 (n=5) and with pristane only (no cells) (n=5).

Throughout the experiment, proteinuria was assessed using Albustix (Bayer Diagnostics, Berks, UK) and weight was assessed every 72 hours. Mice were sacrificed, when the development of ascites resulted in a 20% increase in body weight, or after 1.5 months if ascites had not developed. On sacrifice, sera, ascitic fluid, and organs were collected for further analysis.

2.5.3.3 Immune and biochemistry assays

Human IgG ELISA, anti-cardiolipin ELISA (human and murine), anti-β2-GP1 ELISA (murine), nitrotyrosine ELISA, paraoxonase activity, total anti-oxidant capacity of plasma, total serum nitric oxide, haematoxylin and eosin histological stains, western blot analysis (iNOS and eNOS) and immunohistochemistry (iNOS and eNOS) were performed according to the methods described in sections 2.1, 2.3 and 2.4 of this chapter.
2.5.3.4 Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS), Inc. (Chicago, USA). Non-parametric tests were employed to compare differences between groups (Kruskall-Wallis test) and to evaluate associations between variables (Spearman's rank). A stepwise multiple regression was used to test the independence of the associations detected by univariate analysis.

2.5.4 Antibodies towards HDL and β2-GP1 and PON activity in patients with SLE and primary APS

2.5.4.1 Patients and methods

This cross-sectional study was carried on 68 patients selected at random (32 SLE and 36 primary APS) from the outpatient clinics of the Centre for Rheumatology, the Haematology Department of the Middlesex Hospital, London, UK and the Coagulation unit of the Cardarelli Hospital in Naples, Italy. None of the patients with SLE had APS and all of the patients with APS had primary disease (PAPS). Twenty age and sex matched healthy controls served as a control group. Demographic data are shown in table 5.1. All of the patients with SLE met four or more of the revised American College of Rheumatology criteria for classification of SLE (Hochberg 1997), and all the patients with APS met the Sapporo Revised criteria for primary APS (Wilson 1999). None of the patients in the study were on lipid lowering agents or anti-oxidant drugs. None of the patients with SLE had a history of thrombosis or other atherosclerosis related events. On the day of serum sampling the lupus patients’ disease activity was assessed using the
British Isles Lupus Assessment Group (BILAG) scoring system (Hay 1993). The individual disease activity assessments in the eight organ/systems classification were combined to provide a global score, using the scoring system: A=9 points (the most active), B=3, C=1, D/E=0. Scores in excess of 6 points were regarded as indicative of active disease, less than 6 as inactive. Clinical data from patients with SLE and PAPS included information regarding steroid dose, anti-malarial dose, anti-platelet agents and anticoagulation status. Sera from patients and controls were kept at -80°C until the laboratory tests were performed. The study was carried out according to the declaration of Helsinki and informed consent was given before participation in the study.

Anticardiolipin, anti-β2-GP1, antiprothrombin, and anti-HDL antibodies, total cholesterol, total HDL, HDL sub-fractions 2 and 3, paraoxonase activity and total antioxidant capacity of plasma were tested as described in sections 2.1, 2.3 and 2.4 of this chapter.

2.5.4.2 Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS), Inc (Chicago, USA). Non-parametric tests were employed to compare differences between groups (Kruskall-Wallis test), and to evaluate associations between variables (Spearman’s rank). A stepwise multiple regression, tested the independence of the associations detected by univariate analysis.
2.5.5 Cross-reactivity between anti-CL, anti-HDL and anti-Apo A-I IgG antibodies in patients with SLE and primary APS

2.5.5.1 Patients, monoclonals and immune and biochemistry assays

Fifty consecutive patients (25 SLE and 25 primary APS), attending the Lupus clinic at the Centre for Rheumatology, Middlesex Hospital were selected along with 10 age and sex matched healthy controls (table 1). All patients with SLE fulfilled the revised ACR criteria (Hochberg 1997) and the patients with PAPS met the Sapporo criteria (Wilson 1999). Sera from the patients were collected and stored at –80 C and tested simultaneously for anti-CL, anti-HDL and anti-Apo A-I IgG antibodies. From this cohort, twelve patients (6 SLE and 6 APS) were selected at random for the cross-reactivity studies, based on their high titres of anti-CL and anti-HDL antibodies. The study was carried out according to the declaration of Helsinki and after Ethical Committee approval was obtained. Informed consent was also given before participation in the study.

Three anti-CL IgG human monoclonal antibodies (IS4, CL1 and CL24) obtained through culture of human hybridoma cell lines from patients with APS (Zhu 1999a) (kindly provided by Dr. Pojen Chen, UCLA, USA), were also tested for activity against cardiolipin, HDL and Apo A-I and were initially used to standardise the cross-reactivity assay.
IgG anti-CL, anti-HDL and anti-Apo A-I are described in section 2.1 of this chapter.

2.5.5.2 Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS), Inc (Chicago, USA). Non-parametric tests were employed to compare differences between groups (Kruskall-Wallis test), and to evaluate associations between variables (Spearman’s rank). A stepwise multiple regression tested the independence of the associations detected by univariate analysis.

2.5.5.3 HDL and Apo A-I inhibition of the binding of IS4 to cardiolipin

For this experiment, IS4 was incubated in fluid phase, at different concentrations ranging from 0.8 to 0.004 µg/ml, with human HDL (obtained from a healthy control), at concentrations of 400 to 3.125µg/ml and with Apo A-I (Sigma-Aldrich) at concentrations of 40 to 0.3µg/ml, for 1 hour at 37° C.

Ninety-six well Polysorb plates were half-coated with cardiolipin, (Sigma-Aldrich), at 50µg/ml in ethanol and the samples (IS4 + HDL and IS4 + Apo A-I) were tested using the anti-CL ELISA assay described above.

2.5.5.4 IS4 binding to HDL and HDL+CL in an inhibition assay

Ninety-six well Polysorb plates were half-coated with HDL in ethanol, at 5µg/ml, 100µl/well, overnight, at 4° C. Blocking was performed using PBS containing 2% BSA (Sigma-Aldrich) for 1 hour at room temperature. Plates were then washed once
using PBS. To half of the wells coated with HDL, cardiolipin in decreasing concentrations (125 to 0.5μg/ml in ethanol) was added for 1 hour at 37°C. After five washes with PBS, IS4 (0.8μg/ml) in PBS was added to all the wells. 100μl of alkaline phosphatase conjugated anti-human IgG (1:1000) was added for 1 hour. 100μl p-nitrophenyl phosphate (Sigma-Aldrich) in 5ml of diethanolamine buffer (pH 9.8) was added and incubated at 37°C for colour development and the absorbance read at 405nm after 30'. The results were expressed in OD after subtraction of background in non-coated half of the plate.

2.5.5.5 Cross-reactivity assay

A ninety-six well Polysorb plate was divided in three and coated with CL (50μg/ml in ethanol), HDL (20μg/ml in ethanol) and Apo A-I (10μg/ml in PBS), 200 μl/well, overnight at 4°C. Plates were blocked with 10% foetal calf serum in PBS (for CL) and 2% BSA/PBS for HDL and Apo A-I. IS4, CL1 and CL24 IgG human monoclonals (neat) and sera from 12 patients (1:20) were added in quadruplicate. After washing five times with PBS, 250μl/well of glycine buffer (pH 2.0) was added for 5 minutes to elute antibodies that remained bound to each of the coating antigens. The content of the four wells of each sample were then collected and the pH was normalised by adding TRIS buffer, 1M (pH 9.0), 20μl. Each sample was then added to a second ELISA plate, previously coated with CL (50μg/ml in ethanol), HDL (20μg/ml in ethanol), Apo A-I (10μg/ml in PBS) and anti-Human IgG (2.5μl/ml) (Sigma-Aldrich), 100μl/well in duplicate for each antigen, and blocked as described before. Human IgG
was used as a positive control, to assess the presence of antibody after elution from the 1st plate with glycine buffer. After washing, 100μl of alkaline phosphatase conjugated anti-human IgG (1:1000) was added for 1 hour. 100μl p-nitrophenyl phosphate (Sigma-Aldrich) in 5ml of diethanolamine buffer (pH 9.8) was added and incubated at 37°C for colour development and the absorbance read at 405nm after 1 hour. Results were expressed as a ratio between the binding to each antigen and the binding to total IgG, for each plate, and after subtraction of the background value in the uncoated half of the plate. Samples were considered positive when the referred ratio (ab binding / total IgG binding) was above 1.

2.5.6 Antiphospholipid antibodies induce oxidative stress in infertile women without antiphospholipid syndrome

2.5.6.1 Patients and statistical analysis

Seventy consecutive infertile females undergoing routine IVF treatment were selected. Twenty-eight age matched healthy fertile women (as evidenced by at least one healthy pregnancy) were used as controls. Patients were divided into two groups according to the IVF treatment they received: after one IVF cycle (n = 13) and after three IVF cycles (n = 39). A group of infertile women never treated with IVF (n = 18), was included as a second control. Of those who had three IVF cycles, 11 had a successful outcome after the third cycle and 28 were unsuccessful (18 miscarriages and 10 implantation failures). The 52 patients who had received treatment were also divided into four groups according to fertility diagnosis: male / partner (n = 19), tubal (n = 21),
endometriosis (n = 4) and unexplained infertility (n = 8). Sera were collected on the
twelfth day of the menstrual cycle, before Human Chorionic Gonadotrophin (HCG)
administration and stored at −70°C. The study was carried out according to the
Declaration of Helsinki and informed consent was given before participation in the
study.

Anticardiolipin and antiphosphatidylserine, anti-dsDNA, paraoxonase activity
and total antioxidant capacity of plasma were performed as described in sections 2.1 and
2.3 of this chapter. (Anticardiolipin and antiphosphatidylserine ELISA assays for this
study, were performed by Dr. E. Radway-Bright).

Statistical analysis was performed using the Statistical Package for Social
Sciences (SPSS), Inc (Chicago, USA). Non parametric tests were employed to compare
differences between groups (Kruskall-Wallis test), and to evaluate associations between
variables (Spearman’s rank). A stepwise multiple regression tested the independence of
the associations detected by univariate analysis.
Chapter 3:

INDUCTION OF NITRIC OXIDE SYNTHETASE BY A HUMAN MONOCLONAL IgG anti-CL ANTIBODY

3.1 Production of human monoclonal antibodies derived from fusion of lymphocytes and CB-F7 cells

The aim of these experiments was to attempt the production of human monoclonal anti-CL antibodies, to be used in the following experiments.

3.1.1 Results

Three different sets of fusion experiments were performed using lymphocytes from a patient with SLE and secondary APS (positive for anti-dsDNA and anti-CL antibodies - both IgG and IgM). All the fusion experiments resulted in the generation of human hybridomas, producing IgG or IgM antibodies.
In experiment one, growth of hybridoma cells occurred in 33 wells, out of a total of 136 seeded wells. Of these, 14 wells produced IgG antibodies and 5 produced IgM antibodies.

In experiment two, hybridoma cells were found in 25 out of 103 seeded wells. Only 7 were found to produce human IgG and none produced IgM antibodies.

In experiment three, 14 out of 189 seeded wells presented hybridoma cells and of these 3 produced IgG antibodies and 5 IgM antibodies. (Table 3.1)

Table 3.1: Hybridoma cells derived from fusion of lymphocytes and CB-F7 cells: binding to human IgG and IgM.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>No. of wells seeded</th>
<th>No. wells with hybridomas</th>
<th>No. of IgG positive wells</th>
<th>No. of IgM positive wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>136</td>
<td>33 (24.3%)</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>103</td>
<td>25 (24.3%)</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>189</td>
<td>14 (7.4%)</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

Supernatants of wells that tested positive for either IgG or IgM antibodies were then tested for the specific binding to CL or dsDNA.

None of the tested supernatants were found to be positive for antibodies against these antigens. (Table 3.2)
Table 3.2: Binding of antibodies present in hybridoma containing supernatants to CL and dsDNA.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Anti-dsDNA IgG (mean OD)</th>
<th>Anti-dsDNA IgM (mean OD)</th>
<th>Anti-CL IgG (mean OD)</th>
<th>Anti-CL IgM (mean OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.4 ± 0.7</td>
<td>8.2 ± 0.5</td>
<td>10.5 ± 0.8</td>
<td>7.1 ± 0.5</td>
</tr>
<tr>
<td>2</td>
<td>9.3 ± 0.5</td>
<td>nt</td>
<td>12.4 ± 0.9</td>
<td>Nt</td>
</tr>
<tr>
<td>3</td>
<td>8.5 ± 0.6</td>
<td>4.3 ± 0.8</td>
<td>8.7 ± 0.6</td>
<td>9.8 ± 0.8</td>
</tr>
</tbody>
</table>

Legend: Results are expressed in percentage of the positive controls. OD: optical density; nt: not tested.

3.1.2 Discussion

All fusion experiments performed produced hybridoma cells. Of these, a small proportion was found to produce either IgG or IgM human antibodies. However, none of these showed specific binding to either dsDNA or CL. The limitation of this methodology is that it is not possible to select information for specific antigen-binding antibodies from the patient's lymphocytes.

The possibility that the antibodies produced could present a specific binding towards a different antigen cannot be excluded. However, in the context of this thesis, the
search for any other possibility would be a deviation from the main objectives of these studies.

3.2 Human monoclonal IgG anti-CL antibody induces NOS expression in an *ex vivo* and in an experimental model

3.2.1 Results

*Ex vivo aorta bath model*

The PE concentration-dependent contraction of the rat aorta was markedly decreased in vessels treated with IS4, when compared to IgG and PBS ($n=8$, $p<0.001$; figure 3.1). There were no significant differences in the degree of aorta contraction between the IS4 and LPS treated groups, and between the IgG and PBS control groups.

The Ach response was also diminished in the IS4 treated vessels compared to control and IgG ($n=8$; $p=0.0002$; figure 3.2). The difference in the response to PE by IS4 and IgG treated vessels was abolished by the addition of L-NAME or 1400W indicating that the PE hyporeactivity is secondary to an increased activation of iNOS (figure 3.1). The response to L-arginine was significantly greater in the IS4 treated vessels ($n=6$; $P=0.005$), in keeping with iNOS induction.
Figure 3.1: Phenylephrine concentration response curve following short-term incubation with IS4 leads to hyporeactivity compared to non-specific IgG and saline controls (* P<0.001). This hyporeactivity is reversed by incubation with 1400W.

Figure 3.2: Acetylcholine concentration response curve of percentage relaxation of maximal contraction following short-term incubation with IS4 showing impairment in the vessels relaxation to acetylcholine (* P=0.0002)
The hyporeactivity to PE induced by IS4 was prevented by co-incubation with cardioliopin, but not polymixin, suggesting that the effect is antibody specific and not due to LPS contamination.

Incubation with IS4 did not lead to a reduction in the relaxation to the endothelial-independent NO donor SNP. There were no significant differences in the response of different vessels to KCL.

**In vivo experimental model**

Mean arterial pressure (MAP) in rats injected with IS4 became significantly reduced 135' after antibody administration, when compared with IgG and "saline" control groups (p<0.005, for both). This difference persisted till the termination of the experiment at 240 minutes. Aortic blood flow (ABF) decreased significantly after 90 minutes post-injection in the IS4 group when compared to the IgG control group (p<0.03).

Renal artery flow and renal tissue oxygenation were not significantly different in any the groups throughout the total length of the experiment. There were no differences in the recorded values of all the parameters assessed, between animals injected with control IgG and normal saline (Figure 3.3).
Figure 3.3: Haemodynamic variables in IS4, control IgG and saline groups. Represented values are means and standard error of mean. * p< 0.005 when compared with control IgG and “saline” groups. ** p<0.03 when compared with control IgG and p=ns (0.71) when compared with the “saline” control group.
Oxidation parameters: PON activity, and TAC and NO measurements in plasma of rats injected with IS4, control IgG and normal saline.

There were no changes in PON activity before and after antibody administration in any of the groups. TAC (peroxynitrite quenching) was significantly reduced after administration of IS4 (p<0.02), and control IgG (p<0.02), but not after saline infusion. TAC (superoxide quenching) was increased post-IS4 (p<0.03), and post-control IgG (p<0.03) infusions, but there were no significant differences before and after saline was given. NO levels were significantly increased after administration of IS4 (p<0.007), but there were no differences before and after administration of control IgG and normal saline (table 3.3). When the difference between plasma levels of PON, TAC and NO, before and after administration of IS4, control IgG and saline was considered, only NO showed a significant difference between the IS4 and the IgG control group (p<0.01)(figure 3.4). There was a trend for a decreased TAC (peroxynitrite)(p=0.052), and an increased in superoxide production (p=0.052) in the IS4 group when compared to the control IgG group.
<table>
<thead>
<tr>
<th></th>
<th>PON activity (mmol/ml/min)</th>
<th>TAC (peroxynitrite) (min)</th>
<th>TAC (superoxide) (mV)</th>
<th>NO (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>IS4 (n=6)</td>
<td>54±5</td>
<td>61±6</td>
<td>23.3±2.6</td>
<td>13.3±2.6*</td>
</tr>
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<td></td>
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<tr>
<td></td>
<td>(p=0.02)</td>
<td>(p=0.03)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.72±0.2</td>
<td>2.03±0.8*</td>
</tr>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(p=0.007)</td>
<td></td>
</tr>
<tr>
<td>Ctrl IgG (n=6)</td>
<td>59±3</td>
<td>57±4</td>
<td>23.3±2.6</td>
<td>15.8±2.0*</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>0.75±0.1</td>
<td>3.10±0.2*</td>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(p=0.03)</td>
<td></td>
</tr>
<tr>
<td>Saline (n=3)</td>
<td>55±3</td>
<td>60±2</td>
<td>25.0±0.0</td>
<td>20.0±0.0</td>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.70±0.0</td>
<td>2.90±0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(p=0.03)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3: PON activity, and TAC and NO mean levels before and after IS4, control IgG and saline administration.
Figure 3.4: Difference between NO levels, before and after the administration of IS4, control IgG and saline. * p<0.01 and **p<0.02 when compared with IS4.

**Relationship between haemodynamic variables and oxidative markers**

Variation of MAP at 210’ (from baseline) inversely correlated with plasma NO levels (t= -0.828; p<0.001) and directly with TAC (superoxide quenching) (t= -0.654; p<0.008), in blood samples collected at the end of the experiment. Variation of ABF at 210’ (from baseline) also correlated directly with plasma NO levels (t= 0.529; p<0.05) and TAC (superoxide quenching) (t= 0.514; p<0.05). In a multiple regression model, to
evaluate which variable best associated with a decrease in MAP and ABF, I employed a stepwise multiple regression. NO levels from plasma collected at the end of the experiment were the only independent predictor of both MAP and ABF ($t= -4.673; p<0.001$ and $t= -2.909; p<0.02$, respectively).

**Immunohistochemistry**

Staining for iNOS was significantly increased in kidney vessels of animals injected with IS4, when compared with control IgG and saline (figure 3.5). There was no obvious difference in the staining for eNOS in the same tissues and for the different groups (figure 3.6).

3.5 – iNOS immunostaining in kidney vessels of rats injected with IS4, control IgG and saline.
3.6 - eNOS immunostaining in kidney vessels of rats injected with IS4, control IgG and saline

![IS4](image1)  ![IgG](image2)  ![Control](image3)

### 3.2.2 Discussion

In this study the *ex vivo* model demonstrated that short-term incubation with IS4 leads to phenylephrine and acetylcholine hyporeactivity in rat aortic tissue, with no change in response to the endothelium-independent vasodilator sodium nitroprusside. These changes are reversible following incubation with L-NAME or 1400W and were prevented when the antibody was pre-incubated with its antigen cardiolipin. Addition of L-arginine pre-constricted tissues led to significant relaxation in the IS4 treated vessels, with little effect on control vessels. Lipopolysaccharide contamination was excluded by measurement of the levels by limulus chromogenic assay and by the failure of polymixin to alter the results.

Phenylephrine hyporeactivity that is reversible with L-NAME indicates an increase in NOS expression or activity. The ability of the effects of phenylephrine to be reversed by 1400W indicates that the IS4 leads to an increase in iNOS expression. The effect of the addition of L-arginine supports the view that iNOS is upregulated as only this isoform is
substrate deficient in this setting. Specificity of the effects is suggested by the ability to prevent the effects with cardiolipin and the lack of significant LPS.

The direct effect of the antibody was confirmed in the in vivo experimental model, which also showed an increase of iNOS expression induced by IS4, and resulting in a significant increase of NO plasma levels when compared to a non-specific, control IgG. The haemodynamic changes found in the experimental model between the groups administered with IS4 and control IgG or saline are consistent with an increase in NO production. Thus, a decrease in arterial pressure and flow would be expected. In fact, both MAP and ABF inversely correlated with NO levels from blood collected at the end of the experiment. Furthermore, NO was found to be the only independent predictor of MAP and ABF.

Nitric oxide is antithrombotic (Bouchie 1998; Freedman 1997), by its interaction with platelets, and therefore a reduction in NO production would be expected to correspond to the phenotype of the antiphospholipid syndrome. It has previously been shown that iNOS induction by LPS causes an inhibition of physiological endothelial NOS derived NO production (Lu 1996; Parker 1993). In these experiments hyporeactivity to acetylcholine is observed as this only acts through eNOS, which has been downregulated. Acetylcholine hyporeactivity occurred following incubation with IS4, while the response to SNP was preserved, indicating the downregulation of eNOS activity in this study. Our group reported a reduction of plasma levels of NO in an experimental model, measured 30 days after a maintained level of IS4 in circulation (Delgado Alves 2002a). Interestingly, an increase in plasma nitrotyrosine was also found in these animals, suggesting that high levels of oxidation and NO production might have
been present earlier. This result can be interpreted, in the context of these results, as showing the early iNOS upregulation results in downregulation of eNOS and, in the longer term, causes reduced NO production. In this way the reduced NO production by eNOS can act as the substrate for the prothrombotic phenotype.

The data regarding NO levels in patients with APS is still controversial. Porta et al, reported normal levels of NO in patients with primary APS (Porta 1997), whilst lower levels of NO, assessed by its urinary metabolites, have been reported by others (Ames 2000a). In the later study, an increase in plasma oxidation and coagulation activation was also reported, suggesting that, an increase in “NO catabolism” rather than a decreased synthesis could be present.

Oxidative stress is an important factor in atherosclerosis and thrombogenesis in general, and in APS in particular (Ames 1998). However, it is not clear whether the pro-oxidant changes found in patients with APS are directly related to the presence of anti-CL antibodies. Lipid peroxidation has been reported in patients with anti-CL antibodies in the context of different auto-immune diseases with vascular involvement, e.g. SLE, Beçhet’s Syndrome and systemic vasculitidies (Ames 1999).

PON activity has been shown to be reduced by anti-CL antibodies, both in vitro (see chapter 4), and in patients with APS (Delgado Alves 2002b). PON is an antioxidant enzyme, which prevents oxidation of LDL, and has been considered an important factor in the prevention of atheroma progression (Durrington 2001). However, in this study, there were no differences in the enzyme activity, before and after the administration of IS4. The short period of time of the experiment and the possible differences between rat and human paraoxonase, may account for this finding.
Antiphospholipid antibodies have been shown to activate endothelial cells (Del Papa 1995), inducing the expression of adhesion molecules and E-selectin (Meroni 2000). Furthermore, this activation was found to be mediated by nuclear factor-κB (NF-κB) (Meroni 2001), which has been reported to be a relevant factor in the induction of iNOS expression in these cells (Cooke 2002). This common pathway may explain the overall activation of endothelial cells (both immune and oxidative), found in the presence of anti-CL antibodies.

In conclusion I have shown, in an experimental model, that a human monoclonal IgG anti-CL antibody induces iNOS in isolated rat aortic rings and, when injected in vitro, increases plasma levels of NO that then lowers blood pressure and systemic vascular resistance. Anticardiolipin antibody-induced iNOS expression may lead to feedback inhibition of endothelium-derived NO as shown by impaired relaxation to Ach in this model that, if prolonged, could contribute to the increased risk of thrombosis found in patients with APS.
Chapter 4:

ANTIPHOSPHOLIPID ANTIBODIES INHIBIT PON ACTIVITY AND INDUCE OXIDATIVE STRESS IN VIVO

4.1 In vitro inhibition of paraoxonase activity by a human monoclonal IgG anti-CL antibody (IS4)

This group of experiments was intended to demonstrate an interaction (inhibition) of paraoxonase activity by a human IgG anti-CL monoclonal antibody.

4.1.1 Results

Paraoxonase activity was significantly reduced in the sera of healthy donors after more than three hours of incubation with IgG anti-CL monoclonal antibody (IS4), when compared with irrelevant human IgG or PBS (p < 0.001). There were no differences between the two control groups (Figure 4.1).
Figure 4.1: Incubation of sera from healthy controls with anti-CL IgG antibody compared to irrelevant human IgG and PBS (* p<0.001).

Paraoxonase activity was also measured after incubation of healthy sera with increasing dilutions of anti-CL IgG (IS4). PON activity was significantly higher when sera were incubated with a lower concentration of IS4 (p<0.01 for a 1:4 dilution and p<0.001 for a 1:8 and 1:16 dilutions when compared to IS4 at the original concentration-10μg/ml) (Figure 4.2).
Figure 4.2: Increasing activity of serum paraoxonase when incubated with lower concentrations of anti-CL IgG (IS4). * $p<0.01$ and ** $p<0.001$, when compared to "IS4". (IS4 = concentration of anti-CL IgG of 10µg/ml).

### 4.1.2 Discussion

Paraoxonase activity in serum from healthy subjects was significantly reduced after a 4 hour incubation period with an anti-CL IgG human monoclonal antibody, at the concentration of 10µg/ml, when compared with irrelevant human IgG and normal PBS. Furthermore, there were no differences in the enzymatic activity between the two control groups (irrelevant IgG and PBS), confirming that the reported effect was not a consequence of the incubation with any antibody, but was related to its specificity. This was reinforced by the direct relationship between the anti-CL IgG antibody concentration and the measured activity of the enzyme.
PON is present in plasma only as a part of the HDL complex hence a variation of the enzymatic activity, without changes in the overall concentration of the HDL content, suggests a direct interference of IS4 with the HDL molecule. Possible targets could be cardiolipin or any other phospholipids present in the HDL complex (Deguchi 2000), apolipoprotein A-I (Dinu 1998) or paraoxonase itself. PON activity has been found to be dependent on apolipoprotein A-I constitution (Oda 2001), suggesting that conformation changes of some of the HDL constituents, might interfere with the enzymatic activity.

4.2 Anticardiolipin antibodies are associated with decreased plasma NO and PON activity in a murine model of APS

A murine model was used to address the effect of anti-CL antibodies on PON activity and on the development of a pro-oxidant status.

4.2.1 Results

Antibody titres and characteristics of the different animal groups

All mice injected with human hybridoma-producing cells were found to be positive for human IgG with no significant differences among titres in those groups.
Animals injected with both mouse anti-CL and anti-β2-GP1 producing hybridomas were all positive for the presence of both antibodies. None of the animals belonging to the control groups (TW, 29J3-119, 16B4-2, CB-F7, or “no cells”) had human anti-CL, murine anti-β2-GP1, or murine anti-CL antibodies in their sera. There were no significant differences in weight and survival (time between hybridoma injection and sacrifice) among the different groups.

Characterisation of the oxidative status: PON activity, TAC (peroxynitrite and superoxide quenching), NO, and nitrotyrosine

Values of all the measures of oxidative status are shown in Table 4.1. PON activity was reduced in the IS4 group (human IgG anti-CL), when compared to the TW (human IgG control), CB-F7, and “no cells” groups (p<0.002, p<0.01, and p<0.001 respectively). Mice positive for murine anti-β2-GP1 IgG had a decrease in PON activity compared to the murine positive for irrelevant IgG, but that difference did not reach statistical significance. There were no differences regarding PON activity in the remaining groups.

TAC (peroxynitrite quenching) was decreased in the IS4 group (human IgG anti-CL), when compared to the human IgG control, CB-F7, and “no cells” groups (p<0.01, p<0.01, and p<0.008, respectively). Animals positive for murine anti-β2-GP1 IgG and IgM anti-CL also had a significant decrease when compared to their specific control group (irrelevant murine IgG or IgM), (p<0.03 and p<0.05, respectively). There were no significant differences between the IgM or IgG control groups and the CB-F7 (“non-Ig-producing cells”) and “no cells” groups.
TAC (superoxide quenching) was increased in the IS4 (human IgG anti-CL) group, when compared to human IgG control, CB-F7, and "no cells" controls (p<0.01, for all) It should be noted that, in the case of superoxide quenching, increased superoxide levels are reflected by higher values of plasma TAC. All other groups had similar levels.

NO was significantly decreased in the IS4 group (human IgG anti-CL), when compared to the control groups (p<0.04). In contrast, there was no significant difference between the groups positive for murine IgG anti-β2-GP1 or IgM anti-CL and their relevant control groups.

Nitrotyrosine was found to be elevated only in the IS4 group (human IgG anti-CL). This was significant, when compared to all the control groups (p<0.03).
Table 4.1: Characterisation of the oxidative status: PON activity, TAC (peroxynitrite and superoxide quenching), NO, and nitrotyrosine in the different animal groups.

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>Origin</th>
<th>Ab characteristics</th>
<th>PON (mmol/ml/min)</th>
<th>TAC (perox) (min)</th>
<th>TAC (superox) (mV)</th>
<th>NO (μmol/l)</th>
<th>Nitrotyros (μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS4</td>
<td>Human</td>
<td>IgG anti-CL</td>
<td>74±4*</td>
<td>27±2#</td>
<td>4.0±0.2#</td>
<td>36.9±8.9#</td>
<td>1.50±0.06#</td>
</tr>
<tr>
<td>12A1-A17.3</td>
<td>Murine</td>
<td>IgG anti-β2-GPI</td>
<td>75±5</td>
<td>24±2**</td>
<td>2.3±0.8</td>
<td>110.9±31.5</td>
<td>1.29±0.08</td>
</tr>
<tr>
<td>16A3-14.11</td>
<td>Murine</td>
<td>IgM anti-CL</td>
<td>91±4</td>
<td>29±2***</td>
<td>2.2±0.2</td>
<td>94.3±19.2</td>
<td>1.30±0.04</td>
</tr>
<tr>
<td>TW</td>
<td>Human</td>
<td>IgG control</td>
<td>88±4</td>
<td>45±7</td>
<td>1.9±0.3</td>
<td>93.2±35.5</td>
<td>1.25±0.15</td>
</tr>
<tr>
<td>29J3-119</td>
<td>Murine</td>
<td>IgG control</td>
<td>81±10</td>
<td>34±9</td>
<td>2.3±0.2</td>
<td>102.9±35.4</td>
<td>1.28±0.11</td>
</tr>
<tr>
<td>16B4-2</td>
<td>Murine</td>
<td>IgM control</td>
<td>85±2</td>
<td>36±9</td>
<td>2.3±0.2</td>
<td>107.3±18.3</td>
<td>1.34±0.06</td>
</tr>
<tr>
<td>CB-F7</td>
<td>NA</td>
<td>NA</td>
<td>90±10</td>
<td>37±4</td>
<td>2.3±0.2</td>
<td>97.5±12.4</td>
<td>1.36±0.04</td>
</tr>
<tr>
<td>No cells</td>
<td>NA</td>
<td>NA</td>
<td>101±6</td>
<td>45±4</td>
<td>1.9±0.4</td>
<td>68.9±7.7</td>
<td>1.34±0.07</td>
</tr>
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</table>

Table 4.1: *- significant when compared to TW (p<0.002), CB-F7 (p<0.01), and “no cells” (p<0.001) groups. **- significant when compared to 29J3-119 (p<0.03), CB-F7 (p<0.05), and “no cells” (p<0.05) groups. ***- significant when compared to 16B4-2 (p<0.03), and “no cells” (p<0.05) groups. # - significant when compared to TW (p<0.01), CB-F7 (p<0.01), and “no cells” (p<0.008) groups. #φ - significant when compared to TW, CB-F7, and “no cells” groups (p<0.04 for all).

NA: non applicable; perox: peroxynitrite; superox: superoxide; Nitrotyros: Nitrotyrosine.
Relationship between antioxidant variables

PON activity showed an inverse correlation with both total human IgG titres (p<0.001) and TAC (superoxide) (p<0.008) in mice injected with human IgG-producing hybridomas. There was also a direct correlation between PON activity and TAC (peroxynitrite) (p<0.0009).

NO levels were inversely correlated with both TAC (superoxide) and nitrotyrosine levels (p<0.008 and p<0.01, respectively).

Multiple regression models

To evaluate which factors could be independent predictors of PON activity, NO levels, and nitrotyrosine, stepwise multiple regressions were performed and the following variables were considered: antibody type, total IgG, total IgM, and TAC (peroxynitrite and superoxide).

TAC (peroxynitrite) and total IgG levels were found to be independent predictors of PON activity (p<0.0009 for TAC [peroxynitrite] and p<0.02 for total IgG titres). TAC (superoxide) was the only independent predictor for NO levels (p<0.008) and nitrotyrosine (p<0.002).

Western blot analysis (iNOS and eNOS)

Expression of iNOS in the hearts of mice positive for IS4 (human IgG anti-CL) was significantly reduced, when compared to tissue samples from the human control IgG and CB-F7 groups. Expression of iNOS in the “no cells” group was lower than that of the other control groups, which all showed increased iNOS expression because of
hybridoma cell-induced peritonitis (Figure 4.3). The expression of iNOS in the murine Ig groups showed no apparent differences (not shown).

There were no significant differences in the expression of eNOS in heart tissues collected from both the human (Figure 4.4) and the murine Ig groups.

4.3- iNOS expression in mouse heart

4.4- eNOS expression in mouse heart

Figures 4.3 and 4.4: Western Blot analysis of iNOS (4.3) and eNOS (4.4) in the heart of animals injected with IS4, TW and CB-F7 and “no cells” controls. Mice from the “no cells” group did not have cell-induced peritonitis, hence the decreased iNOS expression when compared to the other control groups.
Histology (haematoxylin and eosin) and immunohistochemistry (iNOS and eNOS)

Haematoxylin/eosin staining of tissue sections from heart, kidney, lung, and brain showed no evidence of fibrin deposition or thrombosis. Immunostaining for iNOS was markedly decreased in vessels of mice positive for IS4, when compared to the relevant control groups (Figure 4.5). In contrast, there were no evident changes in staining for eNOS in any of these groups (Figure 4.6).

4.5 – iNOS immunostaining in kidney vessels of mice injected with IS4, TW and CB-F7, and “no cells” control.
4.6 - eNOS immunostaining in kidney vessels of mice injected with IS4, TW and CB-F7, and “no cells” control.

4.2.2 Discussion

Oxidative stress has been identified as one of the main contributory factors in atherogenesis. In APS, both lipid peroxidation (Ames 1998) and TAC (Delgado Alves 2002b) have been related to anti-CL antibody titres, suggesting a pathogenic role for anti-CL in atherogenesis. A possible explanation is a decrease in PON activity, as a consequence of the cross-reactivity of anti-CL antibodies with HDL or one of its components. In fact, I have demonstrated that some anti-CL antibodies in patients with APS cross-react with the HDL particle and with apolipoprotein A-I (Apo A-I) (Delgado Alves, 2003). Apo A-I is the main protein component of HDL and is important in the
stabilisation of PON (Sorenson 1999). Apart from Apo A-I, the presence of β2-GP1 (Polz 1979) and cardiolipin (Deguchi 2000) in the normal HDL complex may also explain the reported cross-reactivity. Interference with PON function, caused by anti-CL antibody binding to any of these structures, may account for the increase in oxidation seen in these patients.

In the model described, mice injected with human anti-CL IgG-producing hybridoma cells showed a reduction in PON activity when compared to the relevant IgG control group. Furthermore, the antibody titre was shown to be an independent predictor of enzymatic activity, reinforcing the possibility of a causal relationship. The presence of murine anti-β2-GP1 IgG antibodies was also associated with a reduction in PON activity, when compared with the relevant murine IgG control group, but the difference did not reach statistical significance. Mice injected with murine anti-CL IgM did not show a similar reduction in PON activity, consistent with the recognised reduced pathogenic potential of IgM anti-CL. These findings are very similar to those previously described in a population of patients with primary APS, which also presented an inverse correlation between IgG anti-CL and anti-β2-GP1 antibody titres and PON activity, suggesting a possible mechanism to account for the increased plasma oxidation found in patients with APS {Delgado Alves, 2002 1056 /id}.

TAC represents the capacity of plasma to counteract oxidation. One of the major oxidative pathways involves the generation of superoxide and its subsequent reaction with NO to form peroxynitrite (Muijsers 1997). Peroxynitrite, in turn, can oxidise different structures, namely, lipids and lipoproteins (Beckman 1993). Furthermore, the continued presence of peroxynitrite leads to the formation of nitrotyrosine moieties on
proteins. Hence, the presence of nitrotyrosine may be used as marker of a previous or current long-term pro-oxidant environment (Inoue 2002).

TAC, as measured by peroxynitrite or superoxide quenching, is a good indicator of the oxidative potential at any given time, whether it is related to the presence of formed peroxynitrite or to mononuclear and endothelial cell activation and superoxide production. In this chapter, all of the groups injected with hybridomas secreting anti-CL (human or murine) or anti-β2-GP1 (murine) antibodies showed a reduction of TAC (peroxynitrite quenching), suggesting a direct association between these antibodies and peroxynitrite formation. Since PON prevents peroxynitrite-mediated lipid peroxidation (Ahmed 2002), a reduction of PON activity, as seen in some of the groups, would further increase the overall oxidative state.

Further evidence of a pro-oxidant tendency is found in the increased levels of plasma superoxide, as suggested by higher values of TAC (superoxide quenching) in the IS4 antibody group. Human anti-CL IgG have been shown to bind and activate endothelial cells (assessed by an increased expression of adhesion molecules – ICAM, VCAM) (Meroni 2000), but its association with an increase in superoxide production has never been reported.

Although an increase in NO production has been documented both in mouse models and in patients with SLE (Weinberg, 1994; Belmont, 1997), NO levels have not previously been related to the presence of anti-CL antibodies. In contrast, NO has been shown to be reduced in patients with primary APS and to show an inverse correlation with anti-CL antibody titres (Ames 2000a). In this study, plasma NO levels were decreased in the group injected with IS4-producing hybridoma cells. Since anti-CL
antibodies (and IS4, in particular) have been shown to activate endothelial cells (Del Papa 1995), increased NO production, due to an increased expression of iNOS, might have been anticipated. In fact, both western blot analysis and immunohistochemistry confirmed a reduction in iNOS expression, with no significant differences in the expression of eNOS, consistent with the reduced plasma levels of NO.

Nitrotyrosine results from the nitrosilation of proteins by nitrogen radicals present in peroxynitrite. It is therefore a good marker of an increased oxidative status in vivo (Inoue, 2002; Oates, 1999). Hence, the elevated levels of nitrotyrosine found in the IS4 group would strongly suggest that NO production and plasma levels had been elevated earlier.

A down-regulation of iNOS expression following a period of increased activity has been reported previously, but never before in the context of an autoimmune disease (Vallance 1997). In fact, iNOS and eNOS expression are controlled by different “feedback” mechanisms, the most important of which relates to NO itself (Assreuy 1993). Increased local levels of NO have been shown to down-regulate iNOS in macrophages and endothelial cells, resulting in a subsequent decrease in NO production (Sheffler 1995; Swierkosz 1995). This control is achieved through regulation of NF-κB activity, the major pathway involved in iNOS expression (Connelly 2001). This pathway is also associated with the expression of adhesion molecules by endothelial cells (Kim 2001) and can be activated by anti-CL antibodies (Meroni 2000). Recently, Hattori et al have reported a decrease in iNOS expression following inhibition of NF-κB transcriptional activation by 4-hydroxynonenal (HNE) (Hattori 2001). HNE is a lipid peroxidation-derived aldehyde, believed to be largely responsible for the
cytopathological effects observed during inflammatory and oxidative processes (Esterbauer 1991).

The IS4 group demonstrated higher levels of peroxynitrite and superoxide, but a decrease in PON activity. This combination is known to induce lipid peroxidation (particularly LDL oxidation) and an increased formation of HNE, which would down-regulate iNOS and subsequently decrease NO production. These events are consistent with my findings that TAC (superoxide) is inversely correlated with NO levels and suggests that an impaired total antioxidant capacity of plasma, reflecting an increased lipid peroxidation and HNE production, has inhibited iNOS expression and NO production. Absence of thrombosis in the mice may be due to the relatively short exposure to the antibodies (+/- 30 days). The timing of these murine studies was limited by the development of ascites and the consequent need for termination of the experiment.

In conclusion, I have described an experimental model that demonstrates the pathogenic role of anti-CL and anti-β2-GP1 antibodies in the early stages of oxidative stress in vivo. In this model, anti-PL antibodies are associated with a decrease in PON activity, a major anti-oxidant enzyme. This decrease leads to oxidation of LDL and lipid peroxidation, which may inhibit iNOS expression and NO production. The consequence of these effects could be endothelial dysfunction and an increased risk of atherosclerosis and thrombosis. This new model of anti-PL-mediated oxidative stress provides the means to elucidate the role of the oxidative pathways in APS and the mechanisms leading to the thrombosis and atherosclerosis often observed in this syndrome.
Chapter 5:
anti-PL, anti-β₂-GP1 AND anti-HDL ANTIBODIES INDUCE A PRO-OXIDANT STATE IN VIVO

5.1 Antibodies towards high-density lipoprotein and β₂-GP1 are inversely correlated with PON activity in patients with SLE and primary APS

In this section I have explored whether the activity of PON was impaired in patients with SLE and primary APS (contributing to an enhanced atherosclerotic progression in these conditions), and whether the impairment might be dependent on the presence of autoantibodies against cardiolipin, β2-glycoprotein-1, prothrombin and HDL.
5.1.1 Results

Demographic and clinical data of patients and controls are shown in table 5.1.

Table 5.1 Demographic and clinical data on the patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 20)</th>
<th>SLE (n = 32)</th>
<th>APS (n = 36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD years</td>
<td>33.3 ± 3.6</td>
<td>35.5 ± 14.4</td>
<td>31.3 ± 14.2</td>
</tr>
<tr>
<td>No. (%) female/male</td>
<td>17 (85)/3 (15)</td>
<td>28 (87.5)/4 (12.5)</td>
<td>32 (88.9)/4 (11.1)</td>
</tr>
<tr>
<td>Disease duration, median (range) years</td>
<td>NA</td>
<td>5.8 (1-12.3)</td>
<td>5.2 (1-8.5)</td>
</tr>
<tr>
<td>Global score (BILAG), mean ± SD</td>
<td>NA</td>
<td>6.7 ± 4.5</td>
<td>NA</td>
</tr>
<tr>
<td>Taking steroids, no. (%)</td>
<td>NA</td>
<td>23 (71.9)</td>
<td>-</td>
</tr>
<tr>
<td>Steroid dosage, mean ± SD mg/day</td>
<td>NA</td>
<td>10.3 ± 10.0</td>
<td>-</td>
</tr>
<tr>
<td>Taking HCQ, no. (%)</td>
<td>NA</td>
<td>16 (50.0)</td>
<td>-</td>
</tr>
<tr>
<td>Had thrombotic event, no. (%)</td>
<td>NA</td>
<td>-</td>
<td>24 (66.7)</td>
</tr>
<tr>
<td>Had miscarriage, no. (%)</td>
<td>NA</td>
<td>-</td>
<td>14 (38.9)</td>
</tr>
<tr>
<td>Taking aspirin, no. (%)</td>
<td>NA</td>
<td>10 (31.3)</td>
<td>16 (44.9)</td>
</tr>
<tr>
<td>Taking warfarin, no. (%)</td>
<td>NA</td>
<td>-</td>
<td>20 (55.6)</td>
</tr>
</tbody>
</table>

SLE = systemic lupus erythematosus; APS = antiphospholipid syndrome; BILAG = British Isles Lupus Assessment Group (19) (global score of 0-9, with 0 representing no disease activity and 9 representing the most severe disease); NA = not applicable; HCQ = hydroxychloroquine.

Levels of anti-CL, anti-β2-GPI and anti-PT antibodies in patients and controls

Median levels of IgG anti-CL, IgM anti-CL, IgG anti-β2-GPI, IgM anti-β2-GPI and IgG anti-PT were always higher in SLE and PAPS patients than in controls (Figure 5.1 A,B,C,D,E) whereas median levels of IgM anti-PT were not different across the three groups (Figure 5.1 F).
Figure 5.1: Levels of IgG antiphospholipid (anti-CL) (A), IgM anti-CL (B), IgG anti-β2-glycoprotein I (anti-β2-GPI) (C), IgM anti-β2-GPI (D), IgG antiprothrombin (anti-PT) (E), and IgM anti-PT (F) in controls (CTRL), patients with systemic lupus erythematosus (SLE), and patients with primary antiphospholipid syndrome (PAPS). Bars show the medians. P values refer to the overall analysis of variance result, as determined by Kruskal-Wallis test. GPL = IgG phospholipid units; MPL = IgM phospholipid units.
PON activity, TAC of plasma and anti-HDL antibodies in patients and controls

PON activity was low in patients with SLE and even lower in patients with PAPS than controls (p<0.0001) (Figure 5.2 A) but TAC did not differ across the three groups (Figure 5.2 B). anti-HDL antibodies were significantly higher in SLE patients than in controls and PAPS patients (p<0.0001) (Figure 5.2 C).

Level of cholesterol, HDL and HDL sub-fractions and their relationship with PON in SLE patients and controls

Levels of total cholesterol were similar in the SLE group and controls (4.39±1.6 vs 4.55±1.0 mmol/L). Patients with SLE had reduced levels of total HDL (0.51±0.3 vs 1.42±0.9 mmol/L, p<0.001), HDL$_2$ (0.37±0.3 vs 1.01±0.7 mmol/L, p<0.001) and HDL$_3$ (0.14±0.1 vs 0.40±0.2 mmol/L, p<0.004) than controls. In controls, PON correlated with HDL (Figure 5.3 A), HDL$_2$ (Figure 5.3 C) and HDL$_3$ (r=0.44, p=0.04). These relationships were not present in the SLE group (Figure 5.3 B and D).
Figure 5.2: Levels of paraoxonase (PON) activity (A), total antioxidant capacity (TAC) of plasma (B), and IgG anti-high-density lipoprotein (anti-HDL) (C) in controls (CTRL), patients with systemic lupus erythematosus (SLE), and patients with primary antiphospholipid syndrome (PAPS). Bars show the medians. P values shown in the figure refer to the overall analysis of variance result, as determined by Kruskal-Wallis test. Other P values, determined by Dunn’s post hoc test, were as follows: P < 0.01, PON levels in SLE patients versus controls; P < 0.001, PON levels in PAPS patients versus controls; P < 0.001, IgG anti-HDL levels in SLE patients versus controls; P < 0.005; IgG anti-HDL levels in SLE patients versus PAPS patients.
Figure 5.3: Correlation (by Spearman's rank test) between PON activity and HDL levels in controls (A) and SLE patients (B), and between PON activity and HDL₂ levels in controls (C) and SLE patients (D). There were significant correlations in the control group but not in the SLE group.
Relation between oxidation markers, disease activity and drug treatment

HDL, HDL$_2$ and HDL$_3$, anti-HDL titres, PON and TAC levels did not differ between patients with SLE on steroids or anti-malarials and those who were not on those medications. There was no correlation between the global BILAG score, the scores of any of the organ systems considered, C3 levels or ESR with anti-HDL titres, PON activity or TAC levels. In the PAPS group, none of the variables was significantly different between patients on oral anticoagulation and patients on aspirin alone. Likewise, no significant differences in IgG anti-HDL, PON activity and TAC were noted amongst patients with arterial or venous thrombosis and/or with miscarriages.

Relationship between PON and antibody titres

In the SLE group, a strong inverse correlation was noted between PON activity and IgG anti-HDL (Figure 5.4 A), (even after correction for age, steroid and anti-malarials intake) but not with any other auto-antibody investigated. In addition, IgG anti-HDL was negatively correlated to TAC ($r=-0.40$, $p<0.02$), and TAC was positively correlated to PON ($r=0.43$, $p=0.02$). In the PAPS group, PON activity was inversely correlated to IgG anti-CL (Figure 5.4 B) IgG anti-$\beta_2$-GPI (Figure 5.4 C) and IgM anti-$\beta_2$-GPI (Figure 5.4 D).
Figure 5.4: Correlation (by Spearman's rank test) between IgG anti-high-density lipoprotein (anti-HDL) and paraoxonase (PON) activity in SLE patients (A) and between PON activity and IgG anti-CL levels (B), PON activity and IgG anti-β2-GPI levels (C), and PON activity and IgM anti-β2-GPI levels (D) in PAPS patients.

**Multiple regression model**

To evaluate which antibody best associated with a decrease in PON activity in the PAPS group, I employed a stepwise multiple regression. PON activity was entered as the dependent variable and IgG anti-CL, IgG and IgM anti-β2-GPI, which correlated to PON
by univariate analysis, were entered as independent variables and corrected for age, gender and warfin intake. In this model, IgG anti-β₂-GPI was the only independent predictor of decreased PON activity (r=0.483, p = 0.003).

5.1.2 Discussion

Systemic lupus erythematosus and the antiphospholipid syndrome are characterized by an enhanced risk of thrombosis and atherosclerosis. Even though some pathogenic pathways might be shared, there are different factors that ultimately are likely to account for the development of vascular damage found in both conditions.

In patients with SLE, prolonged steroid treatment seems to be a major culprit as it induces an atherogenic lipid profile, characterized by increased levels of very low-density lipoprotein (VLDL) and LDL and decreased levels of HDL, together with hypertension and diabetes (Hochberg 1991). However, the risk of atherosclerosis in patients with SLE has been recognized to be higher than what would be expected if only the traditional risk factors where taken into account, suggesting that other mechanisms are involved (Manzi 1997).

In this study, HDL was reduced in SLE patients when compared to controls, as reported previously (Borba 2000). This reduction was particularly significant for the HDL₂ sub-fraction. A decrease in HDL₂ has never been reported previously in patients with SLE and is important because it contains the higher percentage of Apo A-I, which partly accounts for the protective effect of HDL against atherosclerosis (Williamson 1992). The importance of Apo A-I in the context of SLE is further enhanced by the
recent observation that Apo A-I has anti-inflammatory properties by blocking the contact-mediated activation of monocytes by T lymphocytes (Hyka 2001).

Other pro-atherogenic factors include chronic inflammation (Ross 1999) and enhanced lipid peroxidation (Schisterman 2001). Both conditions have been found in patients with SLE (Ames 1999; Ames 2000b), whereas a strong link between lipid peroxidation and IgG anti-CL titres has also been demonstrated in APS (Ames 1998). Furthermore, anti-CL antibodies have also been related to atherosclerosis (Ames 2002; Roman 2001). This link may occur either via direct activation of the vascular endothelium or by the increased uptake of anti-oxLDL-LDL immune complexes by macrophages (Hasunuma 1997). In this context, anti-β2-GPI antibodies could also be of importance because they hinder the protective role of β2-GPI in preventing oxidized LDL (oxLDL) uptake by macrophages (Hasunuma 1997). Patients with SLE and PAPS had higher titres of anti-CL, anti-β2-GP1 and IgG anti-PT antibodies than controls, as expected. Interestingly, I found higher titres of IgG anti-HDL in patients with SLE than with PAPS. This difference, and the lack of correlation between IgG anti-HDL and anti-CL antibodies, suggest that the former represent a specific antibody subset and are not the result of cross-reactivity with anti-CL antibodies.

Antibodies to lipoproteins have been detected both in patients with SLE (Vaarala 2000) and APS (Horkko 2001). Whether these antibodies are specifically directed to an antigen present in the lipoproteins or are simply cross-reactive anti-CL or anti-β2-GP1 antibodies is still unclear. Previous studies have shown that both situations may coexist (Wu 1999). However, most of the reports to date have studied antibodies to LDL and few have explored HDL as a possible target (Dinu 1998). Dinu et al, reported the
presence of antibodies against Apo A-1 in patients with SLE and these antibodies were more frequent in anti-CL antibody positive patients (Dinu 1998), suggesting that cross-reactivity could be a possibility. More recently, anti-Apo A-1 antibodies were reported by Abe et al and a human anti-Apo A-1 monoclonal antibody has been described (Abe 2001). However, there are no data regarding the impact of these antibodies on clinical and biological markers of atherosclerosis. Of these, one of the most important is lipid peroxidation and one of the main defence mechanisms against lipid peroxidation is paraoxonase. PON is an anti-oxidant enzyme found in the liver, arterial wall and plasma, where it travels associated with HDL (Sorenson 1999). It has an important action in preventing LDL oxidation by peroxinitrite in turn preventing the generation of ox-LDL in plasma (Mackness 1991b). Apolipoprotein A-I stabilizes the enzyme (Sorenson 1999) hence interference with Apo A-I or with the HDL molecule itself could cause a reduction in the activity of the enzyme.

PON activity has never been assessed in patients with SLE before, but a decrease in its activity has been described in patients with different autoimmune conditions and with anti-CL antibodies (Lambert 2000). However, no correlation was detected between clinical and biological variables and the enzymatic activity nor was a mechanism suggested to explain this finding. The data shown in this thesis indicate that PON is reduced in patients with SLE and to an even greater extent in patients with PAPS. In the latter group, PON activity was inversely correlated with IgG anti-CL and IgG and IgM anti-β2-GPI titres. No correlation was found with anti-PT antibodies. This is not unexpected as prothrombin is not associated with HDL or PON, whereas β2-GPI and cardiolipin can be present in the HDL complex (Deguchi 2000). That antibodies against
HDL (or one of its components) may decrease PON activity is suggested by the strong inverse correlation between IgG anti-HDL and PON activity in SLE. Therefore IgG anti-HDL may indirectly affect the oxidant/antioxidant balance in SLE via an inhibitory effect on PON.

Total antioxidant capacity quantifies the overall antioxidant defense of plasma. A higher TAC would suggest an increased resistance to oxidation. Recently, Nuttall et al, showed a decrease in TAC along with a pro-atherogenic lipid profile (elevated cholesterol, triglycerides and lipid peroxides) in SLE (Nuttall 2001). In the present cohort, TAC of patients with SLE and PAPS was only slightly lower than the control group. However, in the SLE group, TAC positively correlated with PON activity and inversely with anti-HDL, suggesting that IgG anti-HDL may decrease TAC by reducing PON activity.

Interestingly, no correlation between anti-CL, anti-β2-GP1, anti-HDL and TAC was found in the PAPS population. This suggests that other mechanisms might be affecting TAC in PAPS. A possible explanation could be that the decrease of PON activity found in PAPS is due to a higher prevalence of the RR polymorphism, known to be associated with a decreased enzymatic activity (Mackness 1999), which could be little affected by the presence of these auto-antibodies. In fact, Lambert et al described an increased incidence of this PON polymorphism in patients with anti-CL antibodies and arterial thrombosis (Lambert 2000).

In conclusion, I have highlighted the relevance of HDL, IgG anti HDL and PON in patients with SLE. Total HDL and particularly HDL₂ are decreased in SLE. This lipoprotein or some of its components may represent target (auto)antigens, as elevated
IgG anti-HDL titers were noticed in the patients investigated. More importantly I have demonstrated that PON activity was markedly reduced both in patients with SLE and PAPS and that that reduction correlated with the presence of IgG anti-HDL, IgG anti-CL and IgG and IgM anti-β2-GP1 antibodies. However, only IgG anti-β2-GP1 was independently associated with decrease PON in the PAPS group. This observation compliments the finding that β2-GPI prevents the oxidation of LDL (Lin 2001), hence antibodies against β2-GP1 may deprive β2-GP1 of its anti-oxidant properties. As PON is important for the prevention of LDL oxidation, we suggest that IgG anti-HDL and IgG anti-β2-GP1, via an inhibitory effect on PON activity, contribute to the oxidation of LDL and thus differential atherogenic routes in SLE and PAPS.
5.2 Cross-reactivity between anti-CL, anti-HDL and anti-Apo A-I IgG antibodies in patients with SLE and primary APS

This section aimed to study the presence of antibodies to cardiolipin, HDL and Apo A-I and at the different patterns of cross-reactivity between these antibodies in patients with SLE and APS. Classical inhibition assays were unable to address this issue because of the affinity between cardiolipin and the HDL complex. I have therefore attempted to develop a novel method to study the cross-reactivity of these antibodies, reliably. For the first time, the existence of two distinct autoantibody populations was shown. One population cross-reacts with both cardiolipin and HDL, whilst the other is relatively specific for HDL. In light of these studies, a different method of studying the affinity and cross-reactive pattern of these antibodies in patients with SLE and APS is proposed.

5.2.1 Results

Demographic data of patients and controls are shown in table 5.2.
Table 5.2: Demographic data and anti-CL, anti-HDL and anti-Apo A-I antibody titres in patients with SLE and APS, and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>Ctrls</th>
<th>SLE</th>
<th>APS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>29 ± 2.9</td>
<td>32.4 ± 4.1 ((p=ns))</td>
<td>31.3 ± 3.8 ((p=ns))</td>
</tr>
<tr>
<td>Gender (F/M)</td>
<td>9 / 1</td>
<td>23 / 2</td>
<td>21 / 1</td>
</tr>
<tr>
<td>Disease duration (y)</td>
<td>na</td>
<td>5.7 (1-10.3)</td>
<td>5.4 (1-8.7)</td>
</tr>
<tr>
<td>Steroids</td>
<td>na</td>
<td>20 (80%)</td>
<td>-</td>
</tr>
<tr>
<td>Warfarin</td>
<td>na</td>
<td>-</td>
<td>22 (100%)</td>
</tr>
<tr>
<td>anti-CL (GPL)</td>
<td>8.5 ± 6.0</td>
<td>14.1 ± 4.7 ((p=ns))</td>
<td>29.0±7.0 ((p=0.008))</td>
</tr>
<tr>
<td>anti-HDL (OD)</td>
<td>29.0 ± 10.7</td>
<td>68.0±7.9 ((p=0.008))</td>
<td>61.4±10.1 ((p=0.02))</td>
</tr>
<tr>
<td>anti-Apo A-I (OD)</td>
<td>22.4 ± 3.9</td>
<td>31.3 ± 3.8 ((p=ns))</td>
<td>26.6 ± 5.0 ((p=ns))</td>
</tr>
</tbody>
</table>

Table 5.2: Values represent mean and standard error of mean (SEM). anti-CL titres are expressed in GPL and anti-HDL and anti-Apo A-I in OD (% of pos. control). \(p\) values refer to comparisons between patients groups and controls. anti-CL antibody titres are also significantly different between patients with SLE and APS \((p<0.03)\). (Ctrls: controls; y: years; OD: optical density; ns: non significant; na: non applicable).

**Binding of IS4, CL1, CL24 and patients sera to CL, HDL and Apo A-I**

Each of the monoclonals bound to cardiolipin as expected. IS4 also bound strongly to HDL and Apo A-I (1.68 and 1.88 OD respectively), after 30 minutes. CL1 reacted strongly with Apo A-I (2.27 OD after 30 min) but not as well with HDL (0.84
OD, 30 min). CL24 had little activity against either HDL or Apo A-I, compared with CL.

Titres of anti-CL antibodies were higher in the samples of patients with APS when compared to patients with SLE and healthy controls (p<0.03 and p< 0.009 respectively). However, anti-HDL titres were higher in both patients with SLE and APS when compared to healthy controls (p<0.009 and p<0.03 respectively), but there was no difference between the two patient groups. Although, anti-Apo A-I titres were generally higher in the samples of patients with SLE, the difference did not reach statistical significance when compared to patients with APS (p=0.3) or healthy controls (p=0.1)(Table 5.2).

Relationalship between antibody titres

IgG anti-HDL antibody titres directly correlated with anti-Apo A-I (r= 0.563, p<0.004) in the SLE population, (Fig. 5.5) but not in the APS population (r=0.295, p=ns) or in the healthy controls. There were no correlations between anti-CL and anti-HDL or anti-Apo A-I titres in the studied population or in any of the disease sub-groups.
Figure 5.5: Relationship between IgG anti-HDL and anti-Apo A-I antibody titres.

Figure 5.5: IgG anti-HDL titres directly correlate with anti-Apo A-I in patients with SLE: $r = 0.563; \ p < 0.004$; OD: optical density.

**Multiple regression model**

To evaluate which antibody best associated with anti-HDL titres, a stepwise multiple regression was employed. IgG anti-HDL titres were entered as the dependent variable and anti-CL, anti-Apo A-I and total HDL cholesterol were entered as independent variables and corrected for age, gender and steroids and warfarin intake. In this model, anti-Apo A-I was the only independent predictor of anti-HDL antibodies ($r = 3.715, \ p = 0.001$).
Inhibition assays

a) HDL and Apo A-I inhibition of binding of IS4 to cardiolipin

Pre-incubation of HDL at increasing concentrations (3.125 to 400 μg/ml) with IS4 (0.4μg/ml), was strongly correlated with increased binding to CL (t=10.777; Rsq=0.95; p<0.0001)(Figure 5.6 A). Pre-incubation of Apo A-I (0.3 to 40μg/ml) with IS4 did not inhibit the binding of this antibody to CL.

b) IS4 binding to HDL and HDL+CL in inhibition assay

IS4 bound to both HDL coated wells and to HDL+CL wells. The binding (OD) directly correlated with the concentration of CL added to the plate (t=8.723; p<0.0001). There were no changes in OD in the wells coated with a constant amount of HDL, to which no CL was added (Figure 5.6 B).
Figure 5.6: A - Incubation of IS4 with increasing concentrations of HDL enhanced the binding of the complex to cardiolipin coated on ELISA plate ($t=10.777; R^2=0.95; p<0.0001$). B - IS4 binding to HDL coated on ELISA plate directly correlated with concentration of cardiolipin added to the antigen ($t=12.427; R^2=0.94; p<0.0001$). (OD: optical density)
**Cross-reactivity assay**

All three monoclonal antibodies showed activity (although at different levels), towards CL, HDL and Apo A-I in the second plate following elution from plate 1 and after incubation with the same antigens (Figure 5.7). All samples were positive for human IgG in the second plate, and there were no significant differences between the different antigens used in plate 1. After removal of samples from plate 1, an ELISA for anti-CL was performed to assess the amount of antibody remaining in the plate. The percentage of removal (based on OD reduction) was approximately 50%, with no significant differences for the different antigens used (data not shown).
Figure 5.7: Cross-reactivity between CL, HDL and Apo A-I of human monoclonals.

Figure 5.7: A - Binding of monoclonals to HDL and Apo A-I after elution from CL coated plate. B - Binding of monoclonals to CL and Apo A-I after elution from HDL coated plate. C - Binding of monoclonals to CL and HDL after elution from Apo A-I coated plate. Total human IgG was used as positive control in all plates. Values (Y axis) are expressed as a ratio between the binding to each antigen / binding to total IgG.
Sera from 12 patients were incubated in ELISA plates with CL, HDL and Apo A-I. After washing and elution of the binding antibodies, a second ELISA assay was performed for each antigen. Eleven samples (91.7%) containing anti-CL antibodies showed activity towards HDL and 2/12 (16.7%) were positive for anti-Apo A-I antibodies. When the samples were first tested for HDL, 7/12 (58.3%) were positive for CL in a second assay and 3/12 (25.0%) were positive for Apo A-I. After incubation with Apo A-I as first antigen, 6/12 (50.0%) had activity towards CL in the second assay. All the samples collected after incubation with Apo A-I had reactivity towards HDL. There were no differences in the cross-reactivity patterns between patients with SLE and APS (Figure 5.8).
Figure 5.8: Cross-reactivity between CL, HDL and Apo A-I of patients sera.

Figure 5.8: A - Binding of patients sera to HDL and Apo A-I after elution from CL coated plate. B - Binding of patients sera to CL and Apo A-I after elution from HDL coated plate. C - Binding of patients sera to CL and HDL after elution from Apo A-I coated plate. Total human IgG was used as positive control in all plates. Results (Y axis) are expressed as a ratio between the binding to each antigen / binding to total IgG.
5.2.2 Discussion

Patients with SLE and APS have antibodies directed against CL, HDL and Apo A-I. Whilst anti-CL antibodies are frequently present in patients with SLE and represent the hallmark of the phospholipid syndrome, there are few reports examining the presence of anti-Apo A-I antibodies in patients with SLE (Dinu 1998) and there is only one previous study of which I am aware, showing the presence of anti-HDL antibodies in SLE and APS (Delgado Alves 2002b). Even though it has been recognized that a possible relationship could exist between anti-CL and anti-Apo A-I in SLE patients (Lahita 1993), and that anti-HDL antibodies showed a different pattern of disease distribution than anti-CL (Delgado Alves 2002b), the possibility of cross-reactivity among these antibodies has not been addressed before. So far, antibody activity against lipoproteins has been considered only in relation to LDL, particularly oxidized LDL (oxLDL) (Vaarala 2000), and cross-reactivity between anti-CL and anti-oxLDL antibodies has been shown (Vaarala 1993). However, anti-CL antibodies isolated from patients with SLE showed different binding patterns to oxLDL and β2-GP1, suggesting that different subtypes might co-exist (Vaarala 1996).

In the initial studies of three monoclonal human IgG anti-CL antibodies revealed that they all bind to HDL and Apo A-I, suggesting that cross-reactivity could occur in human sera. Monoclonal antibodies were used in this study for two main reasons: 1- to standardise the assay, 2- to demonstrate that the identified cross-reactivity was not a consequence of a generalised polyclonal antibody production.

In cohort studied, antibodies against Apo A-I correlated with anti-HDL titres, and were found to be an independent predictor of the latter, even when corrected for
anti-CL titres, total HDL cholesterol, age, gender and treatment. This observation reinforces the concept of cross-reactivity may be the main underlying mechanism to explain these binding patterns. This finding was not unexpected since Apo A-I is present in the HDL complex. However, I could not find a correlation between the anti-CL and anti-HDL titres, even though HDL carries negatively charged phospholipids, including cardiolipin (Deguchi 2000). This finding suggests that anti-CL and anti-HDL belong to different families of autoantibodies. In order to address this possibility, I performed inhibition studies with IS4, a human IgG anti-CL monoclonal antibody. Binding of IS4 to CL was evaluated following its incubation with HDL and Apo A-I. There was no inhibition in any of the cases, however the binding of IS4 to CL was directly correlated with the concentration of HDL used as an inhibitor. HDL actually increased the binding of IS4 to CL instead of inhibiting it. A possible explanation for this finding is that IS4 and HDL formed immune-complexes (IS4-HDL) following their co-incubation, which would in turn bind to CL used as antigen on the plate, via the HDL component of the immune-complex. This hypothesis was tested in the second part of the experiment which suggested that CL was absorbed into the HDL complex coated in the plate, as shown by the strong correlation between the binding of IS4 and the concentration of CL in the system. The affinity between CL and HDL could thus have hindered the study of cross-reactivity of the antibodies against these two antigens.

To overcome this problem, I used a different method for the characterization of the cross-reactivity patterns of these antibodies. Autoantibodies were first isolated from serum by incubation with CL, HDL and APO A-I on a polystyrene ELISA plate. The binding of one autoantigen-specific eluted antibody was then tested against the other
antigens. The data showed cross-reaction between anti-CL and anti-HDL antibodies in the majority of the patients. Few patients demonstrated cross-reactivity between CL and Apo A-I, suggesting that these are a different subset of antibodies. Interestingly, antibodies selected for their binding to HDL on the first plate, cross-react with CL (7/12) and Apo A-I (3/12), but when Apo A-I was used as an initial antigen, all the selected antibodies cross-reacted with HDL. This observation suggests that anti-HDL antibodies (as previously described (Delgado Alves 2002b) and confirmed in this study), could in fact represent at least two different sets of cross-reactivity: one with CL and the other with a different antigen, probably Apo A-I. Finally, there is also a group of antibodies reacting against both CL and Apo A-I, in a similar way to the independent activity against β2-GP1 found in some anti-CL antibodies (Matsuura 1994).

The importance of these findings relies on the increased recognition of the protective role of HDL and Apo A-I against atherosclerosis. HDL has been shown to prevent the oxidation of LDL and its consequent uptake by monocytes, preventing in this way the formation of “foam cells”, one of the most important steps in atherogenesis (Libby 1998). This anti-oxidant mechanism is mainly due to the presence in the HDL particle of an anti-oxidant enzyme called paraoxonase. This enzyme is stabilised by the presence of Apo A-I, contributing to its optimal activity (Sorenson 1999). A significant reduction in Paraoxonase activity has been reported in patients with SLE and APS, re-enforcing the importance of this enzyme in these conditions (Delgado Alves 2002b). Cross-reactivity between anti-CL antibodies and HDL or Apo A-I, by tempering with any of these structures, may contribute to an increased atherogenesis found in these conditions.
In this section I have confirmed the presence of antibodies against both HDL and Apo A-I in patients with SLE and APS and propose a different method to study the cross-reactivity between these antibodies in order to avoid possible misinterpretation due to the affinity between HDL and CL. The full characterisation of these autoantibodies might uncover different antibody subtypes that relate to particular clinical features and/or pathological aspects of these conditions.

Further large-scale epidemiological studies should be put in place to determine the importance of these specific immune markers in relation to the progression of the atherosclerosis.

5.3 Antiphospholipid antibodies are induced by *in vitro* fertilization and correlate with paraoxonase activity and total antioxidant capacity of plasma in infertile women

This study aimed to determine whether anti-PL (anti-CL and aPS) are related to IVF, and to determine whether these antibodies, if present, are associated with changes in the oxidative status of plasma of infertile women. None of these patients had clinical manifestations of APS or any other autoimmune disease, hence the existence of a pro-
oxidant status would suggest that this was related to the presence of the anti-PL antibodies and not to APS itself.

5.3.1 Results

Levels of antiphospholipid antibodies in the sera of infertile patients undergoing IVF treatment, of infertile patients never treated and healthy fertile controls

Of the fifty-two patients undergoing IVF treatment, 56% were positive for IgG anti-CL while 96% were positive for IgM anti-CL. When anti-PS antibodies were considered, 92% of treated patients were positive for IgG, while 27% were positive for IgM. None of the fertile women used as normal controls or the infertile patients yet to undergo IVF treatment were positive for anti-CL or anti-PS antibodies. There was a significant difference between antiphospholipid titres, when the treated patients were compared with untreated patients, and normal controls (p<0.001 for both isotypes of anti-CL and anti-PS). There were no significant differences between anti-PS and anti-CL (IgG and IgM), when fertile controls were compared with untreated infertile patients (Figure 5.9). Antibodies to dsDNA were not detected in the sera of any of the patients or the controls.
Levels of antiphospholipid antibodies in the sera of patients with different types of infertility

There were no significant differences in anti-CL and aPS titres with different types of infertility. Patients undergoing IVF due to their partners’ infertility (male infertility), had similar levels of antibody titres (Figure 5.10).
Levels of antiphospholipid antibodies in the sera of infertile patients submitted to different number of IVF cycles

Patients undergoing IVF treatment were divided into three groups: patients who had undergone one IVF cycle, patients who had undergone three IVF cycles with success after the third cycle and those on their third unsuccessful cycle. Patients submitted to one IVF cycle had significantly higher titres of anti-CL IgG (but not IgM), when compared to infertile patients before treatment (p<0.001). Anti-PS antibody titres were also significantly different between untreated infertile patients and patients who
had undergone one IVF cycle (p<0.01 for IgM and p<0.001 for IgG). When patients submitted to one IVF cycle were compared with patients treated three times, only IgM anti-CL titres were significantly higher (p<0.01). In patients submitted to 3 IVF cycles, there were no significant differences between the successful and unsuccessful groups regarding anti-CL (70.9±13.4 vs 65.9±22.1), anti-PS (38.6±15.7 vs 40.4±22.3), PON activity (232±28 vs 262±50) and TAC (23±4 vs 21±7).

**Paraoxonase activity and Total Anti-oxidant Capacity of plasma in patients and controls**

PON activity was low in the infertile patients after treatment when compared to patients before treatment (p<0.001) and to healthy controls (p<0.001). However, there was no difference between the groups who had had one or three IVF cycles. TAC, as measured by peroxynitrite quenching, was also significantly reduced in the patients after treatment when compared to the group before treatment (p<0.002) and to the control group (p<0.001), but again, there were no differences between groups treated one or three times. TAC as measured by superoxide quenching, was not significantly different in any of the groups considered.

**Relationship between TAC, PON and anti-PL antibody titers**

A strong inverse correlation was noted between PON activity and TAC (peroxynitrite quenching) and each of the IgG antibodies (r= -0.786, p<0.001 for anti-CL and r= -0.732, p<0.001 for anti-PS). In addition, PON activity was directly correlated to TAC (r= 0.721, p<0.001) (Figure 5.11).
Figure 5.11: Correlations between anti-PL antibody titres (Cl and PS), PON activity and TAC.

A - Correlation between anti-CL and PON activity

B - Correlation between anti-PS and PON activity
Multiple regression analysis

To evaluate which antibody was best associated with a decrease in PON activity, I employed a stepwise multiple regression. PON activity was entered as the dependent variable and anti-CL and anti-PS (IgG and IgM), which correlated to PON by univariate analysis, were entered as independent variables, after correction for age. In this model, only IgG anti-CL and IgG anti-PS were found to be independent predictors of decreased PON activity (r= -0.734, p < 0.001 for both). Furthermore, after correction for age and antibody titres, PON activity was the only independent predictor for TAC (r=0.720, p<0.001).
5.3.2 Discussion

Antiphospholipid antibodies have been linked to various aspects of infertility, but this association remains controversial. In this section I compared groups of infertile women, before and after IVF treatment. The prevalence of anti-PL antibodies (anti-CL and anti-PS) was increased in women submitted to IVF treatment when compared to a group assessed before treatment. Furthermore, the anti-PL titres measured before treatment were not different from the levels detected in healthy fertile women. These findings suggest that the presence of anti-PL antibodies might be induced by IVF treatment and not related to the infertile state. These results are in keeping with the findings of Fisch et al who showed in a prospective study, an increased production of anti-PL antibodies after IVF treatment (Fisch 1991). Interestingly, four years later this same group, suggested the opposite (anti-PL are part of the infertile state and not induced by IVF), however, the number of patients in this later study was small and the sera were collected only a few days after oocyte retrieval (Fisch 1995). Anti-dsDNA antibodies were tested in an attempt to determine whether the enhanced immune response found in patients after IVF treatment was non-specific. The fact that none of the women submitted to IVF had anti-dsDNA antibodies suggests at least some degree of specificity (against anti-PL).

The true prevalence of anti-PL among patients treated in IVF clinics is very difficult to determine from the literature. This problem is partly because of differences in assays and interpretation of results. Anticardiolipin testing, though standardised, varies from laboratory to laboratory. Assays for the other anti-PL antibodies (eg, anti-PS, anti-
PI, anti-phosphatidylcholine, anti-phosphatidylethanolamine) are not standardised and different methods have been used to define cut-offs between normal and abnormal antibody levels. Furthermore, some control populations have included males, or females outside the reproductive age range. Some studies have not used control populations at all, and patient populations have varied in the definition of infertility (Branch 1998).

The results presented show a similar prevalence of both anti-CL and anti-PS in patients after IVF, regardless of the type of infertility and even women submitted to IVF treatment due to an infertile partner showed no differences in anti-PL antibody titres. The lack of common factors (apart from IVF treatment), to account for such a finding, would imply a causal relationship between the IVF treatment and the immune response. Birdsall et al, reported similar data, with an increase in antiphospholipid titres in women submitted to IVF, regardless of the cause for infertility (Birdsall 1996), but no explanations for these findings were suggested.

When the group submitted to 3 IVF cycles was compared to the one with only 1 IVF cycle, there were no significant differences in the levels of anti-PL antibodies, suggesting that further IVF treatment would not induce an increase in the immune response. The same findings have been reported by others but there was no reference to the number of IVF courses undergone by the patients in those studies (Fisch 1995; Yron 1992).

In the group of patients submitted to 3 IVF cycles, the absence of major differences in the antibody profile between successful and non-successful treatments suggests that the presence of these antibodies do not influence the IVF outcome reinforcing earlier findings (Chilcott 2000).
PON activity was significantly reduced in patients submitted to IVF (either 1 or 3 cycles), when compared to the control group or to the group of patients before treatment. This suggests that the infertile condition in itself does not affect the activity of the enzyme. Interestingly, there were no differences amongst the groups studied with respect to superoxide anion quenching, suggesting that the amount of superoxide in the plasma of all the groups (submitted to IVF or not) was not significantly different. The discrepancy found between these two oxidative pathways, with an increase of one (peroxynitrite) but not the other (superoxide) reinforces the idea of the involvement of a specific peroxynitrite pathway, rather than a non-specific increase in plasma oxidation. It has been shown that peroxynitrite increase in plasma is directly related to lipid peroxidation (LDL oxidation) (Carr 2000), reinforcing the idea that the inhibition of PON is the prime mechanism. Furthermore, the fact that the only independent predictors of PON were IgG anti-CL and anti-PS and that PON is the only independent predictor for TAC suggests that peroxynitrate related plasma oxidation is conditioned by PON activity and that this is influenced by the titre of IgG anti-PL antibodies.

The reported antioxidant effects of oestrogens, can be overcome by combined progestative therapy (Zhu 1999b). Furthermore, it has been suggested that in fact, at physiological concentrations, oestrogens might bear pro-oxidant activity through myeloperoxidase activation (Santanam 1998). In this way, I suggest that IVF treatment could induce a rise in plasma oxidation that would lead to an increase in oxidation of the membrane phospholipids and the consequent triggering of anti-PL antibody production. These antibodies have been correlated with a reduction in PON activity in patients with APS (Delgado Alves 2002b), enhancing in this way the oxidation levels of plasma that
in turn would further increase the oxidation of membrane phospholipids, hence perpetuating this condition. The fact that this process is time limited and the absence of other potential genetic or environmental factors would account for the lack of clinical features of the antiphospholipid syndrome.

In conclusion, anti-PL antibodies may be related to IVF treatment and do not represent a feature of the infertile state. Even though previous studies have reported an increased production of anti-PL in relation to IVF treatment, none has looked into possible effects of this immune response regarding either clinical or biological changes. I have shown that anti-PL antibodies produced in this way have biological activity by inhibiting PON in a similar way to that described in APS, and this interaction induces an increase in the peroxynitrite related oxidative state of plasma.
Chapter 6:
FINAL COMMENTS AND PROPOSALS FOR FUTURE DIRECTIONS

In this thesis, I have analysed the pathogenic role of antiphospholipid antibodies in general, and anticardiolipin antibodies in particular, in the induction of oxidative stress in the context of patients with SLE and APS.

The first set of experiments was directed towards the study of the direct effect of a human IgG anti-CL monoclonal antibody (IS4) in the vascular endothelium. The ex vivo model which was used allows a comprehensive analysis of the response of the endothelial wall to a known controlled stimulus.

Following a short-term incubation (4 hours), IS4 induced a hyporeactivity of the aorta rings to phenylephrine and acetylcholine, with no response to sodium nitroprusside, an endothelium-dependent vasodilator. These findings strongly suggest that IS4 induced NO production via activation of endothelial cells. There were no significant amounts of LPS in the antibody solution nor did the results alter with the addition of polymixin, confirming the absence of LPS contamination. Furthermore, the prevention of these changes obtained with a pre-incubation with cardiolipin would suggest that the reported effect is specific to IS4.
Endothelial cell activation by anti-CL antibodies has been shown. However, a direct effect on NO production has not been reported before, to the best of my knowledge. The hyporeactivity to phenylephrine that is reversible after incubation with L-NAME indicates an increase in NOS expression, and the fact that it also reverts with 1400W indicates that it is the iNOS isoform that is upregulated. This enzyme is activated through the NF-κβ pathway, and anti-CL has been shown to induce ICAM-1 and VCAM-1 through the same mechanism, hence these results should not be surprising.

To confirm these results in an experimental model, IS4 was administrated in anaesthetised rats and both haemodynamic and biochemical changes in plasma were recorded. After 3 hours, (the average time for the beginning of iNOS expression), a significant drop in blood pressure was found in the IS4 group when compared to the control groups, and the levels of NO in the serum of the IS4 group were also significantly increased, consistent with a systemic vasodilatation caused by NO production.

To analyse whether these findings would still be present in a long-term model, a second set of experiments was performed in which SCID mice would sustain a permanent level of circulating IS4, for a period of 30 days.

Interestingly, a significant reduction of serum NO levels was found in the IS4 group when compared to several controls, however, nitrotyrosine levels were found significantly increased in the study group, suggesting the presence of previously increased levels of NO (which is responsible for protein nitrosylation). This apparent paradox can be explained by the fact that maintained stimulation of iNOS expression (as
expected in this model) would lead to subsequent down-regulation of iNOS resulting in chronic impairment of NO production.

Furthermore, in the long-term model, a constellation of changes regarding pro-oxidant substances was found, which would induce an oxidative state known to further enhance endothelial dysfunction. These changes included the inhibition of PON activity and the increased production of peroxynitrite and superoxide, two powerful pro-oxidant substances.

NO production is a key player in the maintenance of vascular motility, which is an important defence mechanism against atherogenesis and even thrombosis. However, overproduction of NO may result in an increased rate of peroxynitrite formation, a potent oxidant. Furthermore, a continued stimulation of endothelial iNOS would induce a mechanism of down-regulation of the enzyme expression, which in the long run would impair NO production, leading to a decrease in endothelial motility and a high risk of vascular disease (figure 6.1).

These experiments were performed using a human IgG monoclonal anti-CL antibody. The use of a monoclonal antibody allows a more precise study of the interactions between the immune response and the different structures involved in the pathogenesis of these diseases. However, this methodology presents some limitations for one cannot extrapolate directly the results for what is believed to take place in the human system. Even though this antibody was isolated from a patient with APS, it is not possible to define whether this was indeed the cause of disease in that particular patient. Nevertheless, the consistency of the findings throughout the different experiments, using different experimental models, allows a reasonable degree of acceptance of the
pathogenicity of this antibody and therefore of the possibility that the shown mechanisms are a correct reproduction of what may be happening in vivo.

In any case, the complexity of SLE and APS is based on a combination of different pathologic mechanisms that ultimately concur for the clinical picture. In this thesis one of the possible pathways involved is shown. Its overall relevance will be clarified in future work.
Figure 6.1:

Pro-atherogenic mechanisms induced by anti-CL antibodies in patients with APS.

A. GENERAL POPULATION

Pro-oxidant radicals

LDL

HDL

Plasma

Endothelium

Macrophages (normal oxLDL uptake)

Lipid peroxidation

NF-κB

iNOS

Sporadic induction of iNOS with adequate endothelium response (↓ thrombosis and atherosclerosis).

B. ANTIPHOSPHOLIPID SYNDROME

Pro-oxidant radicals

LDL

HDL

Plasma

Endothelium

Macrophages (increased oxLDL uptake)

Lipid peroxidation

NF-κB

iNOS

Permanent activation of iNOS induces long term down-regulation of enzyme expression with chronic reduction of iNOS and consequent endothelial dysfunction.

= stimulation, Θ = inhibition

aCL antibodies
In another set of experiments, I tried to ascertain whether the oxidative changes found in the experimental model could be present in the context of clinical SLE and APS. Two groups of patients (with SLE and PAPS) were studied for different oxidative related variables. Strikingly, I found a significant reduction of PON activity in patients with SLE and even more so in patients with PAPS. Furthermore, this reduction was associated with a parallel reduction of total anti-oxidant capacity of plasma, a known method for assessing increased levels of peroxynitrite and superoxide production. The changes found in the oxidative profile of these patients inversely correlated with anti-CL and αβ2-GP1 antibody titres in serum, reinforcing in an *in vivo* setting the findings of the experimental models.

A direct inhibition of PON activity by anti-CL antibodies was confirmed in an *in vitro* inhibition study where I showed that increasing concentrations of IS4 reduced the activity of PON present in human HDL.

The antioxidant activity of PON is mainly based on its capacity to prevent oxidation of LDL, hence preventing its uptake by macrophages. However, PON activity is highly dependent on the stability of the HDL complex to which it is attached. This attachment is particularly dependent of the presence of Apo A-I.

To assess the possibility of a direct interference of circulating auto-antibodies with the HDL complex, I performed an assay for the presence of antibodies against HDL and Apo A-I in these patients. Not surprisingly, these patients presented with antibodies directed to both HDL and Apo A-I, and these were also associated with reductions in PON activity.
To investigate the possibility that this could be a cross-reaction of the anti-CL antibodies which these patients had, I designed a different protocol for assessing the possible presence of cross-reactivity. Standard inhibition assays are difficult to interpret due to the lipidic nature of the antigens and their reciprocal affinity.

I found the presence of different groups of antibodies in the sera of these patients, directed to CL, HDL and Apo A-I. Even though there is a significant percentage of cross-reaction, each one of these three groups showed independent activity.

These results confirm previous findings of anti-Apo A-I antibodies, but anti-HDL antibodies have never been reported in this context before, and their presence can be of relevance in the understanding of the pathogenesis of both SLE and APS.

Antibodies directed against the HDL complex or one of its components (CL, β2-GP1 or Apo A-I) can interfere with PON activity, leading to an increase in oxLDL. These findings would explain the increased levels of both oxLDL and anti-oxLDL antibodies found in patients with SLE and APS. Furthermore, the increased levels of oxLDL would result in an enhanced uptake from macrophages, leading to a acceleration of the mechanisms of “foam cell” production and atherogenesis, and monocyte/macrophage activation with tissue factor production and thrombosis (figure 6.2).
Figure 6.2: Oxidation in SLE and APS.

--- Direct relation
--- Inhibition
--- Stimulation / Activation
The last set of experiments aimed at confirming these findings in a different clinical setting. Women submitted to IVF have been found to have higher levels of circulating anti-PL antibodies, but without any clinical manifestations of APS. For this thesis I analysed the oxidant profile of this women in relation to their antibody titres. Interestingly, the same pro-oxidant changes with reduced PON activity and total antioxidant capacity of plasma was found, and these findings correlated with the titres if circulating anti-CL antibodies. These results suggest that oxidative stress is in fact induced by the presence of anti-PL antibodies in an independent fashion of the antiphospholipid syndrome itself.

In this thesis, I have shown evidence of a direct relation between anti-PL antibodies and the oxidative stress found in association with SLE and APS. Different pathways related to plasma, lipoproteins and endothelial levels combine to promote a "multiple hit" effect which will ultimately result in an increased atherogenesis and thrombi formation.
**Future directions**

At a cellular level, an important line of research would be to address the molecular and cellular mechanisms involved in the activation of iNOS by anti-CL antibodies, namely the relevance of the activation of NF-Kβ in this context. This would provide important clues as to which antibodies are indeed pathogenic and how to prevent the activation of these mechanisms.

At a clinical level, a complete characterisation of the different families of antibodies (directed against HDL, Apo A-I and CL) would be important in order to understand the cross-reactivity patterns present in these conditions. The clinical relevance of different isotypes (particularly IgG and IgM), the comparison between antibody populations in patients with SLE, APS and in controls, and the prevalence of these auto-antibodies in other auto-immune and non-immune conditions, should be determined.

Furthermore, epidemiological and population-based studies would allow us to determine possible relations between different antibody profiles and clinical manifestations, eventually providing tools for a better classification of these patients regarding disease progress and prognosis.

At a therapeutic level, the understanding of oxidation as a important mechanism to explain the pathogenesis of SLE and APS, would allow research for new therapeutic approaches, either related to anti-oxidants or with the use of lipid-lowering agents.
APPENDIX 1
MEDIA AND BUFFERS

Phosphate buffered saline (PBS)
800g NaCL
20g KCl
114.8g Na₂HPO₄.2H₂O
20g KH₂PO₄
Add to 10 litres of sterile water and adjust pH to 7.4

Bicarbonate (BIC) buffer
8g Na₂CO₃
15.5g NaHCO₃
Add to 10 litres of sterile water and adjust pH to 9.6

Growth Medium (GM)
424ml RPMI 1640 medium HEPES modification (Sigma)
  (RPMI = Rothewell Park Memorial Institute)
50ml heat inactivated Foetal Calf Serum (FCS) - European origin (Sigma)
10ml MEM Non-essential amino acids [100x] (Gibco)
5ml 10000 IU/ml Penicillin / 10mg/ml Streptomycin solution (Gibco)
5ml L-glutamine [200mM/100x] (Gibco)
5ml Sodium pyruvate [100mM] (Gibco)
1ml Gentamicin [10mg/ml] (Gibco)
Freezing Medium
50% v/v RPMI 1640 medium HEPES modification
30% v/v Dimethlysulfoxide (DMSO)
20% v/v FCS

HAT Medium
309ml RPMI 1640 medium HEPES modification (Sigma)
100ml heat inactivated Foetal Calf Serum (FCS) - European origin (Sigma)
50ml NCTC-135 with L-glutamine
10ml MEM Non-essential amino acids [100x] (Gibco)
10ml HAT medium supplement (Sigma)
5ml 10000 IU/ml Penicillin / 10mg/ml Streptomycin solution (Gibco)
5ml L-glutamine [200mM/100x] (Gibco)
5ml Sodium pyruvate [100mM] (Gibco)
5ml OPI medium supplement (Sigma)
1ml Gentamicin [10mg/ml] (Gibco)

HAT Medium (with 2 x HAT)
Hat Medium (as above)
2% v/v HAT medium supplement (Sigma)
i.e. 49ml HAT Medium + 1ml HAT medium supplement.

Post Fusion Medium
347ml RPMI 1640 medium HEPES modification (Sigma)
100ml heat inactivated Foetal Calf Serum (FCS) - European origin (Sigma)
20ml MEM Non-essential amino acids [100x] (Gibco)
20ml L-glutamine [200mM/100x] (Gibco)
7.5ml 10000 IU/ml Penicillin / 10mg/ml Streptomycin solution (Gibco)
5ml Sodium pyruvate [100mM] (Gibco)
COS-7 Medium

440ml Dulbecco's Modified Eagle Medium (DMEM; Gibco)
50ml FCS (Sigma)
5ml 10000 IU/ml Penicillin / 10mg/ml Streptomycin solution (Gibco)
5ml L-glutamine [200mM/100x] (Gibco)
APPENDIX 2

MAIN PUBLICATIONS ARISING FROM THE WORK


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