L'effet des statines sur l'activation des cellules endothéliales par les anticorps antiphospholipides

Dimitrova, Yordanka

UNIVERSITE DE GENEVE

FACULTE DE MEDECINE
Département de Medecine Interne
Division d'angiologie et d'hémostase
Unité d'hémostase

Thèse préparée sous la direction du Professeur Philippe de MOERLOOSE

L'EFFET DES STATINES SUR L'ACTIVATION DES CELLULES ENDOTHELIALES PAR LES ANTICORPS ANTIPHOSPHOLIPIDES

THESE

Présentée à la Faculté de Médecine de l'Université de Genève
pour obtenir le grade de Docteur en Médecine

par

Yordanka DIMITROVA-TIREFORT

de

Bulgarie

Thèse n°10344
GENEVE
2003

UNIVERSITÉ DE GENÈVE

FACULTÉ DE MÉDECINE

DOCTORAT EN MEDECINE

Thèse de :
Madame Yordanka DIMITROVA-TIREFORT
originaire de Bulgarie

Intitulée :

L'EFFET DES STATINES SUR L'ACTIVATION DES CELLULES ENDOTHELIALES PAR LES ANTICORPS ANTIPHOSPHOLIPIDES

La Faculté de médecine, sur le préavis de Monsieur Philippe de MOERLOOSE, professeur associé au Département de médecine interne, autorise l'impression de la présente thèse, sans prétendre par là émettre d'opinion sur les propositions qui y sont énoncées.

Genève, le 21 novembre 2003

Thèse n° 10344

Jean-Louis CARPENTIER
Doyen

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives à la présentation des thèses de doctorat à l'Université de Genève".

ACKNOWLEDGEMENTS

I wish to express my sincere and profound gratitude to **Professor Philippe de Moerloose** who gave me the opportunity to do my thesis and, by his support, competences, and impressive enthusiasm, ensured the realisation of this study. I particularly thank him for his patience and belief in this work and in me during these years. His fascinating professionalism created in me the scientific vision for the medecine.

I also owe my deep gratitude to **Dr Egbert Kruithof** for the work under his guidance. His enormous knowledge about the methods of cellular biology and science in general has greatly helped this study to succeed. I wish to thank him for his continued support during this project being always there in good and in bad days.

In spite of their own busy schedules **Professor Philippe de Moerloose and Dr Egbert Kruithof** gave me invaluable guidelines during the preparation of my manuscript and reviewed it in a short time which helped me to accomplish this study.

Grateful acknowledgements to **Dr Sylvie Dunoyer-Geindre** for her cooperation, indispensable help and assistance in cell culture preparations and all the technics I learned durind last two years.

Special thanks are also due to **Dr Guido Reber** for his collaboration and carrying out the statistical analysis of my data as well as for introducing me to the laboratory diagnosis of antiphospholipid syndrome.

I am sincerely grateful to **Dr Natalie Satta-Poschung and Dr Jia Wei Liu** for the effective coordination of needs, for giving me many valuable tips and for the time devoted trying to solve the problems. Many thanks also to **Hong Yang** for her assistance and friendship and to **Richard Fish** for his helpful suggestions.

Sincere thanks to **Dr Françoise Boehlen** who kindly provided all patients' sera, as well as to **Dr François Mach** and **Dr Brenda Kwak**, Division of Cardiology, University Hospital of Geneva, for for providing the human saphenous vein endothelial cells and all statins I used in this study.

I also thank to **Dominique Wohlwend** and **Susanne Bissat** for their competentence and knowledge on the flow cytometry analysis and FACSSCAN.

I am very thankful to **Professor H. Bounameaux** who accepted me in the Division of Angiology and Hemostasis, as well as to **Isabelle de Haller** for her competence and availability for arranging all the administrative part of my stay.

I wish to thank all the people from **Division of Angiology and Haemostasis** for their interest in my work. In particular, my heartfelt thanks to **Philippe Minazio, Oana Bulla, Christiane Wyler, Yen Lai-Chu, Catherine Vifian** and **Dominique Attias** for the warm welcome, kindness and friendship that I received from them.

Finally I would like to acknowledge the understanding and patience of my daughter, my husband and my family who were enormously supportive at every moment.

Le syndrome des anticorps antiphospholipides a été décrit en 1983. Il s'agit d'une maladie auto-immune caractérisée par des taux élevés d'anticorps antiphospholipides (APLA) associés à des thromboses (veineuses ou artérielles) et/ou à des pertes fœtales à répétition (International Consensus Statement for Definite Antiphospholipid Syndrome). Le syndrome des anticorps antiphospholipides peut se manifester de manière isolée ou accompagner d'autres pathologies, en particulier des maladies auto-immunes comme le lupus érythémateux systémique.

Plusieurs études ont montré que les APLA sont capables d'activer les cellules endothéliales (CE) in vitro. Les mécanismes liés à cette activation sont un des sujets de recherche de la Division d'Angiologie et d'Hémostase. Il a été observé qu'un anticorps monoclonal reconnaît un phospholipide anionique nommé acide lysobisphosphatidique (Kobayashi et al, 1998; Galve-de Rochemonteix et al, 2000). Les études suivantes ont montré que l'acide lysobisphosphatidique est une cible importante pour les APLA, et que les APLA s'accumulent dans les endosomes tardifs des CE isolées des veines ombilicales en redistribuant l'insulin-like growth factor 2/ mannose-6-phosphate receptor (CI-M6PR) de l'appareil de Golgi aux endosomes tardifs. De manière concomitante, il a été découvert que la beta$_2$ glycoprotéine I (β_2GPI) s'accumule sur la surface cellulaire des endosomes tardifs et modifie le trafic des protéines intracellulaires. Ceci a en particulier été démontré par la redistribution du CI-M6PR de l'appareil de Golgi à l'intérieur des endosomes tardifs (Dunoyer-Geindre et al, 2001).

Les mécanismes par lesquels les APLA induisent un phénotype thrombotique ont également été étudiés dans la Division. Nous avons observé que l'incubation des CE avec des anticorps anti-β_2GPI provoque la redistribution du facteur de transcription nucléaire kB (NFkB) du cytoplasme dans le noyau (Dunoyer-Geindre et al, 2002). Cette redistribution du NFkB est importante pour l'activation des CE et est accompagnée d'une augmentation de l'expression du facteur tissulaire et des molécules d'adhésion leucocytaires ICAM-1, VCAM-1 et E-sélectine (Dunoyer-Geindre et al, 2002). Des études récentes ont montré que l'activation des CE par les APLA provoque un phénotype endothélial pro-inflammatoire accompagné d'une adhésion des monocytes à l'endothélium et par la suite une pénétration dans le sous-endothélium (Pierangeli et al, 2000). D'autres

études ont mis en évidence une corrélation entre l'augmentation des molécules d'adhésion par certains anticorps monoclonaux anti-β_2GPI et la résorption fœtale observée chez des souris (George et al, 1998).

Notre travail a porté principalement sur l'effet des statines sur les changements des CE lorsqu'elles sont incubées avec du TNF ou des APLA. Notre hypothèse était que les patients avec APLA pourraient bénéficier d'un traitement par les statines. En effet ces médicaments ont de nombreuses autres actions que la seule inhibition de la biosynthèse du cholestérol. Ces effets dits pleiotropiques pourraient expliquer également leurs effets cliniques bénéfiques sur la régression des lésions athérosclérotiques et sur la réduction des complications cardiovasculaires (Scandinavian Study group, 1994; Hebert, 1997). Nous avons cherché à voir si in vitro l'ajout de statines permettait de diminuer le phénotype pro-inflammatoire des CE incubées avec du TNF ou des APLA. De manière inattendue, nous avons observé que les statines augmentaient l'effet des APLA ou du TNF sur l'expression des molécules d'adhésion. Nous avons ensuite étudié les mécanismes pouvant expliquer cet effet. Nous avons pu démontrer que l'effet des statines était bloqué par une co-incubation avec le mévalonate ou le geranylgeranyl pyrophosphate et mimé par le GGTI-286, un inhibiteur de la geranylgeranyl transférase.

En résumé nos résultats montrent que l'effet des APLA sur les cellules endothéliales in vitro est augmenté par les statines et que l'effet des statines est dû à des modifications de protéines geranylgeranylées.

PLAN

PART II: STATINS AND ANTIPHOSPOLIPID SYNDROME

PART III: EXPERIMENTAL WORK

ABBREVATIONS

ACA	anticardiolipin antibodies
APC	activated protein C
APC-R	activated protein C resistance
APLA	antiphospholipid antibodies
APS	antiphospholipid syndrome
aPTT	activated partial thromboplastin time
β_2GPI	beta$_2$ glycoprotein I
CAM	cell adhesion molecules
CI-M6PR	cation-independent mannose-6-phosphate receptor
CL	cardiolipin
CRP	C-reactive protein
dPT	dilute prothrombin time
dRVVT	dilute Russell's viper venom time
EC	endothelial cells
EDRF	endothelium-derived relaxing factor
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
FCS	foetal calf serum
FPP	farnesylpyrophosphate
FTase	farnesyltransferase
FTI-277	farnesyltransferase inhibitor-277
GGPP	geranylgeranylpyrophosphate
GGTase	geranylgeranyltransferase
GGTI-286	geranylgeranyltransferase inhibitor-286
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HRP	horseradish peroxidase
HSVEC	human saphenous vein cells

HUVEC	human umbilical vascular endothelial cells
INR	international normalized ratio
KCT	kaolin clotting time
LA	lupus anticoagulant antibodies
LFA-1	β2 integrin function antigen-1
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
MMP	matrix metalloprotease
NFkB	nuclear factor kB
NO	nitric oxide
OPD	o-phenylenediamine dihydrochloride
PA	phosphatidic acid
PAI-I	plasminogen activator inhibitor type I
PC	phosphatidylcholine
PE	phospatidylethanolamine
PGI_2	prostacyclin
PI	phosphatidylinositol
PL	phospholipid
PS	phosphatidylserine
Pt	prothrombin
SMC	smooth muscle cells
SLE	systemic lupus erythematosus
TAT	thrombin-antithrombin complexes
TM	thrombomodulin
vWF	von Willebrand factor

SUMMARY

Antiphospholipid syndrome (APS) is an autoimmune disease characterized by the persistent presence of antiphospholipid antibodies (APLA) associated with venous and arterial thrombosis as well as foetal loss. APLA constitute a heterogeneous family of antibodies which play a pathogenic role rather than just being a diagnostic marker of APS. The thrombophilic state has been partially related to the interactions of APLA with endothelial cells (EC). Due to their multiple properties on EC and the numerous favourable clinical trials, the use of statins has also been advocated for patients with APS. Indeed, besides their cholesterol-lowering activity, statins may influence several events in the vessel wall by blocking the mevalonate synthesis and inhibiting the production of down-stream metabolites, such as geranylgeranylpyrophosphate (GGPP) and farnesylpyrophosphate (FPP), that play a major role in the intracellular modification of important signaling proteins as Rho and Ras.

In this study we investigated whether adhesion molecule expression induced by APLA on EC is influenced by various statins, namely simvastatin, fluvastatin and pravastatin. The effect of APLA was compared with TNF-α and LPS. Contrary to what could be expected, we consistently found in our experimental conditions that pretreatment with statins potentiated the APLA-induced expression of E-selectin and VCAM-1 on EC. Mevalonate reversed the potentiating effect of these statins. Studying further the mechanism of these effects of statins revealed that GGPP also reversed the potentiating effect of simvastatin or fluvastatin on adhesion molecule expression, while FPP only partially reversed this effect. Furthermore, we observed that the specific geranylgeranyltransferase inhibitor GGTI-286, but not the farnesyltransferase inhibitor FTI-277, mimicked the effect of simvastatin and fluvastatin by increasing the TNF-α and APLA mediated overexpression of E-selectin and VCAM-1.

In conclusion, statins increase E-selectin- and VCAM-1-induced expression on vascular endothelial cells stimulated with antiphospholipid antibodies. The inhibition of geranylgeranylated protein could contribute to this effect. Our data indicate that other experimental data should be performed before giving statins to APS patients.

PART I: THE ANTIPHOSPHOLIPID SYNDROME

I. INTRODUCTION

The antiphospholipid syndrome (APS) was first described in 1983 (Hughes). APS is an autoimmune disorder characterized by: 1) the presence of antiphospholipid antibodies (APLA): anticardiolipin antibodies (ACA) and/ or lupus anticoagulant antibodies (LA), and 2) clinical complications such as a) venous and/ or arterial thrombosis, and/or b) pregnancy morbidity (International Consensus Statement for Definite Antiphospholipid Syndrome). The syndrome is called primary APS when it occurs without underlying disorder and secondary APS in patients with other diseases such as auto-immune diseases, particularly systemic lupus erythematosus, or malignant diseases. Endothelial cell (EC) activation by APLA seems to be one of the major pathogenic mechanisms of APS. The mechanisms underlying this activation are one of the research topics of the Division of Angiology and Hemostasis. It was observed that a monoclonal antibody to late endosomes recognized the anionic phospholipid lysobisphosphatidic acid (Kobayashi et al, 1998; Galve-de Rochemonteix et al, 2000). Further investigations showed that lysobisphosphatidic acid is an important target for APLA and that APLA accumulate in late endosomes of human umbilical vein endothelial cells (HUVEC) leading to a redistribution of the insulin-like growth factor 2/ mannose-6-phosphate receptor (CI-M6PR) from the Golgi apparatus to late endosomes. It was also found that beta$_2$ glycoprotein I (β_2GPI) accumulate at the cell surface and in late endosomes in HUVEC, resulting in modification of intracellular protein trafficking as shown by a redistribution of the CI-M6PR from the Golgi apparatus to late endosomes (Dunoyer-Geindre et al, 2001). The mechanisms by which APLA induce a thrombotic phenotype were further studied in the Division. It was found that incubation of EC with anti-β_2GPI antibodies resulted in a redistribution of the transcription nuclear factor kB (NFkB), an essential intermediate in the activation of EC, from the cytoplasm to the nucleus after a delay of several hours (Dunoyer-Geindre et al, 2002). This NFkB redistribution was accompanied by an increased expression of tissue factor (TF) and the leukocyte adhesion molecules ICAM-1, VCAM-1 and E-selectin (Dunoyer-Geindre et al, 2002).

Other recent data reported that EC activation by APLA lead to a proinflammatory endothelial phenotype with a monocyte adhesion to the endothelium and penetration into the subendothelial space (Pierangeli et al, 2000). Another study showed that upregulation of adhesion molecules by some murine monoclonal anti-β_2GPI antibodies correlated with foetal resorption in mice (George et al, 1998).

The aim of this study was to investigate the ability of statins, specific inhibitors of cholesterol biosynthesis with clinically proven beneficial effects such as regression of atherosclerotic lesions and reduction of cardiovascular complications (Scandinavian Study group, 1994; Hebert, 1997), to modify adhesion molecule expression in APLA-activated EC and to study the underlying mechanisms.

II. ANTIGENS AND ANTIBODIES IN THE ANTIPHOSPHOLIPID SYNDROME

Antigens

Recent studies demonstrated that APLA recognize not only determinants with anionic PL but also neutral PL as well as proteins (Table 1).

TABLE 1. *Various antigens recognized by APLA*

Phospholipids	cardiolipin phosphatidylserine, phosphatidylinositol phosphatidylethanolamine, phosphatidic acid lysobisphosphatidic acid
Proteins	β_2GPI vitamin K-dependent proteins: *prothrombin, protein C, protein S, thrombomodulin* annexins: *annexin V, annexin II* others

Phospholipids (PL)

Anionic PL are essential cofactors for the blood coagulation system. APLA are supposed to bind the phosphodiester group of negatively charged PL. The length and composition of the polar head groups as well the saturation of the fatty acid side chains contribute to the antigenicity (Levy et al, 1990). Possible antigenic targets of APLA on EC include negatively charged PL such as cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, phosphatidic acid.

- Cardiolipin (CL)

CL has been most often used to study the interaction of APLA with anionic PL. The antibodies that directly bind to CL are characterized by the presence of a larger than average density of positively charged arginine residues in one of antigen binding loops, which mediate the binding to the negatively charged PL.

- Phosphatidylserine (PS), phosphatidylinositol (PI)

PS and PI may be possible antigenic targets of APLA on EC. PS is a PL occurring in abundance in the inner leaflet of the cytoplasmic membrane and is exposed to the outer surface after cell activation or apoptosis (Mevorach et al, 1998). PS promotes the interaction of coagulation factors. PI derivates are important mediators of cell activation.

- Phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidic acid (PA)

Zwitterionic antigens such PE and PA have also been implicated in APS (Berard et al, 1996). PL have a positively charged substituted group that interacts electronically with the phosphodiester, thus blocking APLA access to the phosphodiester. APLA do not usually bind the zwitterionic molecule PC, but removal of the positively charged choline group results in the formation of negatively charged PA, which could be bound by APLA.

- Other PL antigens

Other PL can also be involved. Recently, lysobisphosphatidic acid, a lipid related structurally to CL and present in the membranes of late endosomes, has been identified as another immunologic target for APLA. The team of Division of Angiology and Hemostasis

observed that APLA could react with lysobisphosphatidic acid of internal membranes of late endosomes, resulting in accumulation of APLA in late endosomes and subsequent EC activation (Kobayashi et al, 1998; Galve-de Rochemonteix et al, 2000, Dunoyer-Geindre et al, 2001). The oxidation of PL may be necessary for epitopes recognition by some APLA. Horkko et al (1997) demonstrated that many APLA bound to CL only after it had been oxidized, but not to a reduced CL analogue that could undergo oxidation. They suggested that the reactive groups of oxidized CL, such as aldehydes, generated during the decomposition of oxidized polyunsaturated fatty acids, form covalent adducts with β_2GPI (and other proteins) and that they are epitopes for APLA.

Whether this broad response of APLA indicates polyclonality or crossreactivity could not be determined using sera as a source of antibody. It has been shown that a human monoclonal IgM LA is able to bind to PA, PI and PS. This provided evidence for the polyspecificity of at least some APLA (Thiagarajan et al, 1980).

Binding of APLA to PL is also influenced by the physical state of the PL. Mice immunized with hexagonal versus lamellar phase PL are more likely to develop APLA (Rauch et al, 1990). Protein cofactors like β_2GPI may shift the orientation of some PL from the lamellar to the hexagonal phase.

Proteins

- Beta$_2$ glycoprotein I (β_2GPI)

β_2GPI, also called apolipoprotein H, is a major protein constituent of human plasma, where its concentration is about 200 mg/L. It is the main factor for the recognition of anionic PL by APLA.

Structure

β_2GPI is a single-chain, 5 kDa protein consisting of 326 amino acids. It contains large numbers of Pro and Cys residues, and is highly glycosylated (Figure 1). The protein is a member of the complement control protein or short consensus repeat (SCR) superfamily, characterized by repeating stretches of about 60 amino acid residues, "Sushi domains", each with a set of 16 conserved residues and 2 fully conserved disulfide bonds. It has five repeating SCR domains. The first four homologous repeat regions consist of about 60 amino acids with 2 disulfide bonds in each domain. The fifth domain contains 80 amino

acids and 3 disulfide bonds. The protein binds to phospholipid membranes via the cationic portion of its fifth SCR domain. β_2GPI has a weak affinity for anionic PL but APLA, binding to domains I and V, increase this affinity, due to the bivalency of the antibodies and to conformational changes induced in the β_2GPI protein.

Functions

It was found that β_2GPI interacts specifically with lipoprotein(a) and the endothelial cell protein annexin II (Ma et al, 2000). Although the physiologic importance of β_2GPI anticoagulant activity is still unclear, β_2GPI acts at least in vitro as an inhibitor of the intrinsic blood coagulation pathway due to its ability to interact with negatively charged surfaces, which in turn are necessary for the activation of factor XII (Schousboe et al, 1985). Interestingly Mori et al (1996) have shown that β_2GPI can inhibit the anticoagulant activity of activated protein C. Thus, it remains unclear whether β_2GPI in vivo has anticoagulant or procoagulant properties. β_2GPI has been reported to inhibit adenosine diphosphate-mediated platelet aggregation and the prothrombinase activity of activated platelets (Shi et al, 1993). β_2GPI binds to cells undergoing apoptosis and may be involved in the rapid, noninflammatory clearance of these cells by phagocytes. Recent study showed that the opsonization of apoptotic cells with anti-β_2GPI antibodies may be a proinflammatory event, stimulating the presentation of apoptotic cell antigens by dendritic cells. Thus, anti-β_2GPI antibodies might possibly contribute to an autoimmune response (Rovere et al, 1999). In addition, β_2GPI binds to the cell surfaces by binding to negatively charged molecules such as anionic PL, heparan sulfate proteoglycans and preferentially to oxidized low density lipoproteins. This property has been proposed to be clinically relevant providing a link between anti-β_2GPI antibodies and atherogenesis (Matsuura et al, 1998).

- Vitamin K-dependent proteins

Prothrombin (Pt)

Pt is a vitamin K-dependent glycoprotein with a molecular weight of 72 kDa. It is activated to thrombin by the so-called “prothrombinase complex” (coagulation factors Xa, Va, and calcium on a procoagulant PL surface) by the cleavage of two or possibly three peptide bonds. The first proteolytic cleavage leads to fragment 1,2 and prethrombin 2, which is

further proteolysed to thrombin. The anti-Pt antibodies may react both with Pt and its fragment 1,2, but not with the decarboxylated molecule. Antibodies to Pt are found in 50-90% of patients with LA (Galli et al, 1997; Kandiah et al, 1998) but their clinical relevance is still controversial (Galli et al, 1998). Anti-Pt antibodies may inhibit the activation of Pt into thrombin. They are therefore expected to be anticoagulant. However, Vaarala et al (1996) found that anti-Pt antibodies were associated with a hypercoagulable state and high levels of anti-Pt antibodies predicted a 2.5-fold increase in the risk of myocardial infarction or cardiac death (n =106). In contrast, other investigators (Horbach et al, 1996) reported that the presence of anti-Pt antibodies in LA positive patients (n = 60) does not increase the risk for thrombosis.

Protein C, protein S, thrombomodulin (TM)

These proteins are components of the protein C anticoagulant pathway. In this pathway, thrombin binds to TM and loses its ability to convert fibrinogen into fibrin. Furthermore, it acquires the ability to activate protein C which, in the presence of PL and protein S, degrades the coagulation factors Va and VIIIa and blocks further thrombin generation. Deficiency of protein C and S increases the risk of venous thrombosis (Oosting et al, 1993). Pengo et al (1996) investigated 22 patients with IgG APLA and thrombosis and found elevated levels of anti-protein C IgG and anti-protein S IgG in 18% and 55% of the patients, respectively. Antibodies to TM were found in 30% of the patients with a LA (n = 58) and 10% of the patients (n = 200) with unexplained thrombosis (Carson et al, 2000).

- Annexins

Annexin V (human placental anticoagulant protein 1)

Annexin V is an anionic PL-binding protein, expressed by placental and vascular endothelium. It is postulated that annexin V plays a thromboregulatory role at the vascular-blood interface by shielding anionic PL from forming a complex with coagulation proteins in the circulation. Annexin V could play an important role in the clinical manifestations of APS, particularly in obstetrical complications (Rand et al, 1999; Rand et al, 2002).

Annexin II

Annexin II has not been presently implicated as a target antigen of APLA. However, it was identified as a high affinity β_2GPI-binding protein at the surface of EC, another

mechanism of β_2GPI endothelial cell binding being through the putative PL-binding site located in the fifth domain of the molecule (Meroni et al, 2001). Annexin II does not span the cell membrane, its involvement probably requires an unknown adapter protein (Ma et al, 2000).

- Other proteins

Other potential protein targets for APLA include various proteins such as factor X, high molecular weight kininogen, factor XI, and the protein core of heparan sulfate (Shibata et al, 1994).

In summary, different antigens have been identified in APS. It is now known that antigen targets are mainly proteins such as β_2GPI, Pt or TM forming eventually a complex with various phospholipids.

Antibodies

APLA comprise a broad family of autoantibodies that includes both LA, detected by coagulation tests, and ACA, detected by ELISA methods (Pierangeli et al, 2001).

Immunoglobulin classes

Previous data have shown that the strongest associations of ACA and anti-β_2GPI antibodies with clinical manifestations of APS involve antibodies of the IgG isotype. Cohen et al (1993) determined in vivo that immunisation of mice with pathogenic IgG and IgM ACA, isolated from the serum of a patient with APS, induce the production by the mice of anti-ACA with ACA activity. The mice developed overt APS. IgG ACA were found to have higher pathogenic potential than IgM ACA. However, several works suggest that IgM as well as IgA may also be associated with the disease, although to a lesser extent. Anti-β_2GPI antibodies of IgG, A, and M classes have been reported in 84.8%, 59.3% and 51.5% of patients with primary APS, respectively (Lacos et al, 1999). Amoroso et al (2003) evaluated the prevalence of IgG and IgM antibodies to various antigens in sera from 87 patients affected by SLE. IgG ACA, IgG anti-PA, IgG anti-PI, IgG anti-PS, and

IgG anti-β_2GPI were found in 53%, 37%, 32%, 38%, and 24% of patients, respectively. IgM-ACA, IgM anti-PA, IgM anti-PI, IgM anti-PS, and IgM anti-β_2GPI were detected in 15%, 17%, 18%, 14%, and 16%, respectively.

- IgG

The association of thrombotic risk with high-titer IgG ACA or anti-β_2GPI antibodies, often together with LA, has been confirmed in several studies (Gattorno et al, 1995; Silver et al, 1996). Sammaritano et al (1997) investigated whether the presence of ACA of a specific IgG subclass is associated with clinical complications of APS. They found that IgG_2 was the predominant subclass of ACA, detected in 75% of the patients, and it was significantly associated with thrombotic complications. The IgG subclass distribution of anti-β_2GPI and ACA in patients with primary APS and secondary APS was studied recently (Samarkos et al, 2001). Mean values for anti-β_2GPI antibodies were as follows: IgG_1– 24.4%, IgG_2– 70.2%, IgG_3– 5.0%, IgG_4– 0.4%. The reported IgG subclass distribution for ACA was: IgG_1– 40.1%, IgG_2– 32.8%, IgG_3– 23.7%, IgG_4– 3.3%. Comparing the ranking of IgG subclasses of anti-β_2GPI antibodies (IgG_2 >IgG_1 >IgG_3 >IgG_4) and ACA (IgG_1 >IgG_2 >IgG_3 >IgG_4), IgG_2 was the most prevalent subclass for anti-β_2GPI antibodies whereas for anti-CL antibodies IgG_1, IgG_2 and IgG_3 were all frequently elevated. An association between IgG_2 and IgG_3 anti-β_2GPI antibodies and venous thrombosis has been shown, while IgG_2 and IgG_3 ACA were found to be more specifically associated with arterial thrombosis.

- IgM

In a study determining the distribution of ACA in primary and secondary APS, IgM were found in 26% of secondary APS cases and in 15.8% of primary APS group (Vianna et al, 1994). The antibodies of IgM isotype were related mainly to thrombocytopenia and heart valve disease (Diri et al, 1999). APLA, associated with infections, typically are IgM isotypes and are usually transient (Jaeger et al, 1992).

- IgA

Evidence that IgA antibodies may be important continues to accumulate (Lacos et al, 1999). A significant relationship has been demonstrated between increased IgA levels and a history of venous thrombosis, thrombocytopenia, heart valve disease, livedo reticularis, and epilepsy. Interestingly, black patients with primary and secondary APS appear to have a higher frequency of IgA anti-β_2GPI antibodies, suggesting genetic predisposition for these antibodies (Diri et al, 1999).

- Coexistence of different classes of APLA in the same patient

It has been recently reported that the concurrent presence of IgG, IgM, and IgA appears to increase the frequency of recurrent spontaneous abortions as compared with the presence of a single isotype among the autoantibodies (Guglielmone et al, 1999). Vogel et al (1991) determined APLA in an unselected group of 63 SLE patients. They found APLA in 50.8% of the patients and the simultaneous presence of LA and ACA was associated with an increased of arterial thromboembolic events.

In summary, various studies have demonstrated that APLA are a large and heterogeneous family of immunoglobulins from IgG, IgM, and IgA classes and that the simultaneous presence of APLA increases the risk of clinical complications.

III. LABORATORY DETECTION OF APLA

History

In 1952, Conley and Hartmann published the first description of two patients with SLE and an unique PL-dependent coagulation inhibitor characterized by prolongation of the whole blood clotting time and the prothrombin time. Both patients had a biologic false-positive serological test for syphilis with no evidence of infection. The antibodies responsible for the false-positive serological test for syphilis were ultimately found to recognize CL within the test reagent. In subsequent years, several authors described similar inhibitors that interfered with prothrombin time activation, but without specificity to any known coagulation factors; a number of these patients also had false-positive serological test for syphilis (reviewed in Shapiro et al, 1982). In 1957, Laurell and Nilsson described an association between chronic biologically false-positive serological test for syphilis, a circulating anticoagulant, and recurrent pregnancy loss. A paradoxal association between

PL-dependent coagulation inhibitors and thrombosis was first recognized by Bowie et al in 1964. They described the presence of thrombotic complications in 4 of 8 SLE patients having this inhibitor. The term «lupus anticoagulant» (LA) was proposed in 1972 to describe these inhibitors on the basis of their prevalence in SLE patients (Feinstein et al, 1972). In 1980, the immunologic nature of interaction of LA with anionic PL was demonstrated by isolating an IgM paraprotein with LA activity from a patient with macroglobulinemia (Thiagarajan et al, 1980). This paraprotein inhibited the Ca2+ - dependent binding of prothrombin and factor X to PS-containing liposomes, explaining the extremely prolonged PL-dependent coagulation tests seen in the patient. This paraprotein did not interfere with the binding of factor Xa to platelets, suggesting an explanation for the fact that this patient, like most patients with LA, had no bleeding tendency. Subsequently, it was observed that most LA reacted with CL, which was used as the antigen in serologic tests for syphilis (Pengo et al, 1987). Based on these observations, in 1983 Harris et al developed a radioimmunoassay and, subsequently an ELISA for ACA demonstrating the overlapping specificity of LA, ACA and other APLA.

The complex specificity of ACA became apparent in 1990 when several groups found that actually the majority of ACA in ELISA required the presence of a plasma protein, β_2GPI, in addition to anionic PL (Bevers et al, 1991; Galli et al, 1991; Koike et al, 1991). Subsequently, antibodies to a number of PL-plasma protein complexes have been described, involving among others prothrombin, protein C and annexin V, all binding to anionic PL.

Actual laboratory and clinical criteria for APS were formulated during the workshop in Sapporo, 1998, following the Eigth International Symposium on APLA.

Laboratory criteria for APS

Definite APS is considered to be present in a given patient when at least one of the clinical criteria (see chapter IV) and at least one of the main following laboratory criteria are met:

A) ACA of IgG and/or IgM isotype in blood, present in medium or high titer, on 2 or more occasions, at least 6 weeks apart, measured by a standardised ELISA for β_2GPI-dependent ACA,

B) LA present in plasma on 2 or more occasions at least 6 weeks apart, detected according to the guidelines of the International Society of Thrombosis and Hemostasis (Scientific Subcommittee on Lupus Anticoagulants/ Phospholipid-Dependent Antibodies, 1995), in the following steps (Figure 2):

1. Prolonged PL-dependent coagulation demonstrated on a screening tests: activated partial thromboplastin time (aPTT), kaolin clotting time (KCT), dilute Russell's viper venom time (dRVVT), dilute prothrombin time (dPT), Textarin time.

2. Failure to correct the prolonged coagulation time on the screening test by mixing with normal platelet-poor plasma.

3. Shortening or correction of the prolonged coagulation time on the screening test by addition of excess PL.

4. Exclusion of other coagulopathies, e.g., factor VIII inhibitor or heparin, as appropriate.

The sites of action of the different assays are presented on Figure 3.

Immunologic assays for ACA

ACA react with CL and other anionic PL in solid-phase immunoassays. ACA are commonly detected by ELISA. Microplate wells are coated with CL, blocked with a solution of animal serum diluted in buffer, and then incubated with dilute patient serum. The bound antibodies onto the coated wells are then detected using enzyme-conjugated, isotype-specific secondary antibody and a chromogenic substrate (Loizou et al, 1985).

- Isotypes

Reference sera that are isotype specific (IgG, IgM, and IgA) are now available. IgG and IgM ACA concentrations are expressed in units. By definition, one unit represents cardiolipin binding activity of 1 μg per ml of affinity-purified ACA antibody from reference sera. IgM is measured in MPL units (1 MPL unit = 1 μg of affinity-purified IgM ACA from an original reference serum) and GPL units are used for IgG (1 GPL unit = 1 μg of affinity-purified IgG ACA from an original index serum sample).

- Titer

Reporting the test results, it is important to identify the titer together with the isotype. The titer of ACA can be presented as high, moderate, low, or negative. In many cases of moderate or low titer, ACA are transient antibodies as a result of intercurrent infections. Therefore, it is important that the initial positive result is repeated after 6-8 weeks. Persistence of ACA is one of the criteria necessary to establish the diagnosis of APS. The identification of a positive test result depends upon the laboratory's care in identifying an appropriate normal reference interval. The distribution of the normal range is logarithmic rather than Gaussian. In order to quantify ACA, four house standards from two University Hospital laboratories were compared with the standards provided by the Antiphospholipid Standardization Laboratory by using two different plates and two different buffered protein solutions. Slopes from the serial dilutions of each of the four house standards were found comparable. In contrast different slopes were obtained when using the ASL standards which consist of a mixture of sera. These results indicated that dilutions of single sera are more suitable than mixture of sera when quantification of ACA is required (Rupin et al, 1994). The team from Haemostasis Unit at University Hospital of Geneva studied the positivity variation for ACA in 61 patients and 42 controls by comparing two commercial kits (A and B) with their own assay (C). The results were as follows: 50.8% ACA positivity for A, 57.4% for B and 50.8% for C. As for controls the concordance in patients was better between kit A and assay C than between kit B and assay C (de Moerloose et al, 1990). The performances of nine commercial kits and an in-house method for the quantitation of ACA have been evaluated in a multicenter study (Reber et al, 1995). Marked differences in positivity rate between kits were observed, ranging from 31 to 60% for IgG and 6 to 50% for IgM. This study showed that differences in positivity rates between the commercial kits might contribute to the differences in ACA prevalence rate found in the literature. The choice of cut-off levels might partly explain the moderate concordance between the kits. Sustained efforts on anticardiolipin standardization have resulted in improved agreement between commercial assays.

The ACA ELISA is a sensitive test, but its disadvantage is that it may be positive in a number of disorders other than APS. Alternatively, newer assays that use β_2GPI or a mixture of negatively charged PL (APL ELISA Kit) have been proposed for more specific

measurements of antibodies present in APS (Roubey et al, 1995). The epitope recognized most often by ACA is thought to be β_2GPI. Thus, ELISA kits are using β_2GPI to distinguish autoimmune ACA from true ACA that do not require β_2GPI for direct binding to CL. Comparisons for the sensitivities and specificities of the standard ACA ELISA, anti-β_2GPI ELISA, and APL ELISA Kit were made (Pierangeli et al, 2001). They showed consequently 100%, 74%, and 98% sensitivity of the three assays for APS. Their specificity was, as follows: 60%, 82% and 99%. Inter-laboratory variability of anti-β_2GPI measurements (IgG and IgM) was investigated in the frame of the European Forum on Antiphospholipid Antibodies and its Standardization Group (Reber et al, 2002). They found that the rate of positivity varied from 50% to 93% for IgG and from 13% to 70% for IgM anti-β_2GPI. Excellent concordance between centers occurred only in 13% of cases for IgG and in 6% of cases for IgM, because many selected samples were low-positive. Despite the large variability of anti-β_2GPI measurements between centers, the authors found a good agreement with high- and medium-positive samples. According to an agreement reached during the eighth International Symposium on Antiphospholipid Antibodies (Wilson et al, 1999), the LA test and ACA ELISA should be used primarily in the diagnosis of APS. Moderate-to-high positive ACA or LA test results and well-documented clinical features are enough for diagnosis of APS to be made. If these test results are negative or equivocal, more specific tests, such as anti-β_2GPI ELISA or anti-PL ELISA Kit might be used to confirm the diagnosis of APS.

Coagulation assays for LA

LA are defined as immunoglobulins that inhibit PL-dependent coagulation tests in the absence of specific coagulation factor inhibition. Generally, LA are suspected when one of several screening assays, most commonly aPTT, is prolonged. A suspected LA is further evaluated using an inhibitor screen (mixing study), in which the aPTT of a mixture of the test and normal plasma is measured. LA usually prolong the clotting time of normal plasma immediately, but on occasion prolongation requires incubation (Clyne et al, 1988). If the initial mixing study shows correction of the abnormal clotting time, then an assay for time-dependent inhibition should be performed. A diagnosis of LA should not be made on the basis of multiple abnormal screening assays and mixing studies only. If the mixing

studies indicate the presence of circulating inhibitor, then LA confirmatory studies should be performed. Factor assays may be used when mixing studies show correction, suggesting a factor deficiency, when the LA confirmatory studies are negative or when a specific factor inhibitor is suspected (Brandt et al, 1995).

- Screening assays for LA (Table 2)

TABLE 2. *Main screening assays for LA*

Test	Coagulation pathway
aPTT (activated Partial Thromboplastin Time)	contact activation of the intrinsic pathway
daPTT	
KCT (Kaolin Clotting Time)	contact activation of the intrinsic pathway
dRVVT (dilute Russell's Viper Venom Time)	contact activation of the intrinsic pathway final common pathway (RVV directly activates FX to FXa and to a lesser extent FIX to FIXa
dPT (dilute Prothrombin Time)	VIIa TF activation of FIX, FIXa activation of FX, VIIa TF activation of FX and the prothrombinase reaction
Textarin time	Pt activator (in the presence of FV, calcium and PL)

The presence of LA is often first suspected when the aPTT is abnormal and fails to correct with normal plasma. Among screening coagulation tests, the aPTT is more sensitive than PT, probably because of the lower PL content of the reagent used in aPTT. A common concept is that the amount of PL in the test system is a critical determinant of sensitivity. Test systems with reduced amount of PL such as daPTT, KCT, dRVVT, dPT thus might offer the possibility of increased sensitivity due to their low PL concentration (Working Group on Haemostasis of the "Société Française De Biologie Clinique", 1993). The Textarin time was found to be the most sensitive screening test for LA when compared

with the other test systems. Data show that the PL concentration is not the only determinant of assay sensitivity to LA. Altering the incubation time of aPTT, rather than the PL concentration, was a sensitive way of detecting LA (Robert et al, 1994) but it does function with few cephaloplastins. Furthermore, for partly unknown reasons, some assays appear to be more sensitive to certain subgroups of LA. Therefore, at least two different types screening assays must be performed before the presence of LA can be ruled out. The presence of platelets or platelet fragments in plasma after centrifugation can affect the results of coagulation tests, particularly when plasmas are frozen before testing, and the use of twice centrifuged plasma, or filtration through a 0.2-μm filter, has been advocated to avoid this problem (Ames et al, 1996). This is particularly important for assays without added PL (KCT) or with very low amount of PL (dPT). The residual amount should not exceed 10 G/l (Exner et al, 2000).

The protein dependence of LA may affect the tests differently. For example, it has been shown that KCT is most abnormal in the presence of Pt-dependent antibodies (Galli et al, 1995), whereas the dRVVT is mainly abnormal in the presence of β_2GPI-dependent antibodies.

- Mixing studies for LA

The presence of an inhibitor is usually documented by mixing patient plasma with normal plasma and by demonstrating a persistence of an abnormal clotting time. The sensitivity of LA testing depends on the ratio of patient plasma to normal plasma used to detect the anticoagulant effect, and these variations have not been standardized. The most common ratio of patient to normal plasma, used in mixing studies, is 1:1. However, Clyne et al (1993) showed a relatively high incidence of negative mixing studies using a 1:1 ratio in aPTT system. Some authors have suggested 4:1 mixture for evaluation of mildly prolonged aPTT (McNeil et al, 1991). Other groups have proposed a 1:4 mixture for some cases (Petri et al, 1997).

The problem with negative mixing studies may be related to the observation that some LA show time-dependent inhibition in clotting assays. Up to 15% of LA may show correction of the prolonged clotting time if tested immediately after mixing but a lack of correction if the clotting time is repeated after incubation of patient and normal plasma. These data have

been challenged by Exner (2000), who demonstrated that upon buffering the mixture, the time dependence is abolished.

There is a lack of uniform criteria for the evaluation of mixing studies. One approach is to determine an index of correction. The basic formula for this index is:

Index= 100x(b-c)/a, where a= clotting time of patient plasma, b= clotting time of patient + normal plasma mixture, and c= clotting time of normal plasma.

- Confirmatory assays for LA

A number of confirmatory assays based on the principle of adding to or altering the PL content of the test system have now been described (Table 3). They can be grouped according to the screening assay on which they are based. In the diagnosis of LA, it is important to use a confirmatory study that corresponds to the screening test which is abnormal.

TABLE 3. *Confirmatory assays for LA*

Screening assay	Source of PL
aPTT based assays	Platelet neutralisation procedure, PL dilutions, hexagonal phase PL
dRVVT	Platelet vesicles, platelets
KCT	Platelet vesicles, liposomes
dPT	PL dilutions

The basis of these assays is to determine the effect of altering the PL content of the assay system, by adding platelets, platelet vesicles or hexagonal phase PL. The majority of test systems that are now employed to demonstrate PL dependence use increased concentrations of PL or frozen thawed platelets. The increased PL or platelet membranes "neutralize" or "bypass" LA. The platelet neutralisation procedure uses washed frozen and thawed platelets as a source of PL and is most commonly used in aPTT (Triplett et al, 1983). The hexagonal phase PL test is based on the finding that PE is capable of supporting coagulation test and can effectively neutralize LA activity. Comparing these 2 tests, platelet neutralisation procedure and hexagonal phase PL in systemic lupus

erythematosus plasmas, Reber et al (1994) found that platelet neutralisation procedure gave a higher rate of detection than hexagonal phase PL and other tests of detection. Addition of platelets may also shorten the aPTT in the presence of anti-F VIII antibodies.
As shown in Figure 4, LA and ACA may occur independently or may coexist. LA and ACA activities may be due to the same antibody, or the activities may be physically separable (Champley, 1991). There is another family of antibodies, called reagin antibodies, which are observed in patients with APS. This group of antibodies reacts with the Venereal Disease Research Laboratory (VDRL) reagent. The VDRL reagent denotes a mixture of antigens, including CL, cholesterol and lecithin (Singh et al, 2001).

Indications for laboratory testing

Because of the high risk of thrombosis and miscarriage and the influence of positive APLA tests on therapy, screening can be justified in a many subjects. In relation to venous thromboembolism, all subjects with apparently spontaneous events should be considered for testing. The prevalence of positive tests is likely to be lower in thrombosis secondary to identified events such as surgery or trauma. Recurrent venous thromboembolism, even in the presence of other risk factors, may be an indication for testing for APLA. Subjects with stroke and those with peripheral arterial occlusive events occurring at a young age (for example less than 50 years) should be tested for APLA, especially when risk factors for atheromatous arterial disease are not prominent. The case can be made also for screening older subjects who are non-smokers and are not exhibiting other risk factors such as hypertension, diabetes mellitus or dyslipidaemia. Where recurrent arterial occlusive events occur despite antithrombotic prophylaxis, APS should be excluded. In subjects with SLE, APLA should be sought as part of the assessment of the autoantibody profile.
Because miscarriage is a common phenomenon, screening for APLA is not informative after a single event. In women with three or more consecutive pregnancy losses, testing for APLA should be part of the comprehensive investigation, including gynaecological, hormonal and chromosomal assessments. Unexplained loss of any morphologically normal fetus in the second or third trimester may be an indication for testing for APLA. Consideration should also be given to the possible diagnosis of APS in women with early severe pre-eclampsia or severe placental insufficiency in any pregnancy. Because maternal

antiphospholipid antibodies may be downregulated during pregnancy, tests are best performed preconceptually or early in pregnancy when possible (Godeau et al, 1997).

IV. CLINICAL MANIFESTATIONS OF ANTIPHOSPHOLIPID SYNDROME

Clinical relevance of APLA

Although APLA were first described more than four decades ago, the mechanisms underlying their association with clinical events have not been completely defined.

APLA in healthy population

Estimates of the prevalence of APLA in healthy populations vary depending on the criteria used. It has been reported that ACA exist in approximately 5% of normal individuals, although less than 2% showed persistently elevated levels (Vila et al, 1994).

APLA in patients with no thrombosis or pregnancy morbidity, but with infections, drugs, auto-immune disorders

APLA are commonly found after certain acute or chronic infections. They develop in as many as 30% of children after viral infections. A high proportion of children and adults infected with mycobacteria, malaria, Q fever, hepatitis C, parvovirus B19, cytomegalovirus, etc. develop APLA (McNeil et al, 1991; Mengarelli et al, 2000). In HIV-positive patients, Constans et al (1998) found a 41% frequency of IgG APLA; IgM APLA were positive in 7% and IgG anti-β_2GPI were rare (3-4%). In Geneva University Hospital, ACA were determined by an ELISA assay in 116 HIV-1-infected patients and positive test was found in 23.3% of the patients with a predominance of IgG ACA isotype (Bernard et al, 1990). Another study at Geneva University Hospital included 43 HIV-positive and 29 HIV-negative heavily transfused haemophiliacs. The presence of ACA was detected in 10 patients, all of them infected by HIV (Naimi et al, 1990).

The increase of APLA in patients infected with HIV may be due to disruption of the cell membranes which leads to the exposure of normally “hidden” PL during apoptosis (Clements et al, 1995). APLA from HIV-infected individuals tend to recognize various PL, most commonly PS (Petrovas et al, 1999). In addition, ACA in patients infected with HIV recognize oxidized CL more strongly than reduced CL. This finding, in conjunction with

the increased oxidative stress found in these patients, may further explain the generation of APLA as a result of neoepitope formation by oxidized PL (Tzavara et al, 1997).

Some medications associated with the development of APLA include neuroleptics, quinidine and procainamide (Merrill et al, 1997). APLA could be induced also by phenothiazines, chlorthiazide, ethosuximide, oral contraceptives or alpha-interferon (Kutteh et al, 1997). The duration of APLA after infection or discontinuation of drug exposure is not well established, and the risk of thrombosis is variable.

LA have been identified in 10% to 20% of patients with well-established SLE, and ACA- in 30% to 50% of the individuals with SLE (Sammaritano et al, 1990) who present the majority of cases of secondary APS.

APLA also occur in patients with other autoimmune disorders like Sjögren's syndrome, mixed connective tissue disease, rheumatoid arthritis, systemic sclerosis, ankylosing spondylitis, vasculitis, idiopathic thrombocytopenic purpura, etc. APLA are found also in patients with diabetes mellitus, Crohn's disease and autoimmune thyroid disease. APLA can be detected in some patients with malignancies such as thymoma; carcinoma of the lung, kidney, ovary, cervix uteri, prostate; lymphoma, leukemia and various myeloproliferative disorders (Kutteh et al, 1997).

APLA in patients with thrombosis and/or pregnancy morbidity / primary APS /

According to the Sappporo's criteria for APS, all individuals with APS have by definition ACA and/or LA. Recent data show that LA are the strongest risk factor for thromboembolic events in patients with primary APS and secondary APS (Galli et al, 2000). No clear results have been reported showing that the measurement of ACA defines the patient's thrombotic risk. It has been found that ACA do not recognize anionic PL but are directed against plasma proteins bound to suitable anionic (PL and other) surfaces. Among them, β_2GPI and Pt are the most common antigen targets. β_2GPI is required by the great majority of ACA to react with CL in immunoassays (Galli et al, 1990). A recent study examined the distribution of APLAs among patients with SLE. The most prevalent APLA were found to be anti-β_2GPI antibodies. They were observed in 36.8% of all patients with SLE, being present in 40.4% of SLE patients with secondary APS and 34.9% in SLE patients without clinical features of APS (Bruce et al, 2000).

Clinical criteria for APS

Preliminary criteria for APS were formulated during the International workshop in Sapporo, 1998. Definite APS is considered to be present in a given patient when at least one of the following clinical criteria exist:

A) Vascular thrombosis

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

B) Pregnancy morbidity

1. One or more unexplained deaths of a morphologically normal fetus at or beyond the 10^{th} week of gestation, with normal foetal morphology documented by ultrasound or by direct examination of the fetus, or

2. One or more premature births of a morphologically normal neonate at or before the 34^{th} week of gestation because of severe preeclampsia or eclampsia, or severe placental insufficiency, or

3. Three or more unexplained consecutive spontaneous abortions before the 10^{th} week of gestation, with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded.

Vascular thrombosis

A retrospective analysis of 100 patients with primary APS and secondary APS (Munoz-Rodriguez et al, 1999) reported one or more thrombotic episodes in 53% of the patients: 40% had venous thrombosis, 53% had arterial thrombosis, and 7% had combined arterial and vein thrombosis (Table 4).

TABLE 4. *Clinical manifestations of vascular thrombosis*

Venous thrombosis	deep venous thrombosis superficial thrombophlebitis pulmonary embolism unusual sites- hepatic, mesenteric, axillary, pulmonary, renal veins
Arterial thrombosis	coronary, carotid, aorta and peripheral artery thrombosis cerebrovascular thrombosis retinal vessel thrombosis
Thrombotic complications	cardiovascular manifestations central nervous system manifestations osteoarticular and cutaneous manifestations

Venous thrombosis

Venous thrombosis most commonly involve the deep venous system of the lower limbs and the pelvic region. Cervera et al (2002), studying 1000 patients with primary APS and secondary APS, showed that deep vein thrombosis is the most common clinical complication (38.9%). Deep vein thrombosis occurs most often in high-risk settings, such as pregnancy, prolonged immobilisation, or use of oral contraceptives. A pulmonary embolism was observed in 14%, and superficial thrombophlebitis in the leg in 11.7% of the patients during the evolution of their disease. Thrombi at unusual sites such as the hepatic, mesenteric, axillary, pulmonary, and renal veins, cerebral venous sinus, and inferior vena cava have also been reported.

Less than 1% of episodes of venous thromboembolism are fatal. Although significant morbidity from post-thrombophlebitic syndrome develops in around 30% of individuals with lower limb deep vein thrombosis, a significant proportion of these eventually becomes asymptomatic (Prandoni et al, 1999).

- Recurrence of thrombosis

Munoz-Rodriguez et al (1999) observed that in most of the patients with recurrent episodes of venous thrombosis, the thrombotic event was at the same site as the previous

thrombosis. In recurrent venous thrombotic events in the legs, 45% of the individuals had recurrence in the same leg, 20.7 % in the controlateral and 30.5% in both legs (Margaglione et al, 1999). A prospective four years follow-up study compared the risk of recurrent venous thromboembolism in 412 patients with a first episode of venous thromboembolism, with or without APLA (Schulman et al, 1998). The risk of recurrence was found to be 29% in patients with ACA versus 14% in those without antibodies. The rates of recurrence were 10% per year in patients with ACA and 4% per year in those without such antibodies. The presence of elevated titers of ACA 6 months after an episode of venous thromboembolism was proposed as a predictor for an increased risk of recurrence of thrombosis and death. Recurrences of thrombosis were observed in 19% of the episodes treated with long-term oral anticoagulation with warfarin sodium or dicoumarol, in 42% treated prophylactically with aspirin, and in 91% in which anticoagulant/antiaggregant treatments were discontinued, which indicates the benefit from prolonged oral anticoagulation. Nojima et al (2001) found the presence of anti-protein S antibodies as a significant risk factor for venous thrombosis but not for arterial thrombosis.

Arterial thrombosis

Arterial thrombi can occur in any central or peripheral vessel. The most common arterial locations are the aorta, iliofemoral, cerebral, coronary and retinal arteries. The cerebral arterioles are the most common site of arterial thrombosis in APS. Peripheral arterial occlusion, with gangrene, is less common. In the study of Cervera et al (2002), 19.8% of the patients with APS had a stroke, and 11% had transient ischemic attack. Myocardial infarction was observed in 5.5% of the cases; arterial thrombosis of the legs has been found in 4.3%; from the ophtalmologic manifestations 5.4% of patients had amaurosis fugax and 1.5% had retinal artery thrombosis. Arterial thrombosis carries a much higher risk of morbidity and mortality due mainly to cerebral ischemia. More prolonged and intensive anticoagulant therapy is recommended.

- Recurrence of thrombosis

Krnic-Barrie et al (1997) observed recurrent arterial thrombosis in 55% of patients with a primary and in 38% of the individuals with a secondary APS. The recurrent arterial events

were found mainly in the white race. Schulman et al (1998) reported 28% risk of recurrences of thrombosis in patients with a low positive titer of ACA (5 to 35 GPL units), and 38% risk of recurrences in patients with moderate or high titer (>35 GPL units). In Geneva Vogel et al (1991) investigated a group of 65 patients with SLE and found that half of patients had ACA associated with arterial thromboembolic events. Nojima et al (1997) studied the relationship between arterial or venous thrombosis and the levels of ACA and/or existence of LA. They observed that the prevalence of thrombosis was higher in a ACA and LA positive patients (84%) than in ACA only positive patients (16%), or LA only positive patients (9%). Furthermore, in these patients positive for the two tests, all patients with a high positive level of ACA had arterial thrombosis indicating that a high ACA activity combined with a LA positive result might be a risk factor for arterial thrombosis. In a recent study the authors reported that both anti-β_2GPI and anti-Pt antibodies might be also significant risk factors for arterial thrombosis but not for venous thrombosis (Nojima et al, 2001).

Thrombotic complications associated with APLA

- Cardiovascular manifestations

The most common cardiac lesions described in APS patients are heart valve lesions including thickening, stenosis, vegetation formation and mitral regurgitation. Valvular abnormalities were found in 36% of patients with primary APS and in 48% of patients with secondary APS (Nesher et al, 1997). Deposition of APLA in the subendothelial layer is suggested as the pathogenic mechanism (Durrani et al, 2002). Angina pectoris was found in 2.7% of the cases, myocardiopathy in 2.9% and subclavian vein thrombosis in 1.8% (Cervera et al, 2002).

- Central nervous system manifestations

The cerebral vasculature is a common site of arterial thrombosis in APS (Sheng et al, 1998). Multiple subcortical white-matter infarcts secondary to cerebral ischemia the most frequent finding on magnetic resonance imaging. Levine et al (1995), in their prospective study of 81 consecutive APS patients who developed cerebral ischemia, reported a recurrence rate of 31% during a follow-up period of 3 years. The median time to

recurrence was 7.9 months; an IgG APL titer greater than 100 GPL was associated with an even shorter recurrence time. The reported frequency of neurological complications in APS patients was as follows: stroke in 42%, migraine headaches in 13-20%, epilepsy in 3-7%, multi-infarct dementia in 2%, chorea in 1%, and cerebral vein thrombosis in 0.7% (Munoz-Rodriguez et al, 1999; Cervera et al, 2002). Cerebral venous thrombosis was more common at a younger age and had a more extensive involvement in patients with APS (Carhuapoma et al, 1997). Seizures, transverse myelitis and chorea associated with APS were likely to be due to an interaction between central nervous system cellular elements and APL rather than thrombosis, although a firm link has not been established between these features and APS (Brey et al, 1998).

- Osteoarticular and cutaneous manifestations

As reported by Cervera et al (2002), the most common skin manifestations of APS are livedo reticularis (24.1%), leg ulcers (5.5%), pseudovasculitic lesions (3.9%) and digital gangrene (3.3%). They have been most commonly found on the extremities due to superficial venous thrombosis and thrombo-phlebitis. Avascular necrosis of bone was found in 2.4%, and arthritis in 27% of the patients with APS (Cervera et al, 2002).

Pregnancy morbidity

Pregnancy loss is a defining criterion for APS and occurs with a particularly high frequency in SLE patients. In addition to embryonic losses (before 10 weeks gestation) or foetal losses (after 10 weeks), APS is associated with a number of potential serious obstetric complications, including thrombosis, severe preeclampsia, utero-placental insufficiency, foetal distress and iatrogenic preterm birth (Table 5). These complications have significant maternal consequences, and they also may contribute to foetal loss. They may be associated also with other thrombophilic disorders such as factor V Leiden or G20210A mutations.

TABLE 5. *Obstetrical complications in APS*

Recurrent pregnancy losses
Unexplained second or third trimester loss
Foetal death
Intrauterine growth retardation
Premature birth
Severe preeclampsia
Utero-placental insufficiency
Pregnancy-related thrombosis (venous or arterial)

Cervera et al (2002) analysed 590 women with APS who had 1 or more pregnancies: 74% of them succeeded in having 1 or more live births. The most common obstetrical complications in the mothers were preeclampsia (9.5% of pregnant women), eclampsia (4.4%) and abruptio placentae (2%). The most common foetal complications were embryonic loss in 34.5% of the pregnancies, foetal loss in 16.9% of the pregnancies, and premature birth in 10.6% of life births.

In women with SLE, a previous adverse outcome was identified as the most important risk factor for another miscarriage in a subsequent pregnancy (Finazzi et al, 1996). Faden et al (1997) observed that the presence of anti-β_2GPI antibodies correlates well with some obstetrical complications, mainly eclampsia and preeclampsia. Anti-IgM anti-β_2GPI antibodies correlated well with a history of pregnancy loss (Forastiero et al, 1998).

In 1996, Oshiro et al performed a retrospective study of 366 women with two or more consecutive pregnancy losses, where 79 of them were noted to have LA or ACA, and 290 did not. Both groups had similar rates of pregnancy loss (84%). However, those with APS had 50% foetal deaths compared with 15% foetal deaths in those without APS. Approximately 80% of those with APS had at least one foetal death compared with less than 25% in those without APS. Branch et al (1997) studied 147 women with recurrent pregnancy loss, negative for LA and with medium-to-high levels of IgG ACA, 104 healthy, fertile controls of similar age and gravidity, and 43 women with well-characterized APS. Twenty-six (18%) women with recurrent pregnancy loss and nine (9%)

controls tested positive (above the 99th percentile) for APLA. Sera from five (3.4%) women with recurrent pregnancy loss and four (3.8%) controls demonstrated binding to PL antigens other than CL.

Other manifestation of APS

Thrombocytopenia

Thrombocytopenia was observed in 30-52% of patients with APS (Munoz-Rodriguez et al, 1999; Cervera et al, 2002). It was found in 21% of the cases with primary APS and in 38% of cases with secondary APS. Thrombocytopenia in secondary APS patients was significantly associated with the presence of ACA at medium-high titer (Amoroso et al, 2003). It was usually mild (platelet count above 90 G/l) and, except in very severe cases, no bleeding was observed. Similarly to immune thrombocytopenias, pathogenic antibodies were directed towards epitopes on platelet membrane glycoproteins and were distinct from "antiphospholipid" antibodies (Godeau et al, 1997). Galli et al (1994) measured in 68 patients with APLA also anti-GPIb/IX and GPIIb/IIIa IgG, directed against platelet membrane-associated glycoproteins. Increased plasma levels of these anti-glycoprotein antibodies were found in 40% of cases. Furthermore, APLA have been reported in around 30% of subjects with typical immune thrombocytopenias.

Atherosclerosis

Recent studies demonstrated that EC activation induced by APLA might accelerate atherosclerosis associated with APS (Ross et al, 1999). Several studies have shown a close link between an atherosclerotic process and APS (Harats et al, 1999; Bruce et al, 2000 (b)).

Catastrophic APS

Catastrophic APS is a rare, accelerated form of APS. The patients with catastrophic APS present with wide spread noninflammatory thrombi involving the kidneys, lungs, heart, gastrointestinal tract, liver, the central nervous system as well as adrenal glands, in various combinations. Individuals presented with a clinical picture of acute multi-organ thrombosis with a 60% mortality due to myocardial infarction, acute respiratory distress syndrome, renal failure, or stroke (Asherson et al, 1996; Triplett et al, 2000). In the study of Asherson

et al (2001), a total of 80 patients with catastrophic APS were analyzed. The most important manifestations found at the onset of the episode were cardiopulmonary (25%), neurologic (22%), abdominal (22%), and renal (14%) with a very high mortality (48%).

V. PATHOGENESIS OF THE ANTIPHOSPOLIPID SYNDROME

The pathogenesis of APS is not completely clear. One hypothesis is that the exposure of anionic PL during apoptosis may be the driving antigenic stimulus for the development of APLA (Pittoni et al, 1998). Another hypothesis is that viral or bacterial infections may initiate the production of APLA. Indeed Gharavi and Pierangeli (1998) observed that peptides from adenovirus 2, cytomegalovirus or from bacillus subtilis are homologous to PL-binding region of β_2GPI. Some hypotheses to explain the pathogenic mechanisms in APS rely on the variety of effects of APLA. Indeed PL are involved in the hemostatic reactions and in biological processes in various manners and APLA have been shown to affect coagulation at different steps.

APLA have been shown to play a true causal role in development of thrombosis and obstetrical complications. Indeed immunization of mice with heterologous β_2GPI leads to the development of APLA with recurrent pregnancy loss and thromboembolic complications (Gharavi et al, 1998). Moreover mice infused with APLA developed significantly larger thrombi in femoral veins after experimental injury than mice infused with control antibodies (Pierangeli et al, 1996). Also, a monoclonal human ACA derived from a patient with APS promoted thrombosis in mice (Olee et al, 1996). Atherosclerosis in a susceptible mouse model (LDL-receptor knockout mice) was accelerated by immunization with human ACA from a patient with APS, providing additional evidence for a causal pathogenic effect (George et al, 1997). The recent characterization of chimpanzee β_2GPI with the finding of a high prevalence of anti-β_2GPI antibodies in these animals could propose a possibility of primate models for investigating APS (Sanghera et al, 2001). It is possible that APLA predispose to thrombosis either by causing cells to acquire a procoagulant phenotype or by inhibiting cell surface anticoagulant processes. Additional mechanisms may be involved in the pathogenesis of obstetrical complications.

Effects of APLA on the cells involved in hemostasis

APLA have been shown to interfere with different types of cells (Table 6) involved in hemostasis. Other cells such as fibroblasts may also play a role but they are not detailed in this review.

TABLE 6. *Interactions of APLA with cells of the coagulation cascade*

Endothelial cells
Platelets
Mononuclear cells
Polymorphonuclear cells

Endothelial cells

Endothelium is a metabolically active interface between the blood and extravascular tissues. Resting EC exert anticoagulant and antithrombotic properties by preventing contact of blood with prothrombotic underlying tissues and by producing and presenting on the EC surface molecules that aid in this function (Pearson et al, 2000). When EC are activated by different stimulus such as inflammatory cytokines (TNFα, IL-1), bacterial lipopolysaccharide (endotoxin), viral infections or hypoxia, they express both procoagulant and proinflammatory properties. It is now known that APLA can bind to and also activate EC in a similar manner, thus provoking a procoagulant phenotype in EC. In the next paragraphs we will review the main properties of resting endothelium and the changes after its activation (Table 7).

- Resting EC

Resting EC are mainly antithrombotic and thus thanks to different mechanisms. The main molecules are indicated in Table 7 (next page).

TABLE 7. *Participation of EC to the hemostatic properties of the vessel wall*

Resting EC	Activated EC
Prostacyclin and nitric oxide (+)	Nitric oxide (-)
Tissue factor pathway inhibitor (+)	Tissue factor (+)
Heparan-like sulfate proteoglycans (+)	Surface anionic PL (+)
Thrombomodulin (+)	Thrombomodulin (-)
Endothelial protein C receptor (+)	Plasminogen activator inhibitor type I (+)
Protein S (+)	Surface protease receptors expression (+)
Tissue-type plasminogen activator (+)	Adhesion molecule receptors expression (+)
Annexin V (+)	
Ecto-adenosine diphosphatase and adenosine triphosphatase receptors (+)	

(+) increased EC synthesis / expression; (-) decreased EC synthesis / expression

Prostacyclin (PGI_2) and nitric oxide (NO), also called endothelium-derived relaxing factor (EDRF), inhibit synergistically the platelet aggregation and also act as vasodilators. Besides vasodilatator and antiplatelet properties, nitric oxide has antiadhesive and antioxidative effects. The best-known antioxidant effect of nitric oxide is the impairment of lipid oxidation, mainly free fatty acids, phosphatidylcholine and low-density lipoprotein particles. In view of the proatherogenic effects of oxidized lipids, this antioxidative activity of nitric oxide is likely to be relevant (O'Donnell et al, 2001). The mechanism of the antiadhesive action of nitric oxide could also involve antioxidant effects. Indeed the increased leukocyte adhesion induced by inhibition of nitric oxide synthases was, at least partially, reversed by intracellular oxygen radical scavengers (Niu et al, 1994). Both mediators, PGI_2 and NO, are synthesized and released by EC locally and transiently in response to agonists molecules involved in the coagulation process (e.g. bradykinin and thrombin) or secreted by aggregating platelets (e.g. adenosine triphosphate and adenosine diphosphate). PGI_2 and nitric oxide synthesis are each triggered by increases of intracellular calcium ion concentrations in EC. The elevation of calcium ion, required to activate fully the production of nitric oxide from its precursor arginine is lower than that

needed to drive PGI_2 synthesis (Gryglewski et al, 2001). As well as being triggered by platelet secretory products, PGI_2 synthesis, unlike nitric oxide synthesis, can occur in an agonist/receptor-independent fashion when platelets aggregate. The activated platelets secrete PGH_2 or arachidonate that is directly converted to PGI_2 by endothelium.

Tissue factor pathway inhibitor (TFPI) is the physiological inhibitor of TF/factor VII complex, synthesized by EC and bound to the EC surface (Lupu et al, 1997).

Heparan-like sulfate proteoglycans are localized to the EC surface and they serve to accelerate the inhibition of thrombin by antithrombin.

Thrombomodulin (TM) is a cell surface proteoglycan produced by EC and expressed on their surface. TM binds thrombin and decreases its capacity to cleave fibrinogen but similarly increases its capacity to cleave circulating protein C (Esmon et al, 1995). On thrombin binding, the complex is endocytosed, thrombin is degraded, and TM is recycled to the cell surface.

Endothelial protein C receptor, expressed by EC, enhances the protein C activation by the thrombin-TM complex (Laszik et al, 1997; Esmon et al, 2003).

Protein S, synthesized and secreted by EC, is a co-factor that promotes protein C anticoagulant pathway.

Tissue-type plasminogen activator (t-PA) is a fibrinolytic mediator, secreted from Weibel-Palade bodies in response to thrombin (Emeis et al, 1997; Huber et al, 2001; Rosnoblet et al, 1999). Classically, t-PA is activated by binding to fibrin, hence localizing plasmin generation to the site of a clot. In addition, the EC surface possesses several binding sites for plasminogen and a specific t-PA receptor, which leads to local plasmin generation at the EC surface (Hajjar et al, 1995).

Annexin V, expressed on EC, binds with high affinity to the surface PL of both quiescent and activated EC and inhibits the procoagulant reactions.

Ecto-adenosine diphosphate and adenosine triphosphate receptors are expressed on EC surface. Adenosine diphosphate receptors induce a response on EC initiating PGI_2 and nitric oxide synthesis. The major pathway responsible for ending the pro-aggregatory action of adenosine diphosphate is its sequential dephosphorylation to adenosine monophosphate and then adenonosine (an inhibitor of platelet aggregation), which is due to ectonucleotidase enzymes at EC surface (Zimmerman et al, 1998).

Adenosine diphosphatase, secreted from activated platelets degrades adenosine diphosphate and thereby limits the effect of platelet released adenosine diphosphate.

- Activated EC

Endothelial activation leads to loss of anticoagulant properties (Table 7) and is characterised by several modifications.

Diminution of NO

Recent studies in hypercholesterolaemic animals and humans show that the deficiency in agonist-induced nitric oxide synthesis can be improved by elevating the circulating levels of arginine. This suggests that the intrinsic levels of nitric oxide synthase are preserved but that some aspects of the coupling between agonist receptor and nitric oxide synthesis are disturbed (Maxwell et al, 1998). In contrast with nitric oxide decrease, PGI_2 release is enhanced in patients with atherosclerosis. This has been attributed as a consequence of excessive platelet reactivity.

Tissue factor expression

Tissue factor is a transmembrane protein expressed by stimulated EC. It is the physiological trigger of normal coagulation and a major initiator of clotting in thrombotic disease (Figure 5). Tissue factor initiates the extrinsic pathway of coagulation by serving as a cofactor and receptor for factor VIIa to efficiently cleave its substrates, factor IX and factor X, to their active forms (Roubey et al, 2000).

Increase in cell surface anionic PL

Activated EC have increased exposure of surface anionic PL, which are cofactors for the coagulation system.

Diminution of TM

Activated EC down-regulate the synthesis and cell surface expression of TM and consequently reduce the formation of the thrombin-TM complex, thereby limiting thrombin generation (Laszik et al, 2001).

Secretion of plasminogen activator inhibitor type I (PAI-I)

PAI-I is synthesized and secreted by activated EC. It is the major plasma inhibitor of t-PA, involved in the regulation of fibrinolysis, degradation of the extracellular matrix and angiogenesis.

Release of Von Willebrand factor (vWF)

Activated EC release vWF from storage granules (Weibel-Palade bodies) in response to thrombin, which participates in platelet adhesion.

Cell surface protease receptors expression

Activated EC serve as a site of protease by expressing cell surface protease receptors. Thus EC facilitate the formation of enzyme complexes involved in the regulation of coagulation and fibrinolysis.

Increased adhesion molecule expression

Activated EC express adhesion molecules for leucocytes such as E-selectin, intracellular adhesion molecule (ICAM-1 and -2), vascular cell adhesion molecule (VCAM-1).

- Effects of APLA on endothelial cells

It has been hypothesized that APLA bind to antigens such as PS, TM and heparan proteoglycan on EC surfaces (Pierangeli et al, 1999) and that β_2GPI could be a cofactor facilitating this interaction with EC (Del Papa et al, 1997; Dueymes et al, 1996). Indeed EC activation by APLA is associated with increased expression of adhesion molecules, increased synthesis and secretion of proinflammatory cytokines, tissue factor expression, increased endothelin-1, induction of apoptosis, and EC migration.

Increased expression of adhesion molecules

Activated EC express surface E-selectin, VCAM-1, and ICAM-1 leading to increased monocytes and leucocytes adhesion. (Simantov et al, 1995; Meroni et al, 2001). Pierangeli et al (2000) demonstrated that in a pinch-induced thrombosis model APLA enhance leukocyte adhesion and increase thrombosis. The authors analyzed in vivo leukocyte adhesion to endothelium in venules of exposed murine cremaster muscle. The thrombogenic effects of APLA were reduced in transgenic ICAM-1-deficient mice, ICAM-1/P-selectin-deficient mice and in mice infused with anti-VCAM-1 antibodies.

Increased synthesis and secretion of proinflammatory cytokines

APLA increase EC synthesis and secretion of the proinflammatory cytokines IL-1 and IL-6. This could further contribute to cell activation in an autocrine manner because specific antagonists, such as IL-1 receptor antagonist, can inhibit the process (Del Papa et al, 1997;

Meroni et al, 2000). Thus anti-β_2GPI antibodies can induce an EC activation either directly or by a cytokine autocrine loop.

Tissue factor expression

It has been demonstrated that incubation of cultured EC with anti-β_2GPI antibodies result in an increased production of TF, further supporting the hypothesis that APLA are procoagulant triggers (Branch et al, 1993 ; Kornberg et al, 2000; Dunoyer-Geindre et al, 2001). Amengual et al (1998) demonstrated by reverse-transcription polymerase chain reaction that human monoclonal anti-β_2GPI antibodies upregulate tissue factor mRNA on HUVEC, suggesting that the tissue factor pathway be implicated in the pathogenesis of APLA related thrombosis.

Increased endothelin-1 (ET-1)

Significantly increased plasma levels of ET-1, the most potent endothelium derived contracting factor, were found in patients with APS and arterial thombosis (Atsumi et al, 1998). In vitro incubation of EC with human monoclonal anti-β_2GPI antibodies was shown to upregulate the expression of preproendothelin-1 mRNA.

Induction of apoptosis

A subset of APLA that recognizes annexin V induces apoptosis in EC (Nakamura et al, 1998; Pittoni et al, 1998). In vivo, apoptosis of EC would lead to de-endothelialization and exposure of the thrombogenic subendothelium (Bombeli et al, 1999). It is hypothesized that APLA could also displace annexin V that covers the anionic PL on EC membranes, thus increasing the net quantity of thrombogenic PL exposed their procoagulant phenotype (Rand et al, 2000).

EC migration

APLA may also interfere with EC migration. This activity could potentially interfere with re-endothelialization and prolong the exposure of the thrombogenic subendothelium (Lanir et al, 1998).

Platelets

Platelet binding to activated EC is the next phase in the process of thrombus formation although the precise mechanisms that mediate EC-platelet interaction are not completely clear. It was demonstrated that activated platelets are present in patients with APS but

whether platelet activation in patients with APLA is a direct result of APLA or other autoantibodies or a consequence of vascular injury is uncertain (Emmi et al, 1997). A recent study found that APLA in APS have antiplatelet reactivity but there was no evidence for associated direct platelet-activating ability (Ford et al, 1998). Galli et al (1996) reported that the evidence that human APLA either bind to or activate platelets is still uncertain. Lackner et al (2000) also showed that two human monoclonal APLA had no effect on platelets as determined by flow cytometric analysis of CD62P, CD41, CD42b expression and fibrinogen binding with and without previous activation with adenosine diphosphate or thrombin receptor activating peptide (TRAP-6). One possible explanation for these observations might be the presence of specific antiplatelet autoantibodies that coexist with APLA in patients with APS (Reverter et al, 2000). However, some investigators have shown that APLA may induce platelet activation and aggregation in the presence of low concentrations of agonists such as thrombin, adenosine diphosphate or collagen (Martinuzzo et al, 1993; Campbell et al, 1995; Nojima et al, 1999). A correlation was found between the IgG level of ACA and the CD62-positive platelet percentage in patients with primary APS and, more significantly, in the patients with primary APS and neurological disorders.

APLA-containing plasma promoted platelet aggregation in a perfusion model (Escolar et al, 1992). Wiener et al (2001) suggested that the platelet aggregation in APS is induced by an APLA-complex present in patients' plasma. The initial trigger in this thrombotic process is calcium independent, but probably is followed by release, recruitment, and ultimately fibrin formation by the usual metabolic calcium-dependent fibrinogen binding pathway. Potential targets for APLA on platelets include platelet-activating factor (Barquinero et al, 1994), PS (Vazquez-Mellado et al, 1994; Campbell et al, 1995), and platelet glycoprotein IIIa (Tokita et al, 1996). Recently Ferro et al (1999) characterized 11-dehydro-TXB2 as a sensitive marker of platelet activation, and found it significantly higher in patients with SLE and APLA. A statistically significant correlation was found between plasma levels of vWF and tPA and excretion of this thromboxane metabolite. In a recent study, levels of platelet activation were investigated in 20 patients with primary APS and 30 SLE patients (14 of whom had secondary APS) by measuring CD63 expression on platelets and soluble P-selectin levels. Platelet CD63 expression and soluble P-selectin

levels were significantly higher in patients with primary APS and SLE patients with/without APS than normal controls (Joseph et al, 2001). Robbins et al (1998) hypothesized that APLA/β_2GPI complexes could activate platelets to produce thromboxane A2 (a proaggregatory prostanoid) which could contribute to the prothrombotic state found in patients with APS. Shechter et al (1999) found that platelet serotonin concentration in APS patients was significantly lower than that found in the platelets of normal controls but the reasons of low serotonin levels are still not clear.

Thrombocytopenia is a common finding in patients with APS and a potential association with platelet activation could exist. It was hypothesized that thrombocytopenia is due to platelet activation and consumption of platelets on the damaged vascular endothelium (Walenga et al, 1999). George et al (1999) studied 38 SLE patients and found in 26.3% of them high levels of β_2GPI containing immune-complexes. There was a positive correlation between β_2GPI/ immune-complexes levels and the occurrence of thrombocytopenia.

Monocytes

Monocytes are implicated in the pathogenesis of APLA related thrombosis, mainly by stimulated cell surface expression of tissue factor (Kornberg et al, 1994; Cuadrado et al, 1997). Amengual et al (1998) revealed by flow-cytometry that monocytes from a healthy donor displayed higher tissue factor antigen expression when incubated in the presence of APS plasmas than with control plasmas. Stimulation of monocytes from APS patients with β_2GPI induced substantial monocyte TF, whereas no induction was observed with cells from patients having APLA without APS. Tissue factor induction on monocytes by β_2GPI was dose dependent and required circulating type 1 (Th1) CD4+ T lymphocytes and class II Major Histocompatibility Complex (MHC) molecules (Visvanathan et al, 2000). The authors previously reported that at least 44% of patients with APS possess Th1 CD4+ T cells that proliferate and secrete IFN-γ when stimulated with β_2GPI in vitro (Visvanathan et al, 1999). F(ab)2 fragments of ACA have been reported to induce monocyte tissue factor expression as well, suggesting the contribution of an Fc-independent component to the mechanism of antibody-mediated tissue factor activity in APS (Kornberg, 1994). Increased levels of tissue factor mRNA have been found in the majority of mononuclear cell samples from patients with APS (Dobado-Berrios et al, 1999).

Polymorphonuclear cells

Arvieux et al (1995) investigated the ability of six murine monoclonal antibodies to β_2GPI to induce polymorphonuclear cell functional responses. The six monoclonal antibodies tested in combination with β_2GPI led to a concentration-dependent activation of human polymorphonuclear cells. The activation of polymorphonuclear cells was estimated by their granule release, H2O2 production, and cytosolic Ca2+ increase. The results showed that the process of polymorphonuclear cell activation depends on monoclonal antibody binding to these cells through both Fab (via β_2GPI) and Fc domains.

Effects of APLA on coagulation

The effects of APLA on haemostatic reactions are shown on Table 8.

TABLE 8. *Procoagulant effects of APLA on the coagulation system*

Interference with components of protein C pathway	Inhibition of APC pathway Interference with the activation of protein C by the TM-thrombin complex Inhibition of thrombin formation Binding to cofactors Va and VIIIa
Interference with intrinsic pathway of coagulation Inhibition of antithrombin activity Impairment of fibrinolysis	

Interference of APLA with the components of the protein C/S pathway

The activation of the coagulation and the protein C pathway are shown in Figures 6, 7 and 8. Once activated by the thrombin-TM complex on the surface of EC, activated protein C (APC) exerts an inhibitory effect by cleavage of the factor Va and VIIIa; protein S and factor V are required as cofactors for the APC activity in vivo (Dalhback et al, 1993).

Experimental findings (de Groot et al, 1996) consistently showed that APLA may interfere with protein C axis in multiple ways as described below.

- Inhibition of APC anticoagulant pathway, directly or via its cofactor protein

Several recent studies proposed that APLA cause direct inhibition of the APC pathway and investigated the correlation of APLA specificity and APC pathway inhibition. Bokarewa et al (1994) studied the effect of 38 IgG fractions with either ACA alone or both ACA and LA on the response to APC. Five of eight IgG fractions with LA activity showed a tendency to reduce the effect of APC in the aPTT system and to simulate the activated protein C resistance (APC-R) phenomenon. No correlation was found between protein C activity and ACA levels or the extent of clotting time prolongation. In addition, Nojima et al (2002) found that the co-existence of anti-Pt antibodies and LA activity was a significant risk factor in the pathogenesis of APC-R in patients with SLE. Mali et al (2001) reported similar results from 59 unselected children with SLE showing that acquired APC-R was significantly associated with the presence of LA but not ACA. They proposed that acquired APC-R could be a marker identifying LA-positive patients at high risk of thrombosis. Controversially, Martinuzzo et al (1996) found that the acquired APC-R in patients with APS seems to be associated with ACA and anti-β_2GPI rather than an in vitro interference by LA. Atsumi et al (1998) demonstrated in vitro that ACA antibodies (not anti-protein C autoantibodies) can bind protein C via β_2GPI, and suggested that protein C could be a target of APLA by making a complex of protein C with ACA and β_2GPI, leading to protein C dysfunction.

In addition, patients with APS were often found to have protein S deficiency. Atsumi et al (1997) demonstrated monoclonal ACA binding to protein S in presence of a combination of β_2GPI and CL, with a consequent increase of the affinity of C4b-binding protein for protein S. Protein S could represent one of the targets for ACA when combined with β_2GPI and CL, thus explaining the acquired free protein S deficiency and the attendant risk of thrombosis in patients with ACA. To explore the coagulation/fibrinolytic balance and its relation with free protein S, Ames et al (1996) carried out a cross-sectional study on 18 thrombotic patients with primary APS and 18 apparently healthy subjects with persistence of idiopathic APLA. Low free protein S was found in all non-thrombotic and in

90% of thrombotic patients with defective fibrinolysis. The data are consistent with increased thrombin generation and accelerated fibrin turnover and fibrinolysis abnormalities in asymptomatic subjects with APLA. It indicates also a possible central role for acquired free protein S deficiency in the thrombotic tendency of APS patients.

- Interference with the activation of protein C by the thrombomodulin-thrombin complex

Oosting et al (1993) showed that the anti-TM antibodies inhibiting TM and subsequent protein C activation are directed against the regions containing the epidermal growth factor (EGF) domains in SLE patients with a history of thrombotic complications. When TM was incorporated in PL vesicles, no inhibition by these anti-TM antibodies could be demonstrated. In addition, anti-TM antibodies could not inhibit protein C activation mediated by cultured EC. A conclusion was made that anti-TM antibodies inhibit only soluble TM.

Carson et al (2000) tested 58 patients with LA and found anti-TM antibodies in 30% of the cases. Similar antibodies were found in only 2% of 201 normal controls. Three IgG fractions of the 6 purified IgG from patients with anti-TM antibodies inhibited protein C activation from 40% to 70% compared to no inhibition in 7 healthy controls.

- Inhibition of thrombin formation

Prothrombinase, which consists of factor Xa, factor Va, Ca2+, and PL, converts Pt to thrombin. Thrombin is the final protease of the coagulation system and, in turn, activates protein C in the presence of thrombomodulin on endothelial cells. APC completes a negative feedback loop in the blood coagulation pathway by degrading factor Va and factor VIIIa and thereby inhibits prothrombinase activity (Stenflo et al, 1884). Using chromogenic substance assays and human IgM monoclonal ACA, Ieko et al (1999) found that these monoclonal ACA inhibited both thrombin generation and APC activity. Thrombin generation without APC was inhibited as well by adding these antibodies. This inhibition required the presence of β_2GPI and confirmed the data presented previously by Mori et al (1996).

- Binding to coagulation-activated cofactors Va and VIIIa in a manner that protects them from proteolysis by APC

Borrell et al (1992) studied the effect of purified IgM and IgG from 21 patients with APLA on factor Va degradation by APC on HUVEC. Thirteen of 14 IgM and 8 of 10 IgG from patients showed an inhibitory effect on factor Va degradation by APC when compared with control Ig. Oosting et al (1993) investigated the effect of 30 IgG fractions APLA on APC-mediated factor Va inactivation in the absence and presence of protein S. Three IgG fractions inhibited APC-mediated factor Va inactivation independent of protein S and four IgG fractions APLA inhibited in the presence of protein S. The anticoagulant response of purified APC, added to LA-containing plasmas of 46 patients, was measured through the amount of factor VIII inactivation and an acquired APC dysfunction was found (Potzsch et al, 1995). Thirteen of 14 patients with recurrent thrombotic events and 10 of 19 patients with one single episode of thrombosis showed an abnormal APC response. In contrast, among 13 patients with LA without symptoms, only one showed an abnormal APC response.

A subset of APLA reactive with PE was found particularly active in inhibiting the ability of PE to promote APC-mediated inactivation of factor Va. This effect correlated poorly with LA activity and it was suggested that this "acquired" APC resistance might be a risk factor for thrombosis even in the absence of LA (Smirnov et al, 1995).

APLA also interfered with the fibrinolytic pathway through TM inducible fibrinolysis inhibitor and by increasing PAI-1 activity. These findings suggested that the impairment of fibrinolytic activity by APLA might be one of the causes of thrombophilic diathesis in APS (Ieko et al, 1999).

- Interference of APLA with the components of intrinsic pathway

Recent studies reported the presence of antibodies to factor XII in a significant number of patients with APS suggesting that their presence might lead to acquired factor XII deficiency (Jones et al, 2000). Jone et al (2002) reported that, although factor XII is a member of the family of proteins which include plasminogen and Pt, antibodies to factor XII in patients with APS appear to be distinct from antibodies to Pt. Sugi et al (2001) reported that certain anti-PE antibodies are not specific for PE, but recognize the contact

proteins factor XI and prekallikrein independently or in combination with high molecular weight kininogen. The contact proteins such as high molecular weight kininogen, prekallikrein and factor XII have anticoagulant and profibrinolytic functions and their deficiency was found to be associated with recurrent thrombosis. Several studies confirmed the presence of autoantibodies to the contact proteins in patients with SLE, thrombosis, and recurrent pregnancy loss.

- Inhibition of antithrombin activity by APLA

The formation of thrombin-antithrombin complexes (TAT) in APS was impaired. Ieko et al (2000) did not find an increase in level of TAT in APS, while the level of prothrombin fragment 1+2 increased. Therefore, free thrombin present in patient blood might contribute to thrombosis in APS.

It was demonstrated that at least some APLA cross-react with highly polyanionic heparin and heparinoid molecules and inhibit the acceleration of antithrombin activity (Shibata et al, 1994). Purified IgG from seven patients with APS were reactive with heparin by ELISA, whereas none of five controls had antiheparin reactivity. Specificity studies showed that APS IgG antiheparin antibodies were specifically reactive with a disaccharide present in the heparin pentasaccharide that binds antithrombin. Furthermore, these antibodies inhibited heparin-accelerated formation of antithrombin-thrombin complexes.

Impairment of fibrinolysis by APLA

Ieko et al (2000) investigated the effect of both β_2GPI and APLA on the activity of extrinsic fibrinolysis. The authors found that β_2GPI, without PAI-1, did not affect t-PA activity in a chromogenic assay. When PAI-1 was added to t-PA, the remaining t-PA activity was increased up to 60% by the addition of β_2GPI. The effect of β_2GPI did not require PL. Thus, β_2GPI might protect t-PA activity from the inhibition by PAI-1. When monoclonal ACA were further added to the mixture with a diluted PL, the remaining t-PA activity decreased to 50 and 80%. Monoclonal ACA appeared to inhibit the effect of β_2GPI and to elevate PAI-1 activity. Thus, the impairment of fibrinolytic activity by ACA might be one of reasons for the increased incidence of thrombosis in patients with ACA.

Additional effects of APLA

As PL bear structural resemblance to low density lipoprotein (LDL), several studies (Horkko et al, 1997) showed that APLA might show cross reactivity against oxidized LDL. Oxidized low-density lipoprotein is implicated in atherosclerosis by influencing foam cell formation and cell cytotoxicity. The production of anti-oxidized low-density lipoprotein antibodies results in the formation of immune complexes which are taken up at enhanced rate by macrophages, leading to foam cell formation. George et al (1997) demonstrated in mice immunized with ACA that developed APS, that infusion with oxidized low-density lipoproteins aggravated the manifestations of experimental APS. The authors suggested that cross-reactivity of oxidized low-density lipoproteins with PL might lead to significantly more severe form of APS.

In summary, although many studies were and are still performed to determine how APLA might increase thrombus formation in vitro, their mechanism of action remains unclear. Several studies have proposed that APLA activation of EC, platelets, and monocytes, as well as their effects on fibrinolysis and proteins C and S, may contribute to the prothrombotic state in APS. The large variety of proposed mechanisms makes it difficult to identify either the main primary mechanism. It is possible also that different mechanisms exist or co-exist among patients and even in one single patient.

Potential mechanisms involved in APLA-associated foetal loss

A relation between APLA and pregnancy loss has been formally recognized for almost 30 years (Nilsson et al, 1975). It is now accepted that APLA can lead to foetal loss and probably to recurrent preembryonic and embryonic loss. Studies have reported the presence of thrombi in placenta of patients with APLA-associated foetal loss, as well as other abnormalities, such as decreases in vasculo-syncytial membranes, villous fibrosis and hypovascular villi, and an increase in syncytial knots in placenta from 30% to 50% of these patients (Levy et al, 1998). Donohoe et al (1999) proposed that one of the mechanisms in APS associated obstetrical complications may involve the binding of APLA directly to the placenta where they may initiate placental thrombosis and infarction. By immunofluorescence techniques the authors detected human APLA binding to human placenta. Heterogeneous binding to normal term placenta, involving the trophoblast

microvillous surface, the stromal and the peri-vascular regions was demonstrated by affinity purified APLA from five out of six patients.

However, the frequency and extent of thrombosis might not be sufficient to explain the high incidence of foetal loss in patients with APLA. Autoantibodies from sera of primary and secondary APS patients were found to affect reproductive outcome in pregnant mice in vivo (Matalon et al, 2002). Purified IgG from women with APS and recurrent pregnancy loss was injected to mice and affected directly the embryo and yolk sac reducing their growth. The purified IgG ACA reduced yolk sac and embryonic growth more than sera negative for these antibodies and caused foetal resorptions and growth retardation as well. In their study, Blank et al (1999) converted the anti-β_2GPI monoclonal antibody into single-chain and replaced the H and L chains between the pathogenic and non-pathogenic single-chain anti-β_2GPI. They demonstrated that single-chain of pathogenic anti-β_2GPI are capable of inducing the same clinical manifestations as the whole antibody molecule in actively immunized mice. Elevated titers of mice ACA and anti-β_2GPI, associated with LA activity, thrombocytopenia, prolonged aPTT and a high percentage of foetal resorptions were detected. Vogt et al (1997) observed that monoclonal anti-PS antibodies bind to choriocarcinoma cells as well as to trophoblast cells in histologic sections and, like anti-β_2GPI antibodies, may displace annexin V from trophoblasts. Rand et al (1997, 1998) found that IgG APLA reduced the levels of syncytiotrophoblast apical membrane-associated annexin V in placental villi and the release of annexin V into surrounding media. In addition, trophoblasts and endothelial cells exposed to IgG APLA had significantly faster coagulation times. The authors proposed that the reduction of this anticoagulant protein at the maternal-foetal interface might account for the pregnancy loss in patients with APS.

The effect of APLA on several non coagulation-related placental functions, such as the secretion of human chorion gonadotropin, was also considered (Gleicher et al, 1992). An improved reproductive outcome in animal models of APLA-associated foetal loss in response to nonanticoagulant therapies, such as interleukin-3 or ciprofloxacin, suggested that mechanisms other than or in addition to thrombosis might contribute to placental dysfunction during APLA pregnancy (Blank et al, 1998). Holers et al (2002) found that APLA induce complement activation in the placenta resulting in placental injury, foetal

loss and growth retardation. They used a murine model of APS in which pregnant mice were injected with human IgG containing APLA. The authors found that inhibition of the complement cascade in vivo, using the C3 convertase inhibitor complement receptor 1-related gene/protein y (Crry)-Ig, blocks foetal loss and growth retardation. Furthermore, mice deficient in complement C3 were resistant to foetal injury induced by APLA.

In summary, several mechanisms are involved in APS associated pregnancy loss. They result in uteroplacental insufficiency with usually multiple placental thromboses and infarcts, provoked by the hypercoagulable properties of APLA.

VI. MANAGEMENT OF THE ANTIPHOSPHOLIPID SYNDROME

Treatment and prevention of venous thromboembolism

The initial management of an acute event, with intravenous monitored unfractionated or subcutaneous low molecular weight heparin is not really influenced by the diagnosis of APS. Warfarin therapy should be instituted in the usual way, with a target international normalized ratio (INR) of 2.5 (range 2.0-3.0). The duration of treatment should be determined on an individual basis, taking into account the presence of additional risk factors, the severity of the presenting event and the particular risk of bleeding on warfarin (Prandoni et al, 1996; Prandoni et al, 1999). Recent studies found that short-term warfarin therapy was not sufficient for patients with APS due to the high risk of recurrence of venous thromboembolism. In these cases the use of aspirin did not offer a clear additional benefit. Schulman et al (1998) demonstrated the beneficial effects of prolonged oral anticoagulation on patients with ACA after an episode of venous thromboembolism. Kearon et al (1999), in their study of 162 patients with a first episode of idiopathic venous thromboembolism, proposed also that these patients should be treated with anticoagulant agents for longer than usual because of the increased risk of recurrence of thrombosis. Some other studies (Brunner et al, 2002) reported that patients with APS and recurrent venous thrombosis should benefit from long term warfarin at higher intensity (INR 3.0-3.5). Although the risk-benefit ratio of long-term anticoagulation has not yet been clearly assessed, it is now proposed that for the majority of subjects having persistent APLA, a lifelong anticoagulant treatment (INR 2.0-3.0) should be provided after the first venous thromboembolism (Bauer et al, 2003).

Treatment and prevention of arterial thrombosis

Because of the high risk of recurrence and likelihood of consequent permanent disability or death, stroke due to cerebral infarction in patients with APS should be treated with long-term oral anticoagulant therapy, target INR 2.5 (range 2.0-3.0). Extracerebral arterial thromboembolic manifestations of APS will also warrant consideration of continuation of long-term anticoagulation with warfarin in many instances. Higher-intensity anticoagulation had been recommended and may be appropriate in some cases (Guidelines on oral anticoagulation, 1998), but results of prospective studies are required before the use of a target INR of 3.0 and more can be unequivocally supported.

The use of aspirin and/or hydroxychloroquine may be protective against thrombosis in asymptomatic APLA-positive individuals (Erkan et al, 2002). The addition of aspirin 100 mg to antivitamin K (INR 2.0-3.0) is now discussed in case of arterial thrombosis.

Prevention of pregnancy failure

In women with APS and a history of pregnancy complications, there is a particular need for a close collaboration between specialists. A variety of treatments including corticosteroids, low-dose aspirin, heparin and immunoglobulins have been used either as single agents or in combination in an attempt to improve the rate of live births in women with APLA (Kutteh et al, 1996). Available data are limited by the small number of patients in each study and by the lack of standardization of laboratory assays used to detect APLA. The use of corticosteroids in pregnancy is associated with significant maternal and foetal morbidity and should be avoided (Laskin et al, 1997). In their randomized controlled clinical trial, Rai et al (1997) reported that treatment with low-dose aspirin and heparin is applicable to women with a history of recurrent miscarriage associated with persistent APLA. They recommended treatment with aspirin, 75 mg/d, to be started as soon as the urine pregnancy test becomes positive. Because the majority of miscarriages occur before 14 weeks of gestation, the authors proposed a low-dose heparin treatment to be commenced when foetal heart activity is seen on ultrasonography. In the same study (Rai et al, 1997), unfractionated heparin (5000 IU) was administered twice daily subcutaneously. At present, although largely used, no low molecular weight heparin

preparation is licensed for use in pregnancy in Switzerland. However many studies indicate that low molecular weight heparin preparations are safe alternatives to unfractionated heparin as an anticoagulant during pregnancy (Sanson et al, 1999). Concerning the potential risk of heparin-induced osteopenia, discontinuation of heparin therapy at 34 weeks gestation seems reasonable in women with early pregnancy loss and no history of thrombosis. When late pregnancy complications have occurred previously, continuation of antithrombotic therapy to delivery is reasonable and postpartum thromboprophylaxis is indicated in women with a history of thrombosis. Delivery by caesarean section carries an additional thrombotic risk and it is an indication for perioperative thromboprophylaxis. Although combination treatment with aspirin and heparin leads to a high live birth rate among women with recurrent miscarriage and APLA, successful pregnancies may be complicated by foetal growth retardation, gestational hypertension and premature delivery (Backos et al, 1999). In summary, optimal management, usually with low-molecular weight and aspirin, requires close collaboration between the haematologist and the obstetrician as well as facilities to enable appropriate clinical monitoring and laboratory testing.

Primary thromboprophylaxis in case of APLA

Controversial data exist regarding the prophylactic treatment of patients positive for APLA and without history of thrombosis. Incidental detection of low titers of APLA carries a minimal risk of thrombosis. However, there is convincing evidence to suggest a significant risk associated with the presence of medium to high levels of APLA. Prophylactic therapy is not accepted in clinical practice at present, although there is increasing evidence of the risks of high levels of APLA (Finazzi et al, 1996; Khamashta et al, 1999). Recent guidelines on the investigation and management of APS by Greaves et al (2000) suggested the prophylactic use of low-dose aspirin, which was shown to be protective of deep vein thrombosis in high risk groups (Rai et al, 1997). Prophylactic low-intensity oral anticoagulation with warfarin and low-dose aspirin has been shown to significantly reduce the incidence of ischemic heart disease in patients with APS (General Practice Research, 1998). A decision analysis study of patients with SLE, with and without APLA, also supports the prophylactic use of aspirin (Wahl et al, 2000).

Thromboprophylaxis with statins in case of APLA

Recent literature reported that statins are the principal and the most effective class of drugs to reduce serum cholesterol levels. Statins have been also shown to have anti-thrombotic effects in addition to lower cholesterol and to reduce cardiovascular events, including myocardial infarction, stroke, and death in patients with or without coronary artery disease symptoms. The actions of these drugs extend far beyond cholesterol reduction and involve non-lipid-related mechanisms that modify endothelial functions, immunoinflammatory responses, smooth muscle cell activation, proliferation and migration, atherosclerotic plaque stability and thrombus formation. Thus statins may offer an interesting prophylactic therapeutic approach for APS without the risks associated with anticoagulation. The aim of this study was to evaluate whether statins could modify the APLA-induced adhesion molecule expression by endothelial cells.

PART II: STATINS AND ANTIPHOSPHOLIPID SYNDROME

I. INTRODUCTION

Statins and fibrates are important currently available hypolipemic drug families decreasing the plasmatic concentrations of atherogenic lipoproteins and thereby lowering the risk of coronary heart diseases. Fibrates act as an activator of lipoprotein lipase and increase the catabolism of triglyceride rich lipoproteins. Statins specifically inhibit hepatic cholesterol synthesis and consequently, by enhancing LDL-cholesterol uptake, decrease circulating LDL-cholesterol (Figure 11). Statins act as specific competitive inhibitors of HMG-CoA reductase. HMG-CoA reductase catalyses the 4-electron reduction of HMG-CoA into CoA and mevalonate, which is a rate-limiting step early in the cholesterol biosynthesis.

II. PHARMACOLOGICAL PROPERTIES OF STATINS

Origin and chemistry

Statins were first introduced into clinical practice in the late 1980s after the chance discovery of their lipid-lowering effects in 1976 (Endo et al, 1976). The first statins were produced from fungal metabolites, but now they are synthetically produced. Currently there are at least six statins in clinical use (Figure 9). *Lovastatin* was the first statin to be approved in 1987. Lovastatin, as well *pravastatin*, are natural products of fungal origin. The difference between them is that in pravastatin the methyl group is replaced by hydroxyl group, which differentiate their hydrophilic properties. *Simvastatin* is a semi-synthetic derivative: it differs from lovastatin by one extra methyl group added to it. *Fluvastatin* is the first entirely synthetic statin, derived from mevalonolactone. *Atorvastatin* and *cerivastatin* present a new generation of highly purified, synthetic statins. Lovastatin and simvastatin occur in an inactive closed lactone ring form. They are converted in the liver into the open lactone form. All other statins do not have to be converted to the active hydroxy acid form in vivo. The statins have structural similarities, but they have also structural differences that may influence their cholesterol-independent effects.

Solubility and protein binding

Statins could be divided in two groups: hydrophilic (pravastatin) and lipophilic (atorvastatin, simvastatin, fluvastatin, lovastatin, cervistatin). The lipophilic or hydrophilic nature of statins influences at least in vitro their access to cellular membranes (Figure 10). It has been shown that hydrophilic statins are distributed much more selectively in hepatic than lipophilic cells (Ichihara et al, 2002). Because the membrane of extrahepatic cells consists of lipid bilayers, hydrophilic statins cannot penetrate it, and thus cannot reach the intracellular enzyme; however the hepatic cell membrane contains organic anion transporters, which take hydrophilic substances into the cells. Lipophilic statins can enter extrahepatic and hepatic cells and thus, as shown in Figure 10, inhibit not only the cholesterol synthesis but also the production of metabolic intermediates in many extrahepatic tissues. Protein binding of statins varies from 50% (pravastatin) to 98% (atorvastatin, simvastatin, fluvastatin).

III. STUCTURE AND CHEMICAL INTERACTIONS OF STATINS WITH HMG-CoA REDUCTASE

Human HMG-CoA reductase consists of a polypeptide chains of 888 amino acids with three functional portions: residues 1-339 span the membrane of the endoplasmic reticulum eight times, residues 340-459 connect the membrane portion to C-terminal catalytic portion and residues 460-888 reside in the cytoplasm. A linker region connects the two portions of the protein. The three-dimensional structure of the catalytic portion of HMGR shows a close association of two monomers in a dimer and of two dimers in a tetramer. Two equivalent active sites are located at the monomer-monomer interface of the dimers (Istvan et al, 2000). The HMG-binding pocket is characterized by a loop (residues 682-694). All statins currently in use inhibit HMGR. They are composed of a HMG-like moiety and of different, largely hydrophobic attachments. Crystal structures of six different statins bound to the catalytic portion of human HMGR show that their HMG-like moieties bind in exactly the same way as the HMG moiety of HMG-CoA (Werner et al, 2002). The hydrophobic compounds of statins occupy the HMG-binding pocket and part of the binding surface for CoA. Thus, access of the substrate HMG-CoA to the reductase is

blocked when statins are bound. The tight binding of statins is due to the large number of van der Waals interactions between inhibitors and HMGR. The structurally diverse, rigid hydrophobic groups of the different statins are accommodated in a shallow non-polar groove that is present only when the COOH-terminal residues of statins are disordered. There are differences in the models of binding among various statins, the new synthetic statin rosuvastatin having the greatest number of binding interactions with HMGR.

IV. MECHANISM OF ACTIONS AND EFFECTS OF STATINS

Inhibition of cholesterol synthesis

HMG-CoA reductase catalyzes the 4-electron reduction of HMG-CoA into CoA and mevalonate with oxidation of two molecules NADPH. Statins block this conversion of HMG-CoA to mevalonate, thus inhibiting an early step of the cholesterol biosynthetic pathway (Figure 11).

The primary site of action of all statins is the liver. Their extra-hepatic plasma concentration and permeability, e.g. into vascular cells, differs between statins and depends mainly on their lipophilicity (Lea et al, 1997; McTaggart et al, 2001). Inhibition of cholesterol synthesis in hepatocytes upregulates the expression of the hepatic low-density lipoproteins receptor. As a consequence, low density lipoproteins and its precursors are cleared from the circulation (Goldstein et al, 1990). In addition, statins may inhibit the hepatic synthesis of lipoproteins, an important reason for their ability to lower low density lipoproteins in patients with homozygous familial hypercholesterolemia who have no functional low density lipoproteins receptors. Furthermore, statin treatment lead to a modest increase of plasma concentration of anti-atherogenic high-density lipoproteins (HDL) (Vega et al, 1998) and small reductions (5% to 10%) in triglyceride levels (Bakker et al, 1996).

The beneficial effects of statins in primary and secondary prevention of vascular disease in patients with elevated cholesterol levels have been well established (Hebert et al, 1997; Pedersen et al, 1999; La Rosa et al, 1999; Shepherd et al, 2002; Sposito et al, 2002). Recently, the efficacy of statins has also been extended to the primary prevention of vascular disease in subjects with average cholesterol levels (Downs et al, 1998). These results suggest that statin therapy could reduce the clinical consequences of atherosclerosis

in a large proportion of the population at risk. Recent studies also suggest that statins have benefits in reducing ischemic stroke risk by approximately one-third in patients with evidence of vascular disease (Andrews et al, 1997).

Statins and isoprenylated proteins

By inhibiting L-mevalonic acid synthesis, statins also reduce the synthesis of important isoprenoid intermediates such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) (Goldstein et al, 1990), which mediate many of the cholesterol-independent effects of statins (Figure 12a, 12b). The isoprenoid intermediates serve as important lipid attachments for the posttranslational modification of proteins. Numerous proteins undergo prenylation and are thus converted to a more lipophilic state that allows a protein to interact with cellular membranes. Rho GTPases, including RhoA, Rac, and Cdc42, are major substrates for post-translational modification by isoprenylation. A key step in the activation of Rho, which cycles between a GTP-bound and a GDP-bound state, is the attachment of the isoprenoid geranylgeraniol. The post-translational lipid modification allows the translocation of inactive Rho from the cytosol to the membrane. Therefore statins, which block geranylgeranyl synthesis, inhibit Rho membrane translocation and activity. Experimental evidence suggests that inhibition of Rho isoprenylation mediates several of the cholesterol-independent effects of statins not only in vascular wall cells (Amerongen et al, 2000) but also in leucocytes and bones. Each member of the Rho family serves specific functions in terms of cell shape, motility, secretion and proliferation, although overlapping functions between the members could be observed in overexpression systems. Furthermore, Rho family may participate in the regulation of gene expression of many proteins. Other important prenylated proteins include the Ras family that requires attachment of a farnesyl group for its actions and plays a crucial role in cellular differentiation and proliferation, the Rab proteins which are necessary for vesicle transportation within the cell and the Rap family which is known to play a role in cell replication, platelet activation and the generation of oxygen radicals (Laufs et al, 2000).

Many recent studies reported that the effects of statins extend beyond their cholesterol-lowering capacity (Werner et al, 2002; Takemoto et al, 2001; Case et al, 2002) and most of

their multiple activities (so called pleiotropic effects) are mediated by the ability to block the synthesis of isoprenoid products.

Pleiotropic effects of statins on vascular cells

Experimental evidence suggest that statins may influence several events in the vessel wall that are relevant for the progression of atherosclerosis (Koh et al, 2000)

- Statins and endothelial function

Stimulation of eNOS and inhibition of endothelin-1 (ET-1)

An important characteristic of endothelial dysfunction is the alteration in either the expression or function of the endothelial vasoactive factors, ET-1 and NO. ET-1 is an isopeptide synthesized by EC, with powerful vasoconstrictive effects among others. Atorvastatin and simvastatin inhibited pre-proET-1 mRNA expression and reduced immunoreactive ET-1 levels (Perera et al, 1998). Seeger et al (2000) demonstrated that fluvastatin significantly enhanced prostacyclin synthesis and significantly reduced ET-1 production in cell cultures of human umbilical endothelial veins. Since prostacyclin is a vasodilator and endothelin a vasoconstrictor, fluvastatin might have a significant effect on hemodynamics by favoring the balance towards vasodilation.

Endothelial nitric oxide has been shown to mediate vascular relaxation and inhibit platelet aggregation (Radomski et al, 1992), vascular smooth muscle cells (SMC) proliferation and endothelium-leucocyte interactions (Gauthier et al, 1995). Laufs et al (1998) have shown that inhibition of HMG-CoA reductase in vascular EC upregulates the expression and activity of eNOS and prevents their downregulation by ox-LDL. This effect occurs through an increase in eNOS mRNA stability.

Effects on the EC fibrinolytic activity

Lovastatin and simvastatin have been shown to induce an increase in the local fibrinolytic activity of EC by stimulating tPA-expression and activity, an effect that was potentiated by the inhibition of PAI-1 expression (Essig et al, 1998). Wiesbauer et al (2002) showed that statins decreased mRNA levels for PAI-1 in EC and SMC and increased mRNA levels for t-PA in SMC.

Inhibition of TF

Eto et al (2002) demonstrated that simvastatin prevents the induction of tissue factor by thrombin in human aortic EC and block the increase in tissue factor activity on the cell surface. Simvastatin also prevented the upregulation of tissue factor expression and activity in human aortic SMC. Simvastatin prevents tissue factor induction through inhibition of Rho/Rho-kinase and activation of Akt.

Statins decrease MHC class II antigen expression

Simvastatin selectively decreases interferon-gamma (IFN-gamma)-induced MHC class II expression (mRNA and protein) in human primary EC through actions on the CIITA promoter, general regulator of both constitutive and inducible MHC class II expression. In contrast, simvastatin does not affect the expression of MHC class I, pointing to specific actions in the MHC class II signaling cascade. In repressing induction of MHC-II and subsequent T-lymphocyte activation, statins therefore provide a new type of immunomodulation (Kwak et al, 2001).

Statin effects on angiogenesis

Urbish et al (2002) have shown a double-edged, dose-dependent effects of statins in angiogenesis signaling by promoting the migration of mature EC and endothelial progenitor cells at low concentrations of 0.01 to 0.1 μmol/L, and antiangiogenic effects at high concentrations (> 0.1 μmol/L). Promigratory and proangiogenic effects of atorvastatin on mature EC were correlated with the activation of the phosphatidylinositol 3-kinase-Akt pathway. Weis et al (2002) also showed biphasic dose-dependent effects of statins on angiogenesis that were lipid independent and associated with alterations in endothelial apoptosis and vascular endothelial growth factor signaling.

- Statin effects on SMC migration, proliferation and apoptosis

Kaneider et al (2001) observed that cervistatin inhibited proliferation of SMC and also led to an induction of programmed cell death by increasing caspase-3 activity. Muller et al (1999) demonstrated that lovastatin inhibit SMC proliferation in association with induction

of apoptosis. Lovastatin induced arrest of cells in G0/G1 phase of the cell cycle and DNA synthesis was reduced. A significant induction of p21WAF1/Cip1 protein expression was found by western blot analysis. This led to a strong inhibition of cyclin dependent kinases resulting in a cell cycle arrest.

- Statin effects on platelet adhesion and aggregation

Platelet activation and aggregation are crucial initial events in the development of cardiovascular disease and aggravate pathological alterations in the vessel wall. Adenosine diphosphate release from activated platelets induces further platelet recruitment, followed by cell aggregation. Adenosine diphosphate causes granule release and thromboxane A2 generation (Daniel et al, 1999). Neutrophils express locomotive activity in response to adenosine diphosphate and adenosine triphosphate released from activated platelets via activation of P2Y4 and P2Y6 receptors (Di Virgilio et al, 2001). Direct interactions of neutrophils and platelets are mediated through P-selectin (CD62P), the expression of which represents the first step in the formation of a leukocyte-platelet thrombus in vivo (Konstantopoulos et al, 1998). Statins have been shown to inhibit platelet function. A potential mechanism might include a reduction in the production of thromboxane A2 (Notarbartolo et al, 1995). Romano et al (2000) reported that statin treatment reduce the expression of P-selectin on platelets. Thrombin-activated platelets decreased their adenosine diphosphate and adenosine triphosphate release by coincubation with statins (Kaneider et al, 2002). Linjen et al (1994) found that pravastatin reduced the membrane cholesterol content of platelets, suggesting that certain properties of these membranes are altered in a manner that renders them less prone to participation in thrombosis.

- Statin effects on monocyte/macrophages

Macrophages are important for the development of atherosclerotic plaques. Secretion of proteolytic enzymes, such as matrix metalloproteases (MMPs), by activated macrophages may weaken the fibrous cap of the plaque, leading to plaque instability, rupture and ensuing thrombosis and arterial occlusion (Uzui et al, 2002). Cerivastatin treatment has been shown to decrease macrophage expression of MMP-1, MMP-3, MMP-9 and TF, as well to reduce the number of macrophages expressing histone mRNA, a sensitive marker of cell proliferation (Aikawa et al, 2001).

Statins decreased the expression of adhesion molecules on monocytes isolated from patients with hypercholesterolemia (Weber et al, 1997) and also decreased significantly monocyte chemoattractant protein-1 (MCP-1), which plays a major role in recruiting monocytes into the vessel wall (Kothe et al, 2000). Lovastatin was found to inhibit bacterial lipopolysaccharide and cytokine-mediated production of nitric oxide and expression of iNOS in macrophages probably by inhibiting farnesylation of p21(ras) or other proteins that regulate the induction of iNOS. Statins also decrease the production of cytokines TNFα, IL-1, IL-6 by activated monocytes (Pahan et al, 1997; Rosenson et al, 1999; Grip et al, 2000).

Pleiotropic effects of statins on extravascular system

Observational and experimental studies have implicated potential benefit from the administration of statins for other noncardiovascular diseases, including osteoporosis and dementia. Statins might have effects on bone formation. Cell culture experiments showed that murine osteoclast formation can be inhibited by lovastatin (Fisher, 1999). Furthermore, Sugiyama et al (2000) described that statins increase the expression of bone morphogenic protein-2, which plays an important role in osteoblast differentiation and bone formation. Jick et al (2000) presented a case-control study of individuals with dementia suggesting that those who were prescribed statins had a substantially lowered risk of developing dementia, independent of the presence or absence of untreated hyperlipidaemia. Recently, the data of clinical trials revealed also the antineoplastic potential of statins, particularly with the use of lovastatin in patients with cancer, anaplastic astrocytoma and glioblastoma multiforme (Larner et al, 1998). Stimulation of apoptosis could be involved in the beneficial effects of statins for these processes (Muller et al, 1998).

In summary, statins constitute the most powerful class of lipid-lowering drugs. However, the benefits demonstrated in clinical practice with statin therapy appear to be related, at least in part, with effects that are independent of their cholesterol-lowering effects. Extensive research in the last decade suggests that statins have additional beneficial

effects, related to an improvement in endothelial function, a reduction in blood thrombogenicity, antiinflammatory properties as well as immunomodulatory actions.

Other effects of statins

Inhibition of dolichol synthesis

The mevalonate pathway also leads to the formation of dolichols, having an essential role in lipoprotein synthesis, and of ubiquinone, involved in electron transport (Corsini, 1999).

Activation of protein kinase Akt

Phosphorylation of the protein kinase Akt is important in several cellular cascades, including the endothelial nitric oxide synthase activation and endothelial cell survival. Akt is activated by several growth factors including VEGF, fibroblast growth factor as well as shear stress (Edwards et al, 2000). Statins activate the protein kinase Akt via a phosphoinositol-3-kinase dependent pathway resulting in enhanced eNOS activity, inhibition of endothelial apoptosis and increased release of endothelial progenitor cells (Kureishi et al, 2000).

Regulation of caveolin

Caveolae are small plasma membrane invaginations that play an important role in signal transduction and compartmentalization of signaling molecules. For example, caveolin forms an inhibitory complex with endothelial nitric oxide synthase (eNOS). Recently, inhibition of caveolin expression in the presence of statins has been demonstrated, resulting in an upregulation of nitric oxide release (Feron et al, 2001).

Statins and HMG-CoA independent effects

Until recently, all cholesterol-independent effects of statin treatment were shown to be mediated by inhibition of mevalonate synthesis. Recently, Weitz-Schmidt et al (2001) reported that statins bind to a novel allosteric site within the ß2 integrin function antigen-1 (LFA-1), independent of mevalonate production. LFA-1 is important for the adhesion and co-stimulation of lymphocytes. The authors optimised statins for binding to LFA-1 and developed selective and orally active LFA-1 inhibitors that suppressed the inflammatory

response in murine model of peritonitis. These findings, independent not only of cholesterol but also of HMG-CoA, open up a new area of research and treatment options.

PART III: EXPERIMENTAL WORK

As indicated in the previous sections, EC activation by APLA was shown to be a crucial event in APS. The activated EC express procoagulant and proinflammatory phenotype. EC activation is associated especially with increased endothelial surface adhesion molecules expression, thus favoring mononuclear leukocyte adhesion. The aim of my study was to investigate to what extent statins influence the expression of adhesion molecules by EC activated by APLA. The working hypothesis was that statins could prevent EC adhesive properties induced by APLA. A secondary aim was, in case a statin's effect was observed, to investigate the mechanism explaining the effect.

The work was started by studying the effect of statins on EC activated by TNF-α. Indeed TNF-α is well characterized, available in sufficient amounts and previous experiments have shown a reproducible effect of this cytokine to induce adhesion molecules by EC.

I. PATIENTS AND METHODS

Patients

Sera were collected from 13 patients with clinical symptoms of APS. All patient sera contained IgG ACA and anti-β_2GPI antibodies at high titer and were LA (+) or LA (-). In preliminary experiments the ability of purified IgG APLA from these patients to induce a proadhesive phenotype in HUVEC was tested. APLA from 8 patients were found to be able to activate HUVEC increasing surface adhesion molecules and these 8 APLA were used in this study.

Reagents

TNF-α was from R&D Systems (Mineapolis, MN, USA). Simvastatin, fluvastatin and pravastatin were obtained from commercial source. Simvastatin in lactone form was activated by dissolving in ethanol and treatment at 50°C for 2 hours with 0.1 M NaOH. Then, the pH was adjusted to 7.0 with HCl. The stock solutions of simvastatin, fluvastatin and pravastatin were stored at a concentration of 10 mM at –20°C. Mevalonic acid lactone, farnesyl pyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) were obtained

from Sigma (St. Louis, MO, USA). To obtain the active mevalonate salt, 13 mg of mevalonic acid lactone was mixed with 0.1 M NaOH (2 hours, 50°C) and the pH was then adjusted to 7.4. Farnesyltransferase inhibitor-277 (FTI-277) and geranylgeranyltransferase inhibitor-286 (GGTI-286) were obtained from Calbiochem (San Diego, USA) and dissolved in DMSO containing 10 mM dithiothreitol. Stock solutions were kept 1 week at –70°C (FTI-277) or –20°C (GGTI-286). Monoclonal antibodies anti-VCAM-1 (anti-CD106), and anti-E-selectin (anti-CD62E) were purchased from Serotec (Oxford, UK). FITC-conjugated goat anti-mouse antibody, used as secondary antibody for FACS analysis, was from Cappel Organon Technika (Durham NC, USA), and goat anti-mouse IgG, conjugated with horseradish peroxidase (HRP) used for the ELISA method, was from Bio-Rad Laboratories (Hercules CA, USA). O-phenylenediamine (OPD) tablet used as substrate for bound HRP was obtained from Sigma. Protein A-Sepharose CL-4B was purchased from Amersham Pharmacia Biotech AB. Endotoxin levels in the statin solutions and IgG preparations were measured using the Limulus Amebocyte Lysate Endochrome Assay (Charles River Laboratories, Charleston SC, USA). Bicinchoninic acid (BCA) protein assay reagent for evaluating IgG content in the purified patient fractions was obtained from Pierce Europe BV, Oud-Beijerland, The Netherlands.

Cell cultures

Human umbilical vascular endothelial cells (HUVEC)

HUVEC were isolated from collagenase-perfused umbilical cord veins as previously described (Jaffe et al, 1973; Galve-de Rochemonteix et al, 2000). Briefly, the veins were rinsed with Krebs-Ringer bicarbonate buffer (120 mM NaCl, 4.75 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 5 mM glucose, pH7.4) to remove the red blood cells and incubated for 10 minutes at 37°C with 1 mg/ml collagenase (CLS type 1, Worthington Biochemical, Lakewood NJ, USA) in Krebs-Ringer bicarbonate buffer with 25 mM HEPES. Cells were collected by flushing the vein with 50 ml of RPMI 1640 supplemented with 5 % foetal calf serum (FCS) (Seromed Biochrom, Berlin, Germany). After centrifugation (10 minutes, 1200 rpm), cells were cultured in Petri dishes coated with 0.1 % gelatin at 37°C in a humidified atmosphere containing 5 % CO_2. The

culture medium was RPMI 1640 supplemented with 10 % FCS, 15 μg/ml EC growth supplement (Upstate Biotechnology, Lake Placid NY, USA), 10 mM HEPES, 90 μg/ml heparin (Boehringer Ingelheim, Germany), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco BRL-Life Technologies, Rockville MD, USA). After reaching confluence, HUVEC were harvested with trypsin-EDTA, splited 1:2 and cultured in tissue-culture dishes coated with gelatin. The cells were used at passages two to four for all experiments.

Human saphenous vein cells (HSVEC)

HSVEC were isolated by collagenase treatment and cultured in gelatin coated dishes as previously described (Kwak et al, 2000). Cells were maintained in medium 199 (Gibco BRL-Life Technologies, Rockville MD, USA) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco BRL-Life Technologies, Rockville MD, USA), 10% foetal calf serum (FCS) (Seromed Biochrom, Berlin, Germany), 100 μg/ml heparin (Boehringer Ingelheim, Germany), 15 μg/ml EC growth supplement (Upstate Biotechnology, Lake Placid NY, USA), and 25 mM HEPES.

Methods

Purification of patient IgG

Patients sera and sera from normal subjects were purified by protein A-Sepharose CL-4B affinity chromatography. The binding of IgG was performed using 0.1 M and 0.01 M Tris buffer, pH 8.0 as a binding buffer. The available binding capacity was 20 mg human IgG per ml of drained gel and the binding efficiency was approximately 90%. IgG were thereafter recovered by acid elution with 0.1 M glycine HCl buffer pH 3.0. The protein content of the eluted fractions was evaluated by BCA (bicinchonic acid) protein assay reagent. The purified IgG fractions were found to have bacterial lipopolysaccharide < 0.06 ng / ml as determined by Limulus Amebocyte Lysate Endochrome Assay.

Analysis of adhesion molecule expression on EC by flow cytometry (FACS)

HUVEC and HSVEC were grown in 24-well plates. After confluence, the cells were incubated in complete medium overnight with different concentrations of simvastatin (0.1 -

5 μM), fluvastatin (0.1 - 15 μM) or pravastatin (5 - 15 μM) with or without mevalonate (400 μM), GGPP (15 μM) or FPP (15 μM). Then, the cells were stimulated with TNF-α (10 ng/ml) or bacterial lipopolysaccharide (20 ng/ml) for a period of 4 hours to measure the effect on expression of E-selectin, and for 6 hours to measure the VCAM-1 expression. When the cells were stimulated with purified APLA (0.5 mg/ml), overnight preincubation with these antibodies in complete medium with 20 % FBS and without heparin was performed. For negative controls cells were incubated with medium alone and normal human serum IgG (0.5 mg/ml), respectively. Thereafter, the cells were washed in RPMI and incubated for 1 hour at 4°C with primary mouse monoclonal antibody (anti-E-selectin, anti-VCAM-1) at 10 μg/ml in PBS (Gibco, BRL-Life Technologies, U.K.), 5 % FCS, 0.02 % sodium azide. Cells were washed in PBS-FCS-sodium azide and incubated for 1 hour at 4°C with the secondary antibody, polyclonal FITC-conjugated goat anti-mouse antibody, dissolved in PBS/FCS/sodium azide. Cells were washed in PBS-sodium azide, harvested with trypsin-EDTA and, after centrifugation at 800 g for 5 minutes, fixed in 2.5 % formaldehyde, 2 % glucose, 0.02 % sodium azide in PBS. Propidium iodide (10 μl of 50 μg/ml) was added to each sample. Cell fluorescence was analysed in a Becton Dickinson FACScan flow cytometer (San Jose CA, USA). A total of 10'000 viable cells were analysed per experimental sample. Data were analysed using the CELLQUEST software (Becton Dickinson). The same experiments were performed with HUVEC incubated for 48 hours with GGTI-286 (10 μM) or FTI-277 (0.5- 2.5 μM). Fresh GGTI-286 or FTI-277 solutions were added after 24 hours. The concentration of GGTI-286 (10 μM) was five-fold higher than its IC_{50} (2 μM) for the inhibition of geranylgeranyltransferase; the concentration of FTI-277 (2.5 μM) was twenty five-fold higher than its IC50 (0.1 μM) for the inhibition of farnesyltransferase and about twenty five fold lower than its IC_{50} (50 μM) for the inhibition of gerenalygeranyltransferase (information provided by Calbiochem, San Diego, USA). After the preincubation, cells were stimulated with TNF-α (10 ng/ml).

Cell Enzyme Linked Immunosorbent Assay (ELISA)

A modified ELISA was used to measure E-selectin and VCAM-1 content at the cell surface. HUVEC and HSVEC were grown in 96-well coated plates and exposed to simvastatin with or without mevalonate at same concentrations and incubation times as

described for FACS analysis. After 4-6 hours incubation with TNF-α (10 ng/ml), cells were washed 3 times with RPMI containing 2.5 % FCS and fixed in 4 % paraformaldehyde for 15 minutes. After 3 washes with RPMI/FCS, cells were incubated for 1 h at room temperature with primary mouse antibody (anti-E-selectin, anti-VCAM-1), diluted 1:1000 in RPMI/FCS. After 3 washes in RPMI / FCS and 1 wash in PBS, cells were incubated with secondary antibody, goat anti-mouse IgG-horseradish peroxidase (HRP)-conjugated, diluted 1:4000 in RPMI / FCS. After 1-hour incubation at room temperature, the wells were washed 4 times in RPMI/FCS and once in PBS. Then 100 µl of 1 mg/ml o-phenylenediamine dihydrochloride (OPD) solution was added and after 10 minutes incubation at room temperature, the color development was stopped by addition of 50 µl of 3M H_2SO_4. The absorbance was read at 490 nm with an ELISA reader.

Statistical analysis

For ELISA assays, the effect of simvastatin on adhesion molecules expression was analyzed with Kruskal-Wallis ANOVA and its reversal by mevalonate with Mann&Witney test.

II. RESULTS

Induction of E-selectin and VCAM-1 expression by IgG from patients with APS

In preliminary experiments we tested the ability of purified IgG from 13 patients with APS to activate HUVEC. IgG derived from 6 patients with APS were found to induce a proadhesive phenotype on HUVEC. We investigated the induction of adhesion molecule expression by these APLA. As seen in Figure 13, HUVEC incubated for 24 hours with purified patient IgG induced a moderate increase in VCAM-1 expression as measured by flow cytometry analysis. Adhesion molecule expression of TNF-α stimulated cells was defined as 100% cell activation. The APLA-induced VCAM-1 expression varied between 30% - 60% when compared with TNF-α. The induction of an adhesive phenotype on endothelial cell by APLA was not attributable to contaminating endotoxin as all IgG preparations had levels of endotoxin below 0.06 ng/ml as measured by the Limulus lysate assay. Incubation of HUVEC with IgG derived from 6 healthy donors had no effect on expression of VCAM-1 and E-selectin when compared with control non-treated cells. Mean values (n=3) for E-selectin expression were 6.7 ± 3 and for VCAM-1 8.4 ± 1 expressed by cells treated with IgG from healthy donors. In the nontreated cells these values were 6.3 ± 2.2 and 8.2 ± 2.2, respectively.

Effect of statins on the expression of E-selectin and VCAM-1 on EC

Effect of statins on TNF-α activated EC

1A) Simvastatin effect on HUVEC

ELISA method

First we investigated the effect of statins on the stimulation of HUVEC with TNF-α. Compared to cells without TNF-α, in TNF-α treated HUVEC we observed a six fold increase in E-selectin expression by cell-based ELISA. Pre-treatment of the cells with different concentrations of simvastatin resulted in a dose dependent increase in their response to TNF-α (Table 9). At concentrations of 1 to 2.5 μM of simvastatin, as compared to non-pretreated cells, a 80% ($p<0.001$) and a 40% ($p<0.001$) increase were observed for E-selectin and VCAM-1 expression, respectively. At higher simvastatatin

concentrations, cell detachment and cell death were frequently observed. Addition of 400 μM mevalonate to the preincubation solution reversed the effect of simvastatin.

Flow cytometry analysis

We investigated E-selectin and VCAM-1 expression by flow cytometry analysis as well. Figure 14A shows that after TNF-α stimulation the expression of E-selectin was strongly increased. Strikingly, a very heterogeneous response was observed, a majority of cells showing a high level of E-selectin expression and a minority a lower expression. A similar heterogeneous response was observed when ten-fold higher TNF-α concentrations (100 ng/ml) were used (data not shown). This implies that the suboptimal response in some cells was not due to insufficient TNF-α concentrations. For the remainder of our work, a TNF-α concentration of 10 ng/ml was used. In cells pre-treated with simvastatin and then with TNF-α, almost all cells exhibited a high level of E-selectin expression. The effect of simvastatin was reversed by pre-treatment with mevalonate (Figure 14B).

1B) Simvastatin effect on saphenous vein EC

Figure 15 shows that simvastatin pre-treatment had similar effects on VCAM-1 expression in response to TNF-α both in HUVEC (A, B) and human saphenous vein EC (C). Table 10 summarises the mean (± SD, N= 10 independent experiments) fluorescence values obtained by flow cytometry for TNF-α treated HUVEC. It shows that E-selectin expression varied between 150% and 223% in simvastatin pre-treated HUVEC as compared to non-pre-treated cells. VCAM-1 expression was 118% to 156% in the simvastatin pre-treated cells as compared to non-pre-treated cells. Addition of 400 μM mevalonate to the preincubation solution reversed the effect of simvastatin.

2. Fluvastatin effect on EC

Similar results were obtained by flow cytometry with HUVEC preincubated with fluvastatin and stimulated with TNF-α (Figure 16A). At fluvastatin concentrations of 5 μM, E-selectin expression was increased (210% in one experiment, 321% in another one) and VCAM-1 expression was increased 143% and 160% (n=2) when compared with HUVEC treated with TNF-α alone. This effect was completely reversed by mevalonate (Figure 16B).

3. Pravastatin effect on EC

Preincubation of HUVEC with pravastatin, a hydrophilic statin, similarly augmented the TNF-α response of these cells. At pravastatin concentrations of 10 μM and 15 μM, a further 28 % increase in E-selectin expression was observed by flow cytometry analysis in TNF-α treated HUVEC and this effect was completely reversed by mevalonate (Figure 17). Mean values (n=6) for E-selectin expression were expressed as a percentage of mean value of non-statin pre-treated, TNF-α stimulated cells. The mean percentage values were: cells treated with TNF-α alone: 100 % (control); pravastatin pre-treated and TNF-α stimulated cells: 128 % ± 13 % ($p < 0.02$ vs control); cells treated with pravastatin, mevalonate and TNF: 97 % ± 11 %. Since the pravastatin effect was weaker than simvastatin, we focused on simvastatin and fluvastatin for the remainder of our work.

Effect of statins on bacterial lipopolysaccharide activated EC

Simvastatin pre-treatment similarly increased E-selectin expression in cells stimulated with 20 μg/ml bacterial lipopolysaccharide (Figure 18).

Effect of statins on APLA activated EC

We next performed flow cytometry analysis to study the effects of simvastatin and fluvastatin on HUVEC, activated with patient derived APLA. Contrary to what could be expected from clinical data, we observed an increase in adhesion molecule expression (Figure 19A) and this effect was completely reversed by mevalonate (Figure 19B). Figure 20 summarises the mean fluorescence values obtained by flow cytometry for HUVEC treated with APLA. It shows that APLA induced a 1.5 to 6 fold increase in E-selectin expression when compared to control (non-treated) cells. Fluvastatin pre-treatment gave a further 1.5- 2 fold increase in E-selectin as compared to non-pre-treated HUVEC. VCAM-1 expression was between 3 and 8 fold higher in APLA-activated cells as compared to non-activated cells and pre-treatment with fluvastatin led to another 2 to five fold increase in VCAM-1 expression as compared to non-pre-treated cells. Addition of 400 μM mevalonate to the preincubation solution reversed the effect of fluvastatin. These results

suggest that statins induce an additional increase of VCAM-1 and E-selectin in HUVEC activated by human affinity purified IgG APLA.

In the absence of stimulation of HUVEC by TNF-α, bacterial lipopolysaccharide or APLA, pretreatment with simvastatin and fluvastatin had no effect on the expression of E-selectin (mean fluorescence 7.5 ± 2 vs 5.5 ± 2 in control cells; n= 6) and VCAM-1 (8 ± 3 vs 6 ± 2). Also, mevalonate pre-treatment had no effect on the basal expression of E-selectin (mean fluorescence 10 ± 3 vs 5.5 ± 2; n= 6) and VCAM-1 (12 ± 3 vs 6 ± 2, n=7). Statin effects on APLA-stimulated HUVEC were comparable with statin effects on TNF-α and bacterial lipopolysaccharide activated HUVEC.

The effect of statins on adhesion molecule expression is reversed by isoprenoid intermediates

By inhibiting L-mevalonic acid synthesis, statins reduce the synthesis of intermediates from the mevalonate pathway. In Figures 14B, 16B and 20 as well as in Tables 9 and 10, we have shown that mevalonate reverses the increasing effect of simvastatin and fluvastatin on adhesion molecule expression to values comparable to TNF-α and APLA alone. This demonstrates that the statin effect is due to inhibition of HMG-CoA reductase.

Mevalonate is an essential intermediate not only for cholesterol biosynthesis but also for farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), which are important lipid attachments for the post-translational modification of proteins. We investigated the effect of GGPP and FPP on simvastatin mediated TNF-α-induced E-selectin overexpression. GGPP (15 μM) completely reversed the increasing effect of simvastatin (Figure 21) and fluvastatin (Figure 22) while with FPP (15 μM), only a partial reversal was achieved. With higher doses of FPP we did not obtain a complete reversal effect (data not shown). Mean values (n=3) for E-selectin expression were expressed as a percentage of mean value of non-pre-treated, TNF-α stimulated cells as follows: TNF-α stimulated cells 100%, simvastatin pre-treated cells 186% ± 40 %, simvastatin and GGPP preincubated cells 94 % ± 8 % and finally simvastatin and FPP preincubated cells 125 % ± 11 %. For HUVEC preincubated with 5 μM fluvastatin the mean value (n=2) for E-selectin expression in percentage was as follow: TNF-α stimulated cells 100%, fluvastatin pre-treated cells 200% ± 11%, fluvastatin and GGPP preincubated cells 110% ± 20% and

fluvastatin and FPP preincubated cells 150% ± 5%. These results suggest that statin effect is due to the inhibition of protein geranylgeranylation.

Similar results were obtained when HUVEC were activated by APLA. The overnight preincubation with GGPP completely reversed the potentiating effect of fluvastatin on E-selectin and VCAM-1 (Figure 20). These results demonstrated that geranylgeranylation might be involved in the potentiating effect of statins on TNF-α and APLA-induced adhesion molecule expression.

Inhibition of protein geranylgeranylation, but not protein farnesylation mimicks the effect of statins

The farnesylation or geranylgeranylation of proteins are catalysed by farnesyltransferase (FTase) or geranylgeranyltransferase (GGTase), respectively. To study the role of these enzymes, we investigated whether FTI-277 (2.5 μM) and GGTI-286 (10 μM), selective inhibitors of FTase and GGTase respectively, could mimick the effect of simvastatin.

Pre-treatment of HUVEC with GGTI-286 caused a significant increase in TNF-α-stimulated E-selectin expression, comparable to the effect of simvastatin (Figure 23A). In contrast, FTI-277 pre-treatment had no effect on E-selectin expression in TNF-α treated cells (Figure 23B). These results were confirmed on APLA-activated HUVEC where the potentiating effect of fluvastatin on VCAM-1 and E-selectin was mimicked by GGTI-286 (Figure 20). This effect on protein prenylation indicates that inhibition of protein geranylgeranylation but not farnesylation increase adhesion molecule expression in TNF-α and APLA-stimulated endothelial cells.

PART IV: DISCUSSION and CONCLUSION

Interaction of leukocytes with endothelial cells is an important part of the inflammatory pathway leading to vascular disease (Ikeda et al, 1998; Cines et al, 1998). Several studies have shown that APS, a hypercoagulable state associated with high titers of circulating APLA, may be the result of an APLA-dependent endothelial cell activation. This may lead to an enhanced expression of the adhesion molecules ICAM-1, VCAM-1 and E-selectin on EC and consequently an increased monocyte adherence to EC (Simantov et al, 1995). Indeed Pierangeli et al (1999) have demonstrated that upregulation of expression of ICAM-1, VCAM-1 and E-selectin on EC by APLA correlated directly with an increased adhesion of leukocytes to endothelium of mouse cremaster muscle (an indication of EC activation in vivo) and with enhanced thrombosis. In another study, George et al (1998) have shown that the upregulation of adhesion molecules by murine monoclonal anti-β_2GPI antibodies correlated with foetal resorption in mice producing APLA. As additional support for the hypothesis that APLA antibodies activate EC and may create an hypercoagulable state in APS patients, Kaplanski et al (2000) demonstrated that the levels of soluble ICAM-1 and VCAM-1 were significantly increased in the plasma of patients with APS and recurrent thrombosis.

In our study we confirm that purified IgG from patients with APS are able to induce VCAM-1 and E-selectin expression on HUVEC. Induction of an adhesive phenotype on endothelial cell is not attributable to contaminating endotoxin since purified IgG from healthy donors are not able to induce adhesion molecule expression and Limulus assays on sample preparations were always negative. We can thus conclude that the effect of APLA on EC is specific.

As the so-called pleiotropic "anti-inflammatory" effects of statins represent an attractive mechanism for their beneficial clinical effects in addition to their well known cholesterol lowering effects (Koh et al, 2000; Werner et al, 2002; Takemoto et al, 2001; Case et al, 2002), we examined whether the statins decrease the expression of adhesion molecules by endothelial cells stimulated with APLA. We investigated the statin effects on the expression of leukocyte adhesion molecules by HUVEC or saphenous vein stimulated first by TNF-α and bacterial lipopolysaccharide, and next by IgG from APS patients.

Contrary to what was expected, we consistently demonstrated that preincubation with fluvastatin and simvastatin led to a moderate increase in the E-selectin and VCAM-1 response to TNF−α as well as bacterial lipopolysaccharide in HUVEC. This effect was HMG-CoA-reductase dependent since it was reversed by mevalonate. Other studies have investigated the effects of statins on adhesion molecule expression by HUVEC but the results were quite divergent. Our results are in agreement with those of Sadeghi et al (2000) and Schmidt et al (2002) who found a potentiating effect of simvastatin or lovastatin on the cytokine-mediated increase in adhesion molecule expression by using flow cytometry analysis or a cell-based ELISA methods. Our results are also comparable with the data of Bernot et al (2003) who found that atorvastatin significantly enhanced surface VCAM-1, ICAM-1, E-selectin and fractalkine in TNF−α activated HUVEC as measured by flow cytometry and confocal microscopy.

Our findings, however, are in contradiction to the results of Meroni et al (2001) who reported that fluvastatin and simvastatin decreased E-selectin and ICAM-1 response to TNF-α and bacterial lipopolysaccharide measured using an ELISA method or with those of Wagner et al (2002), who reported an inhibition of VCAM-1 expression by atorvastatin when EC were stimulated with TNF-α and interferon-γ. Finally, Rasmussen et al (2001) reported that inhibition of HMGR in EC attenuated VCAM-1 expression, but increased E-selectin expression, after stimulation with cytokines.

Our data also show that simvastatin has a similar effect on endothelial cells from saphenous vein as that on HUVEC, thus indicating the simvastatin effects of adhesion molecule-induced expression are similar in different types of endothelial cells.

By flow cytometry, we observed that the E-selectin response to TNF-α or bacterial lipopolysaccharide was heterogeneous, with part of the cytokine-stimulated cells expressing a high level of E-selectin and part of the cytokine-stimulated cells expressing an intermediate level of E-selectin. After pre-treatment of the cells with simvastatin, fluvastatin or the geranylgeranyl-transferase inhibitor GGTI-286, the large majority of the cytokine-stimulated cells exhibited a strong E-selectin response that was comparable to that of the high responders to TNF-α in the control group. This suggests that simvastatin, fluvastatin or GGTI-286 act on a subgroup of cells that respond poorly to TNF-α. Because an increase of the TNF-α concentration by ten folds did not further increase the adhesion

molecule response, we can conclude that the poor response of part of the control cells was not due to suboptimal TNF-α concentrations.

We next investigated the statin effects on endothelial cell adhesive properties induced by APLA. We demonstrated that stimulation of HUVEC with patients' APLA led to a moderate increase of the surface adhesion molecule levels. Again, contrary to what could eventually be expected from clinical data, preincubation of the cells with simvastatin or fluvastatin led to an increase in the E-selectin and VCAM-1 response to APLA. Taken together our results show that statins increase rather than decrease adhesion molecule expression by APLA, TNF-α or bacterial lipopolysaccharide activated EC.

Statins block L-mevalonic acid synthesis and consequently its conversion into farnesylpyrophosphate (FPP), which is a precursor for geranylgeranylpyrophosphate (GGPP). Both FPP and GGPP are essential substrates for protein isoprenylation. This posttranslational modification is required for the cellular localization and biological function of small G-proteins such as Ras or Rho (Takemoto et al, 2001; Allal et al, 2000; Matozaki et al, 2000). In EC, Ras translocation from the cytoplasm to the plasma membrane is dependent on farnesylation whereas Rho translocation is dependent on geranylgeranylation (Laufs et al, 1998a; Laufs et al, 1998b). Statins, by inhibiting Ras and Rho isoprenylation, lead to the accumulation of inactive Ras and Rho in the cytoplasm. Studies have shown that inhibition of Rho isoprenylation mediates some effects of statins in vascular cells (Laufs et al, 1998a; Laufs et al, 1999). To determine which isoprenyl group is involved in the statin effect on leukocyte adhesion molecule expression, we determined whether FPP or GGPP could reverse the effects of statins. The enhancing effect of simvastatin or fluvastatin on TNF-α-induced E-selectin expression was reversed by GGPP and partially by FPP, indicating that the inhibition of protein isoprenylation, but not of cholesterol synthesis, is associated with the effect of these statins. Accordingly, statin effects on APLA-activated EC were reversed by GGPP as well. As FPP is a precursor for GGPP, but GGPP cannot function as a precursor for FPP, our results suggest that these statins act by inhibition of protein geranylgeranylation. These reversal effects of GGPP and FPP have been also shown by Sadeghi et al (2000). They observed an inhibition of IL-1-mediated adhesion molecule induction by direct G protein activator NAF and proposed that geranylgeranylated G proteins might be involved in the cytokine-induced

increase in adhesion molecule expression. Further support for the idea that the statin effect is on protein geranylgeranylation and not protein farnesylation is provided by our results which show that the geranylgeranyl transferase inhibitor (GGTI 286) mimicks the effect of simvastatin or fluvastatin in TNF-α or APLA activated EC, whereas the farnesyl transferase inhibitor FTI-277 had no such effect.

Taken together our results suggest that the enhancing effects of statins on cytokine-mediated adhesion molecule be due to an inhibition of protein geranylgeranylation. A large number of geranylgeranylated proteins are known. Most likely one (or more) of these proteins downregulates the adhesion molecule response of EC to TNF-α and APLA. The identification of such proteins may improve our understanding of the factors that modulate the response of EC to inflammatory mediators.

Our data also indicate that the beneficial effect of statins observed in clinical studies is probably not due to a suppression of the EC response to TNF-α, bacterial lipopolysaccharide or APLA. Several studies have shown that statin treatment could improve endothelial cell function in multiple ways, for example by decreasing the synthesis of endothelin-1, by increasing the synthesis of eNOS or the capacity of these cells to inhibit platelet adhesion and aggregation (Hernandez-Perera et al, 1998; Takemoto et al, 2001; Sposito et al, 2002; Seeger et al, 2000; Kaneider et al, 2002). The complexities of the anti-inflammatory effects of statins have been the subject of a recent review emphasizing that statins have also HMG-CoA reductase-independent effects, for example by a specific inhibition of lymphocyte-function-associated antigen 1 binding to ICAM-1 (Weitz-Schmidt et al, 2002).

Divergent results were also obtained when adhesion molecules were measured before and after statin treatment. Indeed some studies demonstrated a reduction of ICAM-1 after pravastatin (Blann et al, 2001) or fluvastatin (Romano et al, 2000) in hypercholesterolemic patients. However several other studies (Hackman et al, 1996; Rauch et al, 2000; Koh et al, 2000; Sardo et al, 2001) were unable to find a decrease of different adhesion molecules. Very recently, Jilma et al (2003) compared in a randomized trial the effects of three-month treatment with standard doses of atorvastatin, simvastatin and pravastatin on plasma levels of ICAM-1 and E-selectin in 75 hypercholesterolemic patients. None of the statins lowered plasma adhesion molecules.

At the present time, we can only speculate on the conflicting literature data on the effect of statins on the response of EC to inflammatory agents and APLA. It is known that statins inhibit farnesyl biosynthesis. Farnesyl is a precursor not only for cholesterol biosynthesis but also for protein farnesylation and geranylgeranylation and, as a precursor for dolichol, it is important for protein glycosylation. As an example there are already more than hundred small GTP binding proteins that depend for their biological activity on a farnesyl or geranylgeranyl anchor. Several of these small GTP binding proteins play key roles in gene expression and cytoskeletal organization. It is possible that among the different proteins that are affected by statins, some have a co-stimulatory effect on TNF-α and APLA treated cells, whereas others have an inhibitory effect. The balance between the stimulatory and inhibitory activities, and thus the effect of statin treatment, could depend on subtle differences in cell culture conditions, timing of addition of agents, subendothelial matrix or other experimental conditions.

In conclusion, our results show that statins potentiate the adhesive properties of endothelial cells in response to APLA. Our findings suggest that a geranylgeranylated protein limits the response of endothelial cells to APLA. The identification of this geranylgeranylated factor may lead to novel approaches to control the inflammatory activation of endothelial cells. Obviously these in vitro data do not exclude a possible favourable effect in vivo of statins in a clinical trial. However, if such a benefit is shown in APS patients, this should be explained by other mechanisms than by the inhibition of adhesion molecules by endothelial cells.

Figure 1

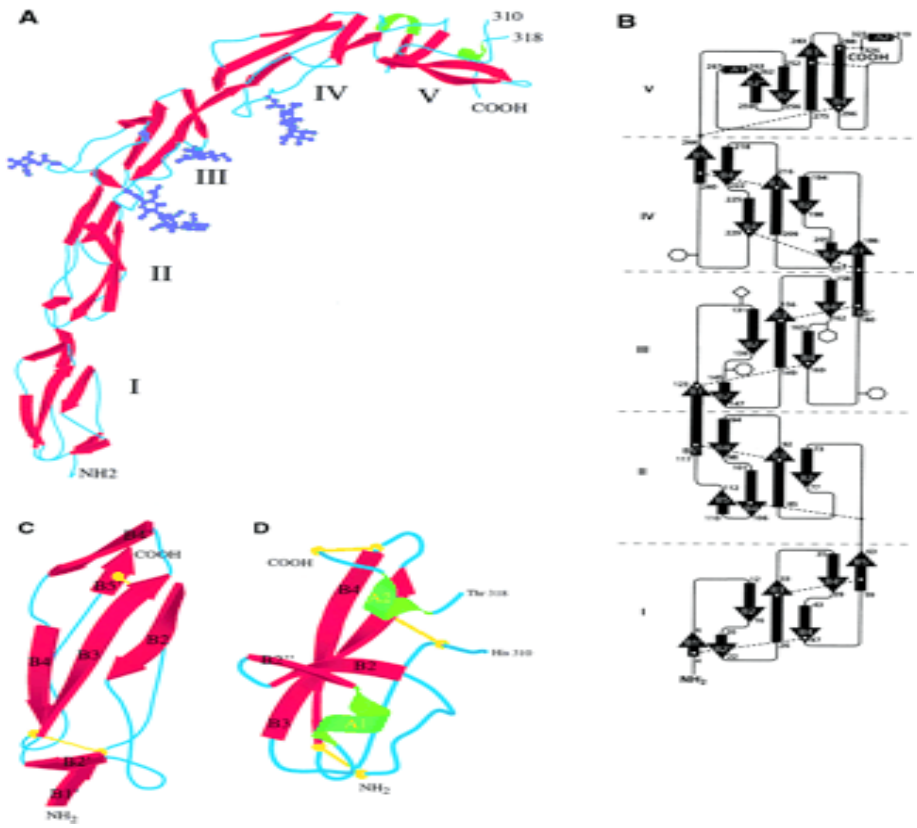


Figure 1. Structural representations of human blood plasma β_2GPI revealing the extended chain of the five SCR domains. (A) Ribbon drawing of β_2GPI with consecutive domains labelled I-V. N-linked glycans, as well as the position of the putative O-linked glycan, Thr130, are indicated by a ball-and-stick model. The strands are shown in red and helices in green. (B) Topology diagram of β_2GPI. The central sheets of all five domains are labelled B2(-B2)-B3-B4(-B5), the N- and C-terminal -sheets are labelled B1'-B2' and B4'-B5', the -helix and the 3/10 helix are denoted A1 and A2 and numbers of residues delimiting secondary structure elements are given. Disulfide bonds are indicated with dashed lines. The positions of N-glycosylation are given by hexagons; a diamond indicates the putative O-glycan. Horizontal dashed lines mark domain boundaries. (C) Ribbon representation of domain III of β_2GPI with labelled secondary structure elements. The two fully conserved disulfide bonds are shown in yellow. (D) Ribbon representation of domain V of β_2GPI with labelled secondary structure elements. The three disulfide bonds are indicated with yellow lines. The aberrant face, which contains the membrane-binding site, is located on the right-hand side.

EMBO 1999; 18: 5166

Figure 2

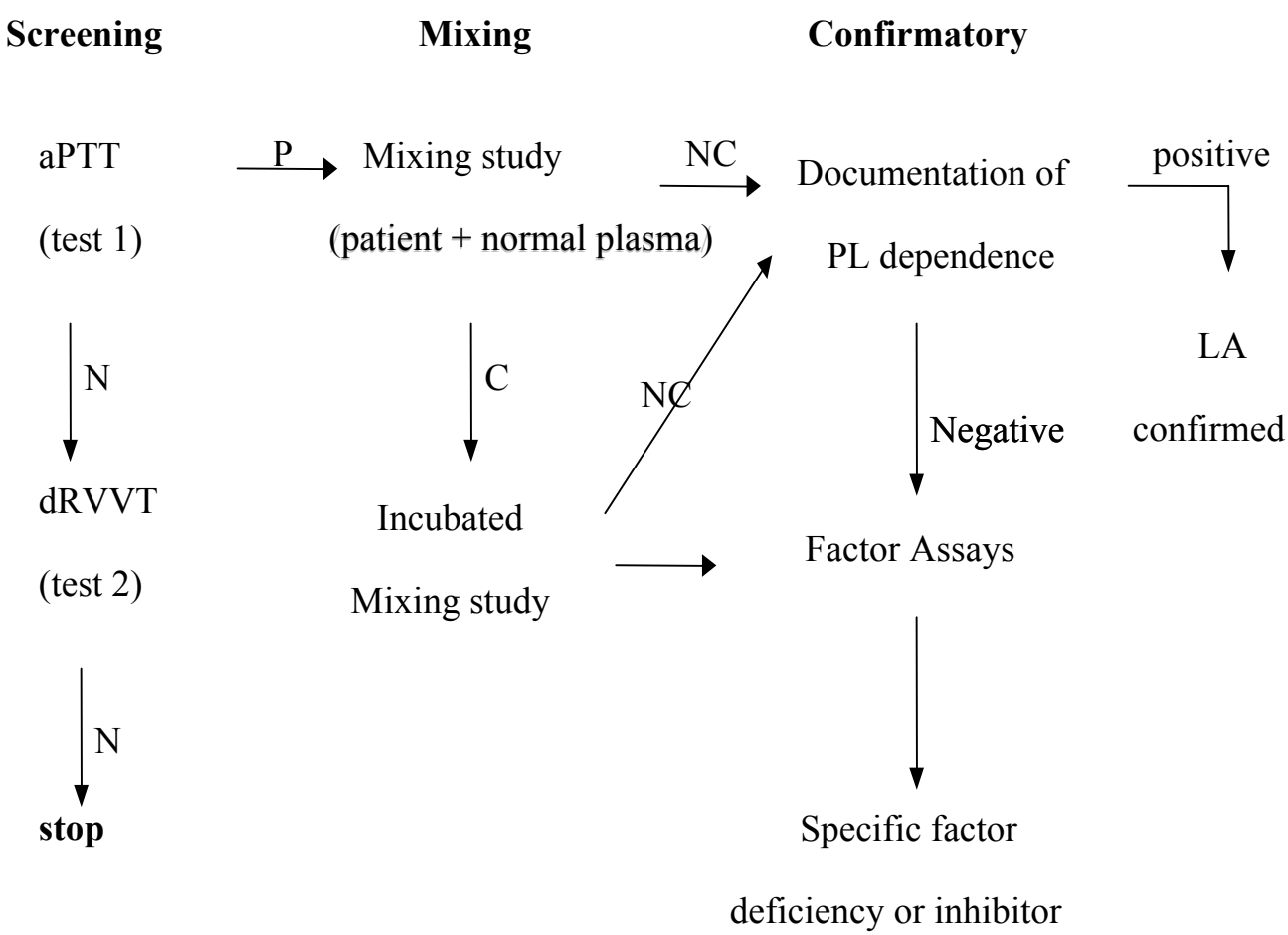


Figure 2. Approach to the diagnosis of LA. More information: Guidelines of the International Society of Thrombosis and Haemostasis. Tests need to be repeated on 2 occasions, at least 6 weeks apart.

N: normal, P: prolonged, C: correction, NC: no correction

Figure 3

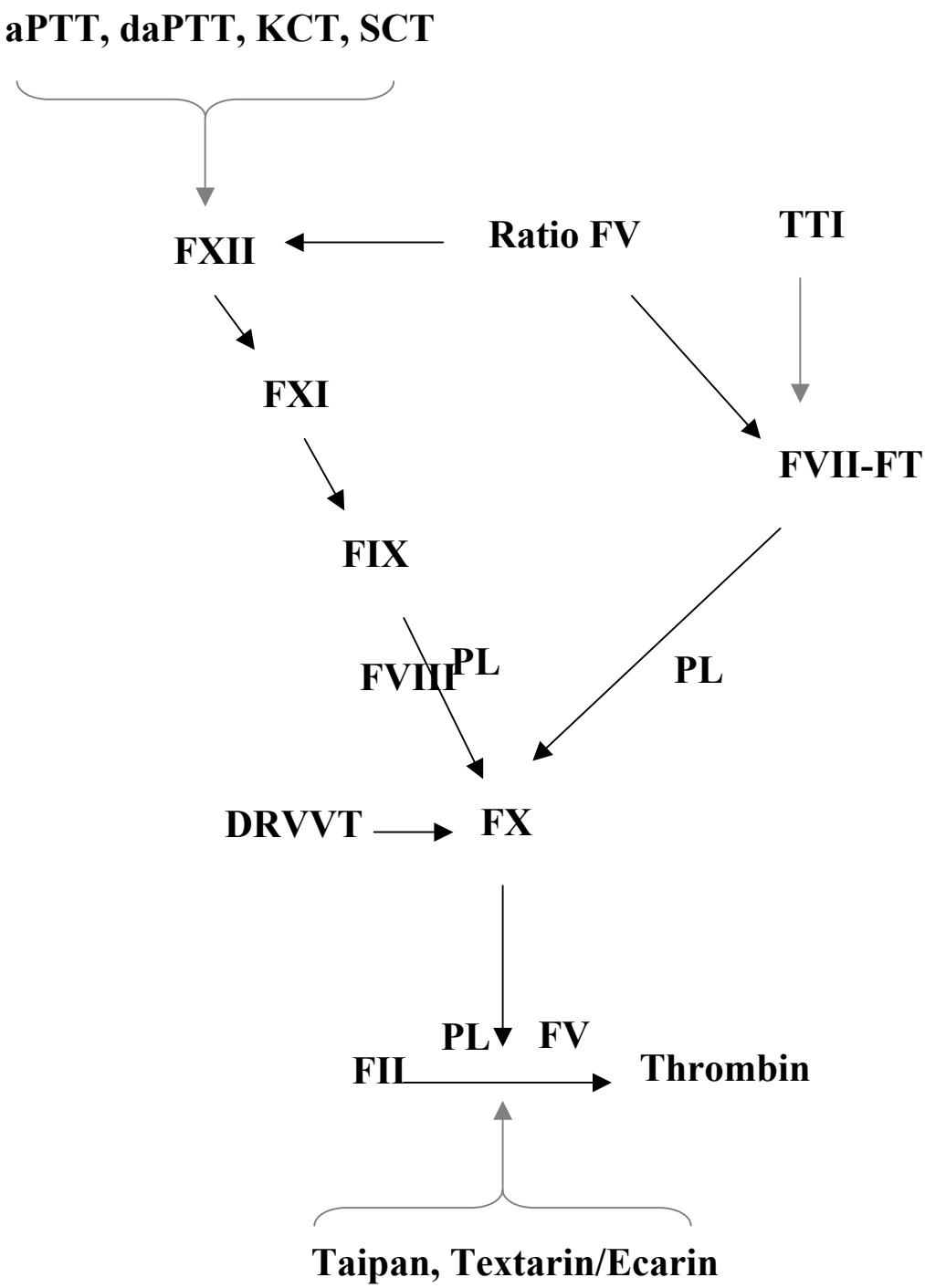


Figure 3. Sites of action of the essays in the cascade

PL: phospholipids, aPTT: PL:activated partial thromboplastin time, daPTT: diluteactivated partial thromboplastin time, KCT: kaolin clotting time, SCT: silica clotting time, DRVVT : dilute Russell's viper venom time, TTI: tissue thromboplastin inhibition

Figure 4

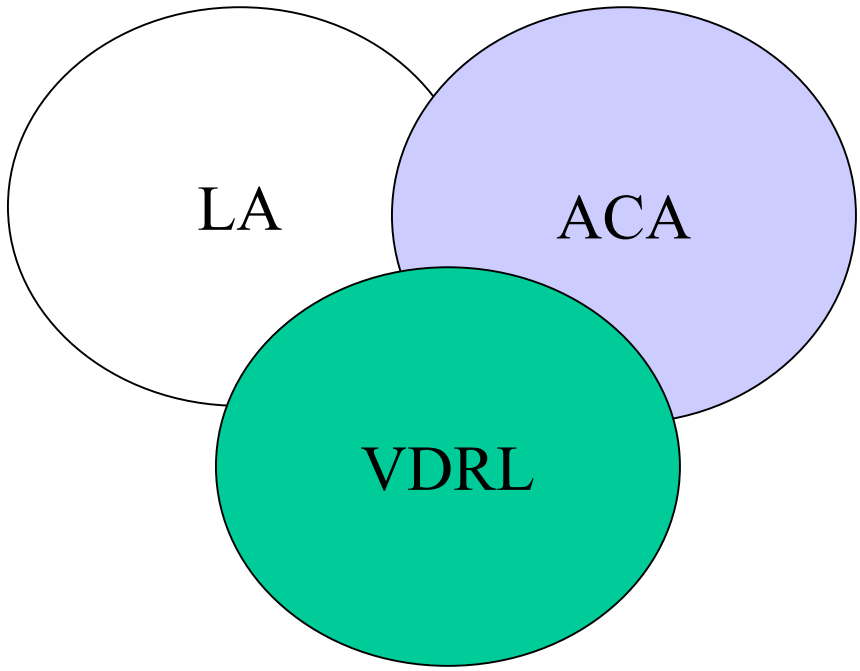


Figure 4. Coexistence or independent occurrence of LA, ACA and VDRL antibodies in APS.

LA: lupus anticoagulant, ACA: anticardiolipin antibodies, VDRL: antibodies reacting with the Veneral Disease Research Laboratory reagent

Figure 5

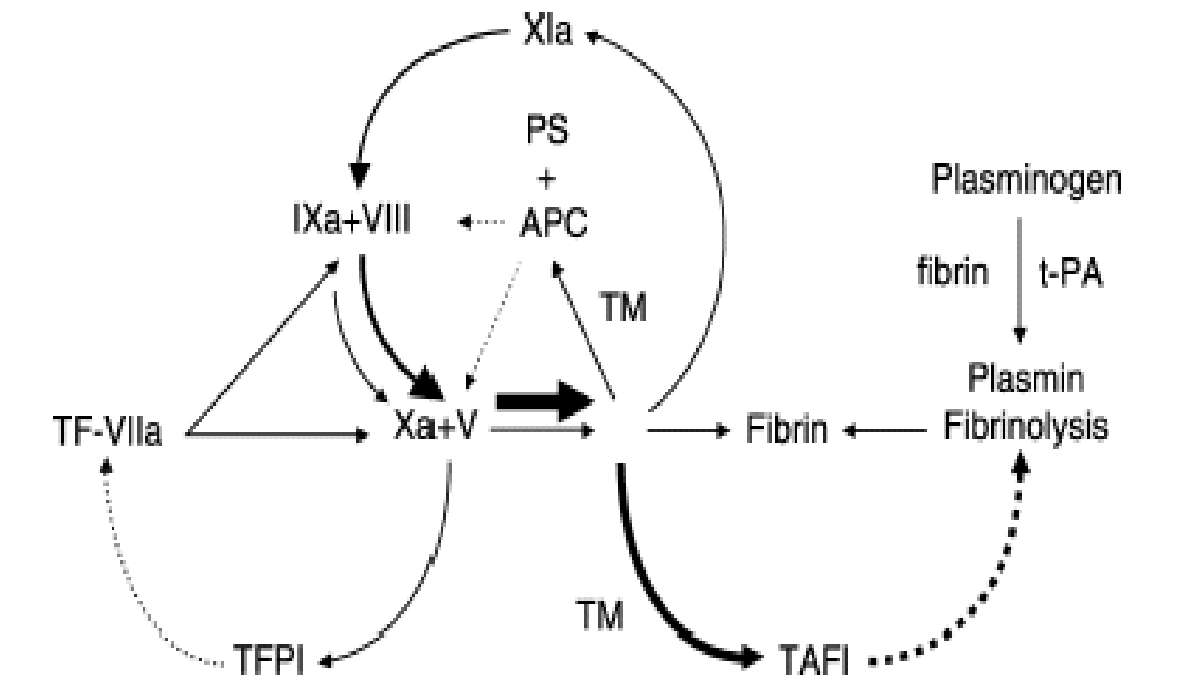


Figure 5. Model of blood coagulation.

TF-VIIa: tissue factor-FVIIa complex, TFPI: tissue factor pathway inhibitor, APC: activated protein C, PS: protein S, T: thrombomodulin, TAFI: thrombin-activatable fibrinolysis inhibitor, t-PA: tissue-type plasminogen activator. An uninterrupted line indicates activation, while an interrupted line indicates inactivation. The uninterrupted line between Xa and TFPI indicates that FXa has to form a complex with TFPI, and that this complex then inhibits TF-FVIIa.

J Thromb Haemost 2003; 1: 1566

Figure 6

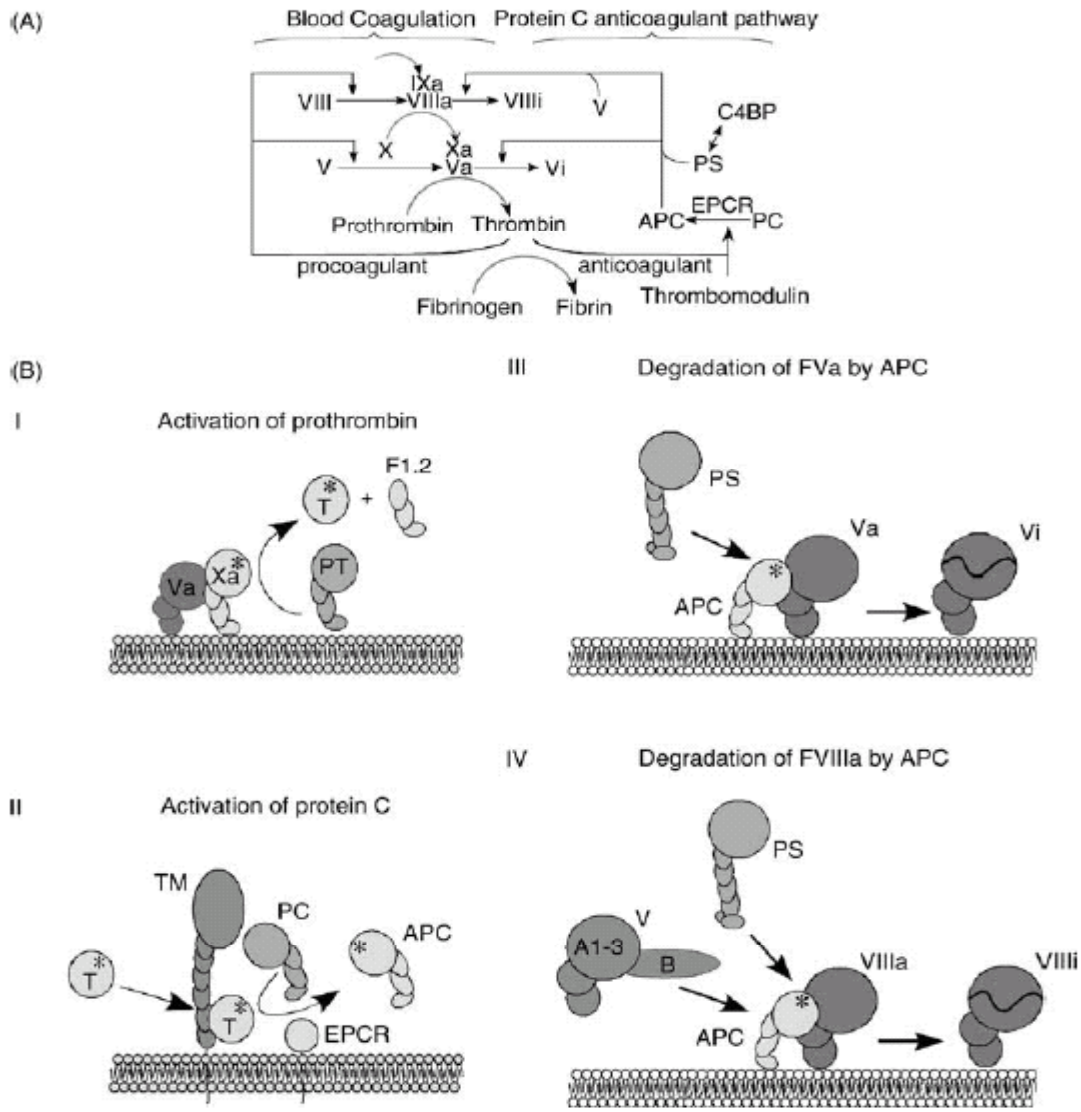


Figure 6. Schematic representation of blood coagulation and the protein C anticoagulant system.

Section A demonstrates an incomplete scheme of blood coagulation reactions together with the balancing anticoagulant reactions of the protein C pathway. In section B (I–IV), the membrane-bound molecular events of selected reactions are shown in cartoon-like fashion. I: activation of prothrombin (PT) to thrombin (T), a reaction that also generates the F1.2 prothrombin fragment. II: thrombomodulin (TM) and the endothelial protein C receptor (EPCR) are proteins that span the membrane. The role of EPCR is not fully understood, but it has been shown to be able to bind the Gla-domain of protein C, which results in stimulation of protein C activation. III: the degradation of FVa by APC is enhanced by protein S (PS). IV: degradation of FVIIIa by APC is stimulated by the synergistic cofactor activity of protein S and factor V. The large B domain that protrudes from the triangularly arranged A1–A3 domains of FV is in the linear sequence located between A2 and A3 domains.

Figure 7

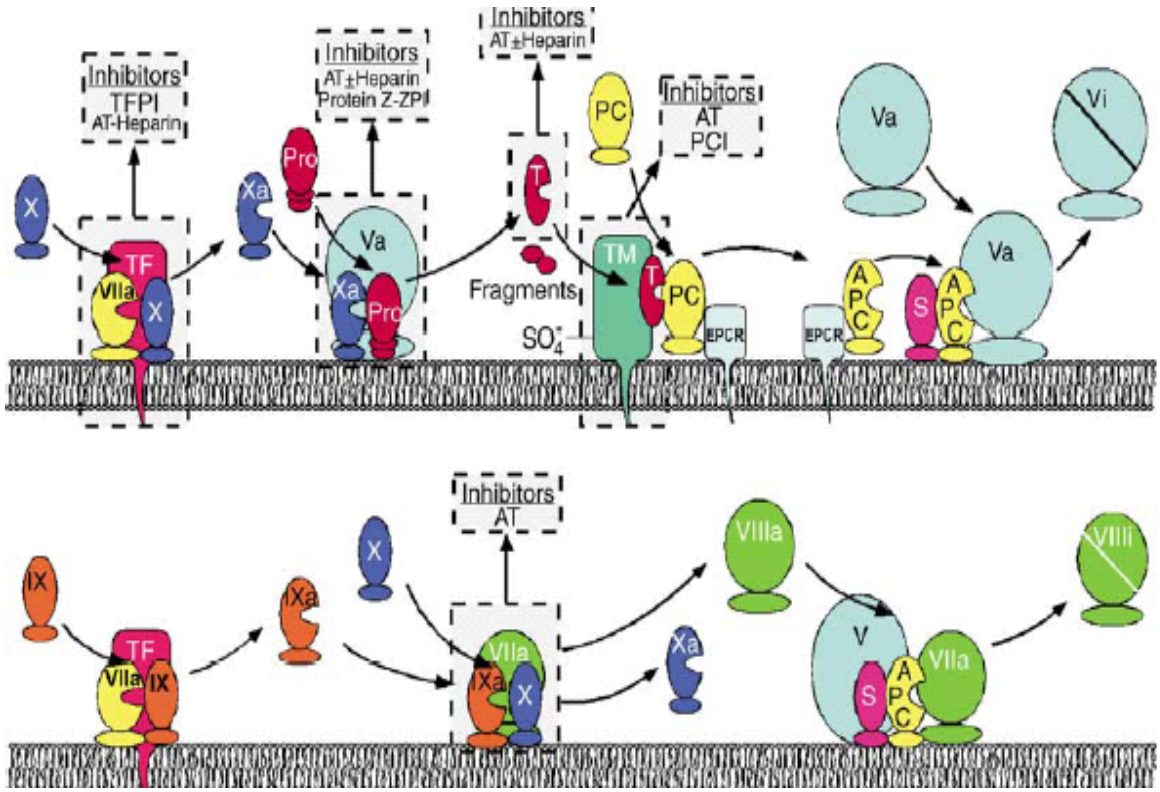


Figure 7. Regulation of blood clotting.

Inhibitors that control coagulation are shown in gray boxes above the complex or factor they regulate (AT: antithrombin, ZPI: protein Z-dependent protease inhibitor, PCI: protein C inhibitor). Top panel: FVIIa binds to tissue factor (TF) to activate FX, generating FXa. FXa then binds to FVa. The complex of FXa–FV converts prothrombin to thrombin (T). Thrombin can then either bind to TM or carry out procoagulant reactions like fibrin formation or platelet activation. When bound to TM, thrombin can activate protein C (PC) to APC. This process is enhanced when protein C is bound to the EPCR. APC bound to EPCR cleaves substrates other than FVa. APC dissociates from EPCR and can then interact with protein S to inactivate FVa. Bottom panel: The FIXa–FVIIIa complex is inactivated by APC. In this case, FV participates with APC and protein S in the inactivation of FVIIIa. For simplicity, the activation of FVII, FV and FVIII are not shown.

J Thromb Haemost 2003; 1: 1343

Figure 8

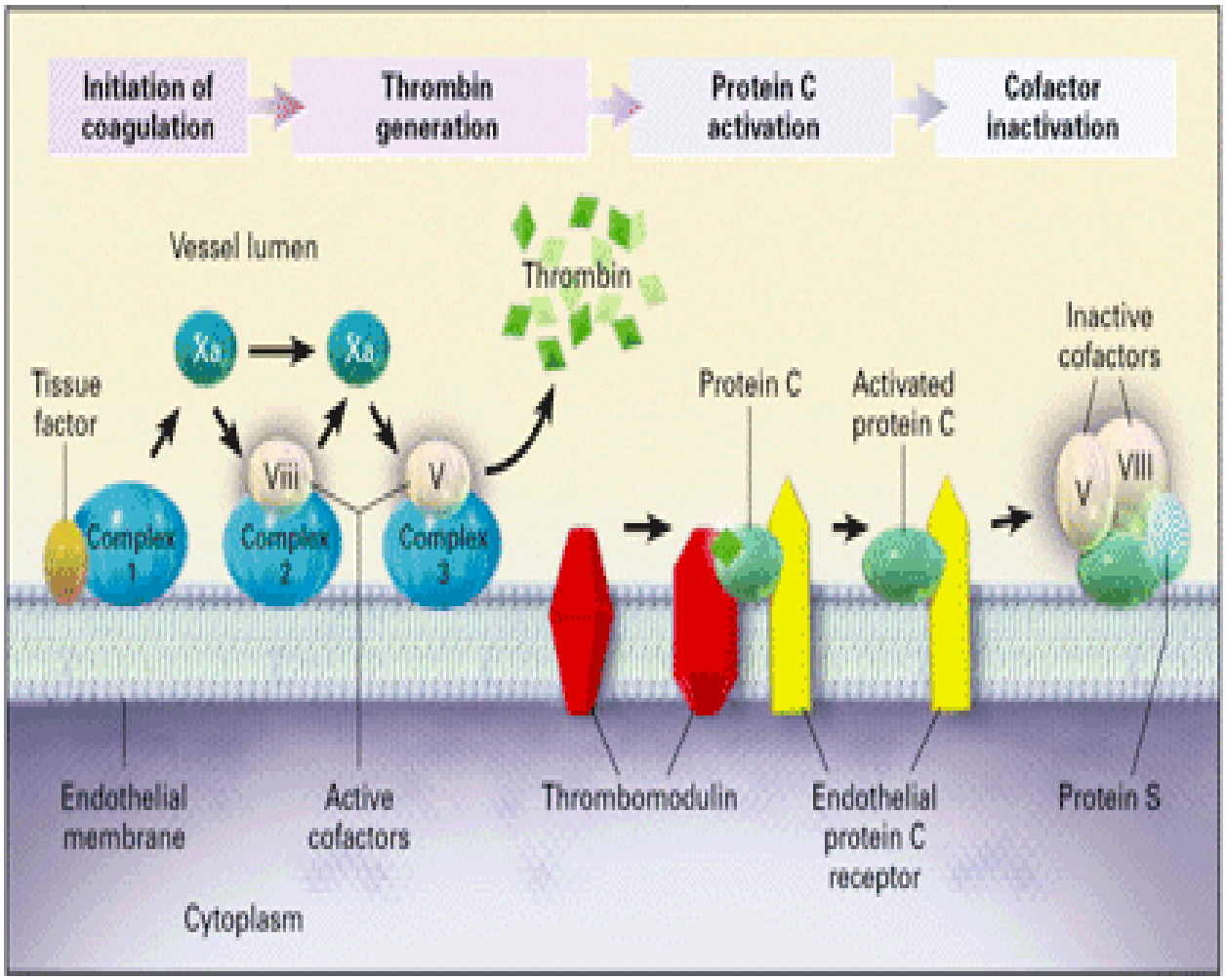


Figure 8. Protein C Pathway
Protein C activation takes place by way of interaction between the thrombomodulin–thrombin complex and the endothelial protein C receptor. Activated protein C, together with its cofactor, protein S, inactivates factors V and VIII to provide negative feedback to the generation of thrombin. Complex 1 comprises TF and coagulation factors VII, IX and X; complex 2 comprises factors IX and X and cofactor VIII; and complex 3 comprises factor X, prothrombin and cofactor V.

N Engi J Med 2001; 345: 409

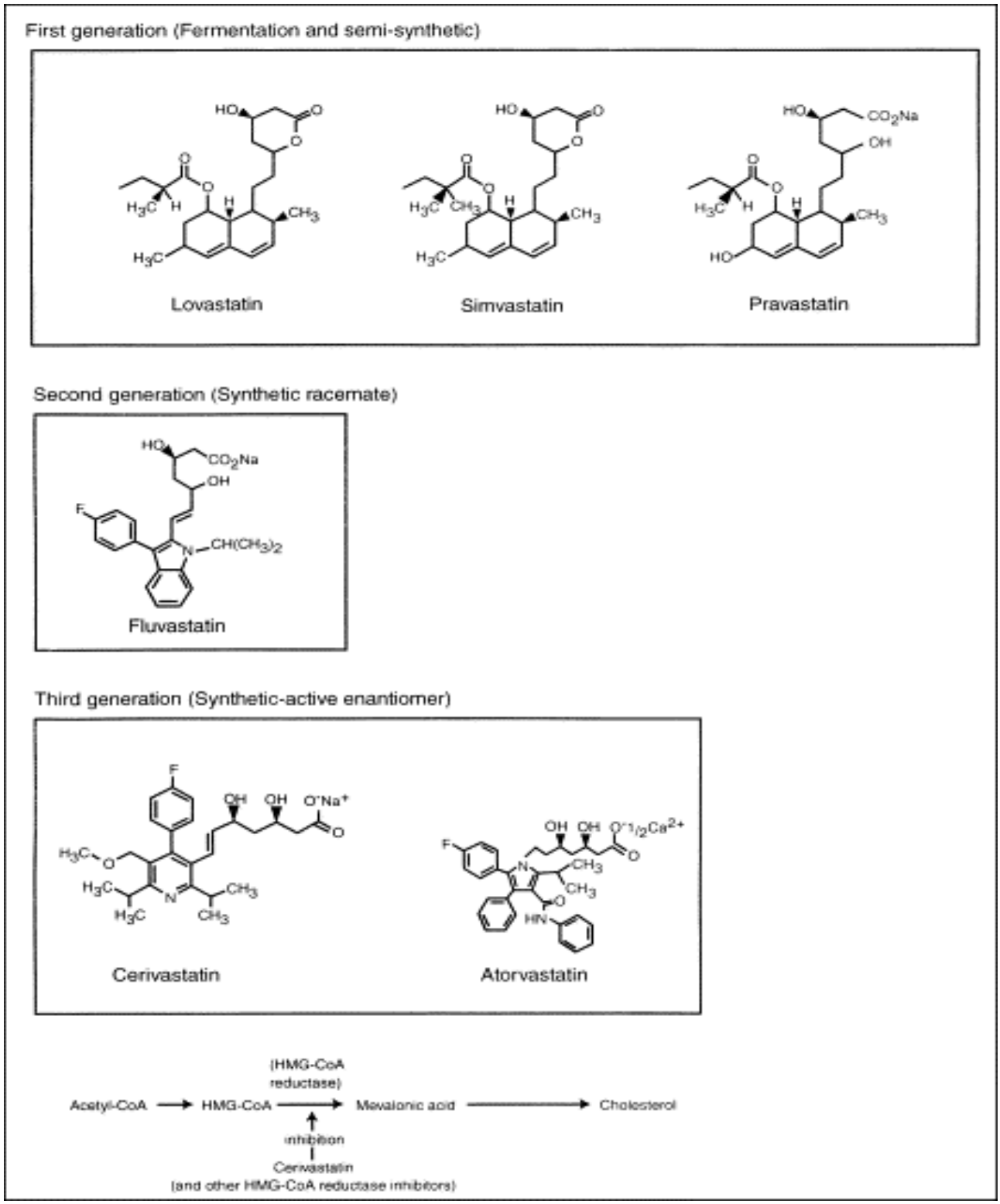


Figure 9. Structural formulas of statins and their site of action in the cholesterol biosynthetic pathway

HMG-CoA = 3-hydroxy-3-methylglutaryl coenzyme A.

Figure 10

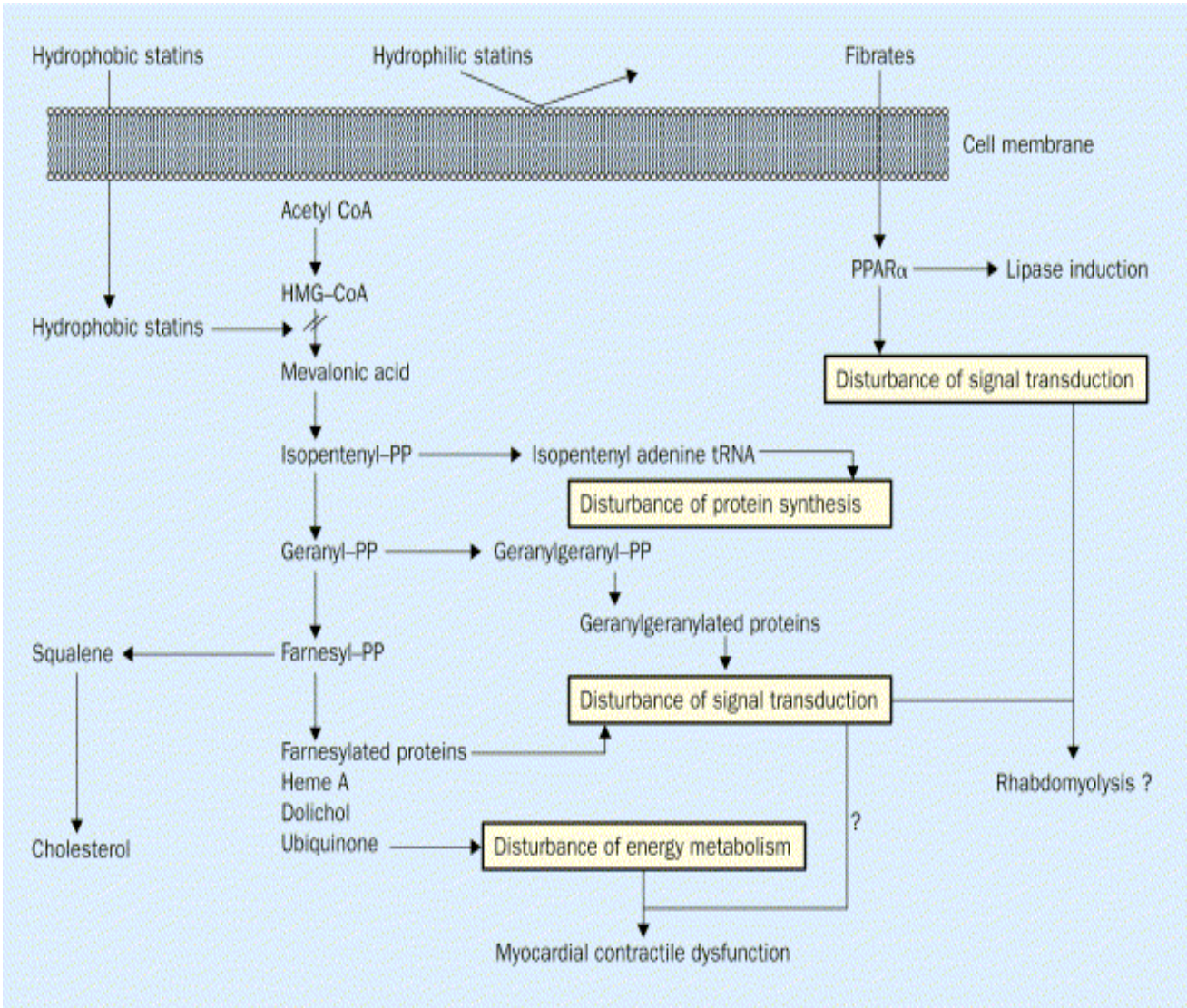


Figure 10. The mevalonic pathway and possible effects of hydrophilic and hydrophobic statins. In liver cells hydrophilic statins gain access by a receptor mediated process

Lancet 2002; 359: 2195

Figure 11

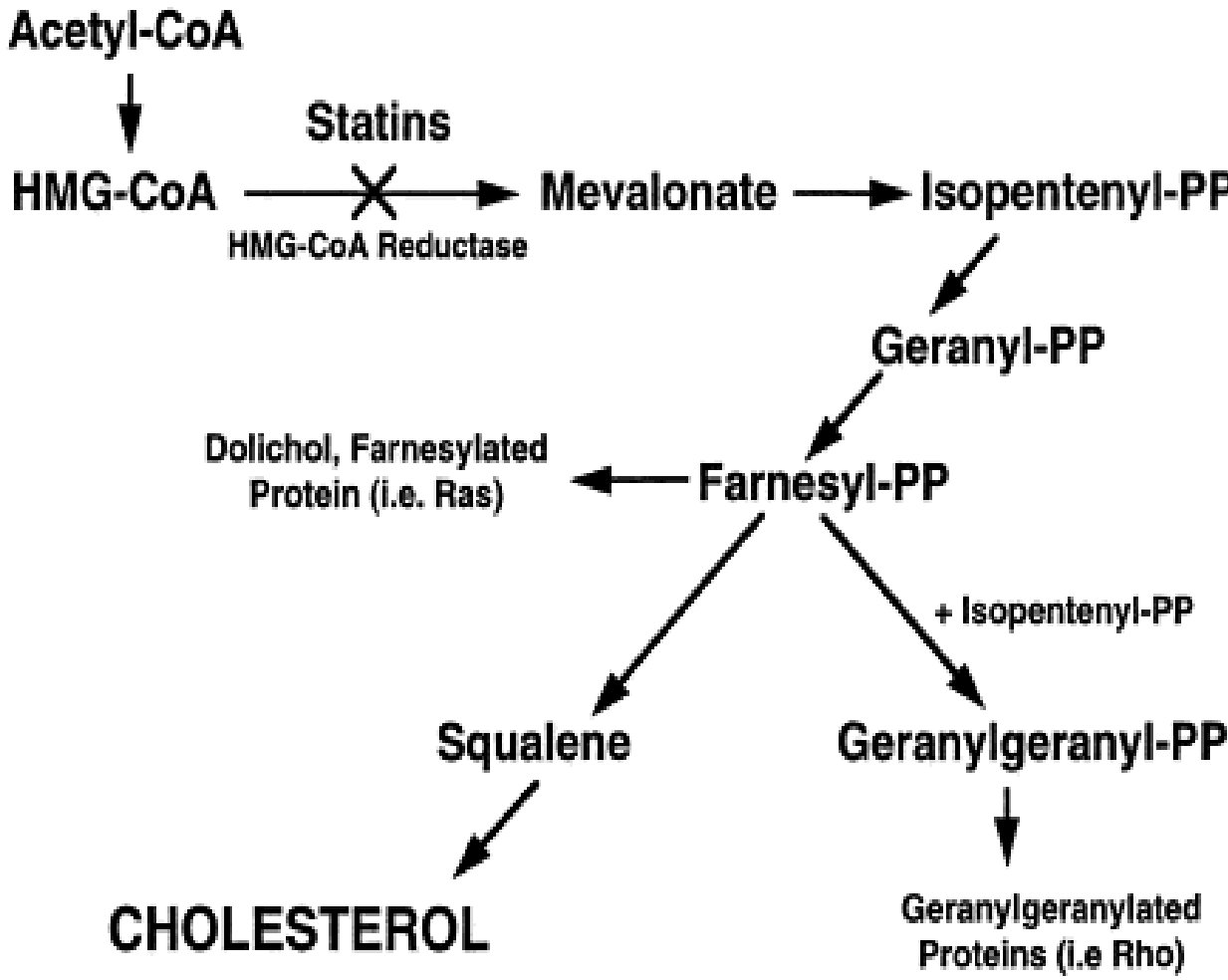


Figure 11. Pathway for cholesterol biosynthesis. Inhibition of HMG-CoA reductase by statins decreases the synthesis of isoprenoids and cholesterol

Trends Cardiovasc Med 2000; 10: 143

Figure 12a

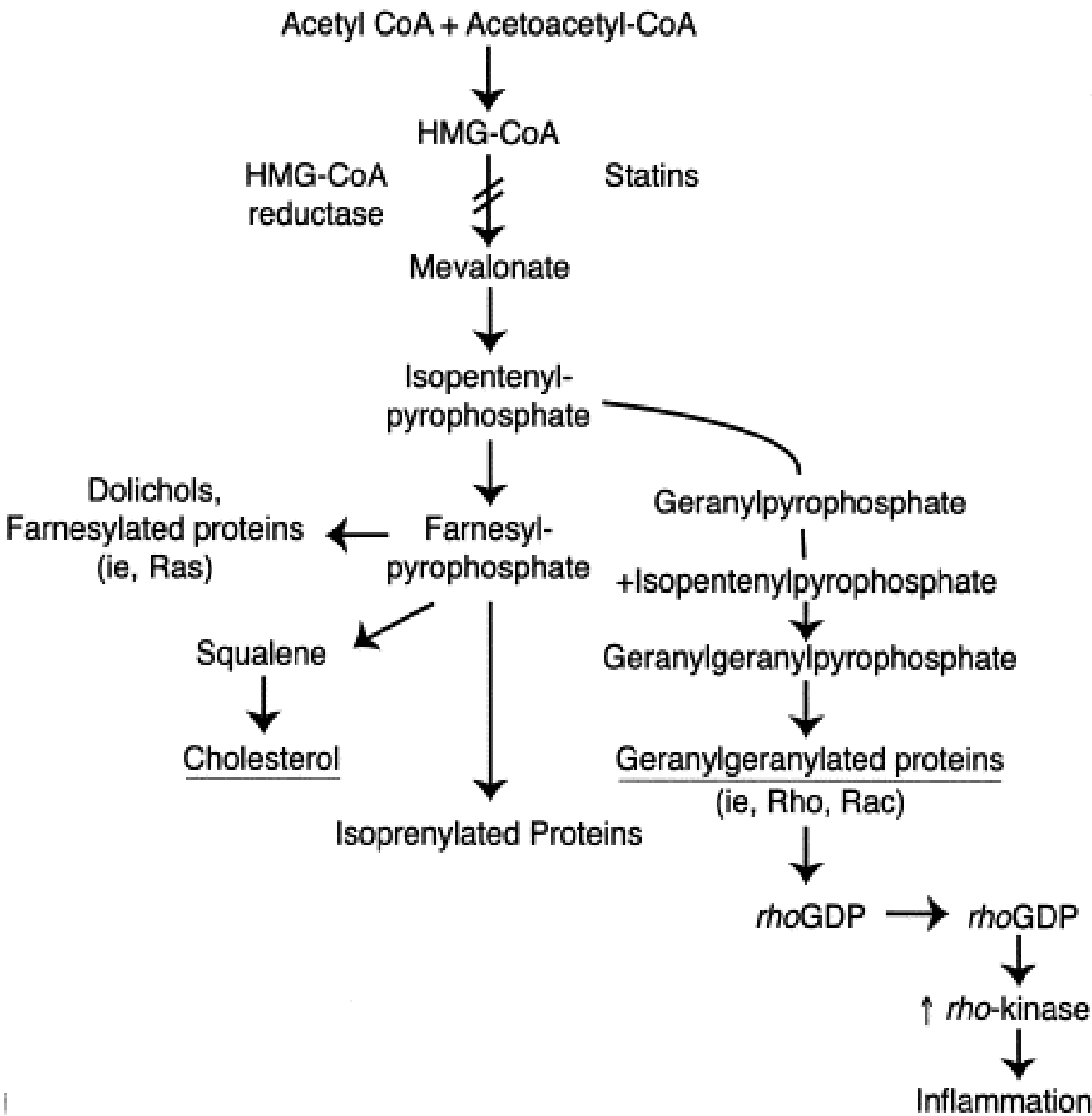


Figure 12a. Effects of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibition (statins) on biosynthesis of cholesterol and isoprenoids. Many pleiotropic effects of statins are thought to be mediated by a reduction in protein isoprenylation

Am J Cardiol 2003; 20: 91

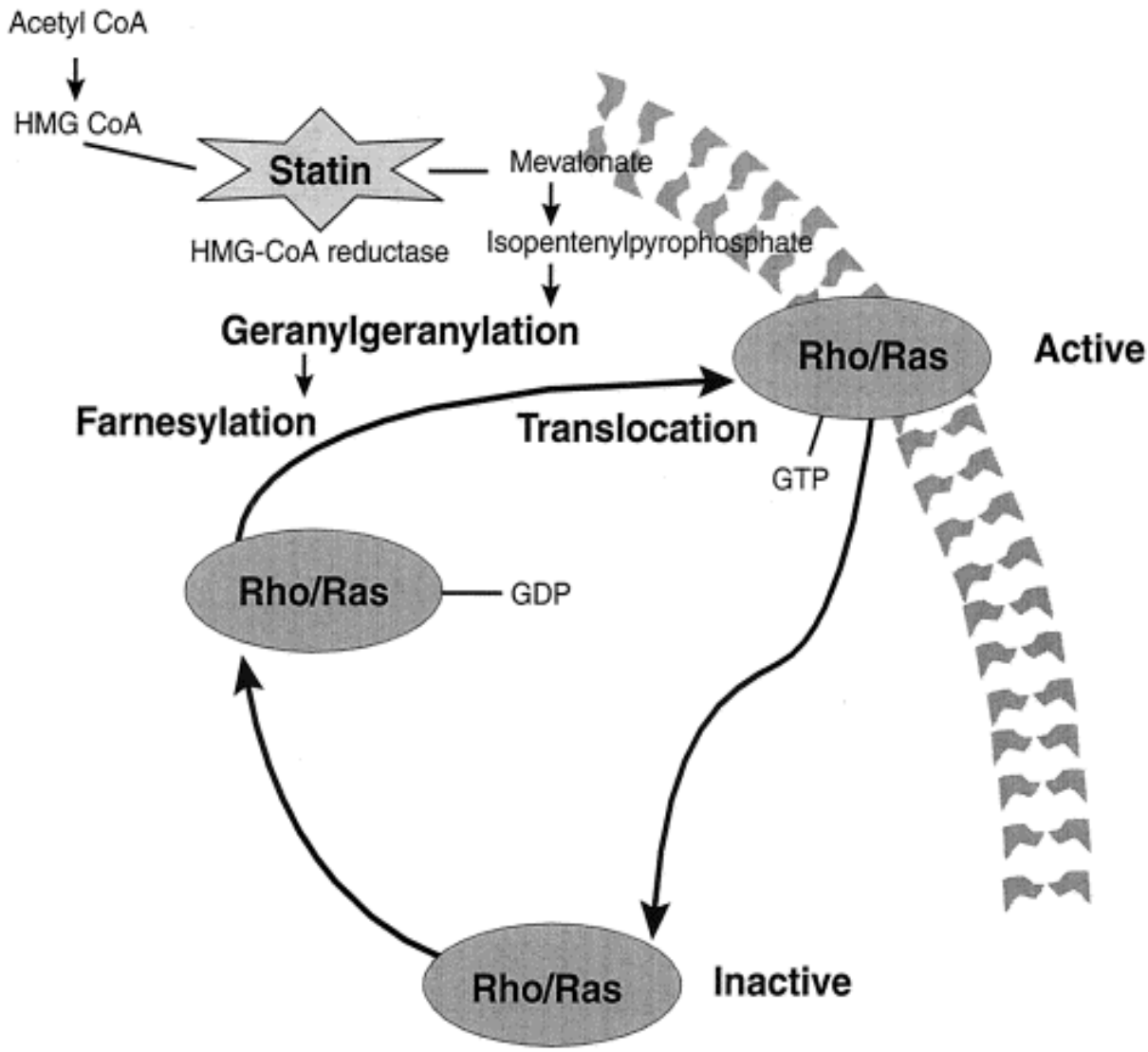


Figure 12b. Inhibitory effects of statins on small G-proteins. Isoprenylation is a necessary step in activation of members of the Rho and Ras families. Statins decrease the synthesis of isoprenoid intermediates, thereby preventing activation.

CoA: coenzyme A; GDP: guanosine diphosphate; GTP: guanosine triphosphate; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A

Am J Cardiol 2003; 20: 91

Figure 13

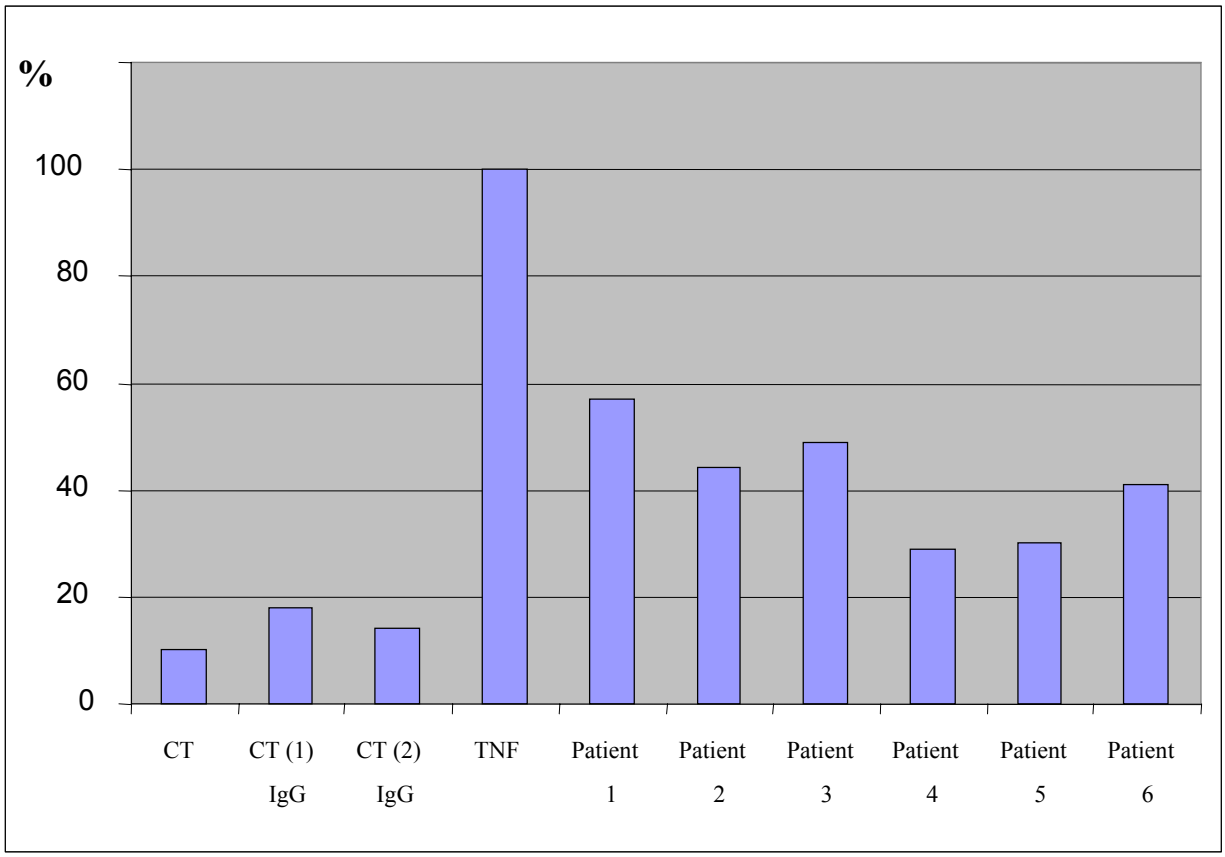


Figure 13. Flow cytometry analysis of APLA-induced VCAM-1 expression Confluent HUVEC were incubated with purified patient APLA (500 μg/ml) or TNF-α 10 ng/ml for 24 hours and surface VCAM-1 adhesion molecules were measured by flow cytometry analysis. Mean value for VCAM-1 expression induced by TNF-α was taken for 100%. The results were expressed as a percentage of mean value of TNF-α 10 ng/ml treated cells.

Table 9

Simvastatin (μM)	E-selectin Mean +/- S.D		VCAM-1 Mean +/- S.D	
	Simvastatin	Simvastatin with 400 μM mevalonate	Simvastatin	Simvastatin with 400 μM mevalonate
0	100*	102 ± 3§	100*	105 ± 3§
0.1	150 ± 21*	119 ± 15§	118 ± 23*	89 ± 13§
0.5	178 ± 20*	96 ± 10§	140 ± 60*	86 ± 20§
1	223 ± 36*	125 ± 10§	154 ± 62*	117 ± 38§
2.5	186 ± 40*	109 ± 10§	156 ± 64*	119 ± 55§

Table 9. Cell ELISA analysis of simvastatin effect on TNF-α induced E-selectin and VCAM-1 expression
Mean values (n=5) for each simvastatin concentration were expressed as a percentage of mean O.D. value of TNF-α 10 ng/ml treated cells without simvastatin. * $P<0.001$ for E-selectin and VCAM-1 expression mediated by simvastatin. § $P<0.001$ for mevalonate

Table 10

Simvastatin (μM)	E-selectin Mean +/- S.D		VCAM-1 Mean +/- S.D	
	Simvastatin	Simvastatin with 400 μM mevalonate	Simvastatin	Simvastatin with 400 μM mevalonate
0	100	102 ± 3	100	105 ± 3
0.1	150 ± 21	119 ± 15	118 ± 23	89 ± 13
0.5	178 ± 20	96 ± 10	140 ± 60	86 ± 20
1	223 ± 36	125 ± 10	154 ± 62	117 ± 38
2.5	186 ± 40	109 ± 10	156 ± 64	119 ± 55

Table 10. Flow cytometry analysis of simvastatin effect on TNF-α induced E-selectin and VCAM-1 expression.
Mean values (n=10) for each simvastatin concentration were expressed as a percentage of mean value of TNF-α 10 ng/ml treated cells without simvastatin.

Figure 14

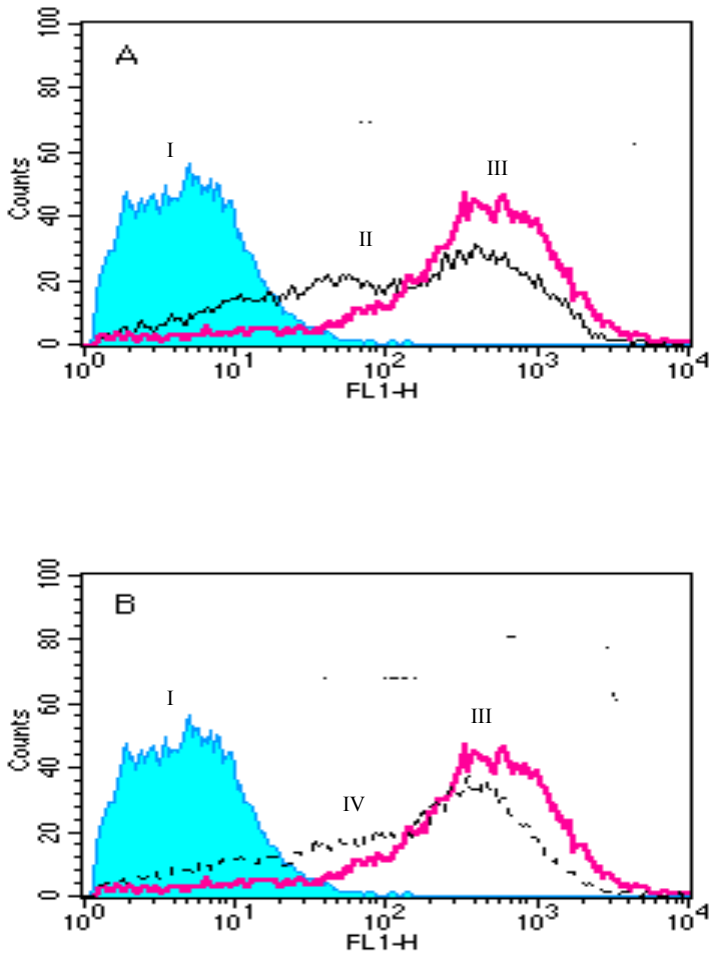


Figure 14. The effect of simvastatin on TNF-α-induced E-selectin expression (A) and its reversal by pretreatment with mevalonate (B). Confluent HUVEC were pretreated overnight with 2.5 μM simvastatin (A) or 2.5 μM simvastatin with 400 μM mevalonate (B). TNF-α (10 ng/ml) was added to the medium for 4 hours and E-selectin expression at the cell surface was measured by flow cytometry analysis as described in the Methods section. I represents E-selectin expression in control HUVEC; II represents E-selectin expression after TNF-α treatment; III represents TNF-α induced E-selectin expression after an overnight preincubation with simvastatin; and IV represents the TNF-α induced E-selectin expression after an overnight preincubation with simvastatin and mevalonate.

Dimitrova et al. J Thromb Haemost, in press 2003

Figure 15

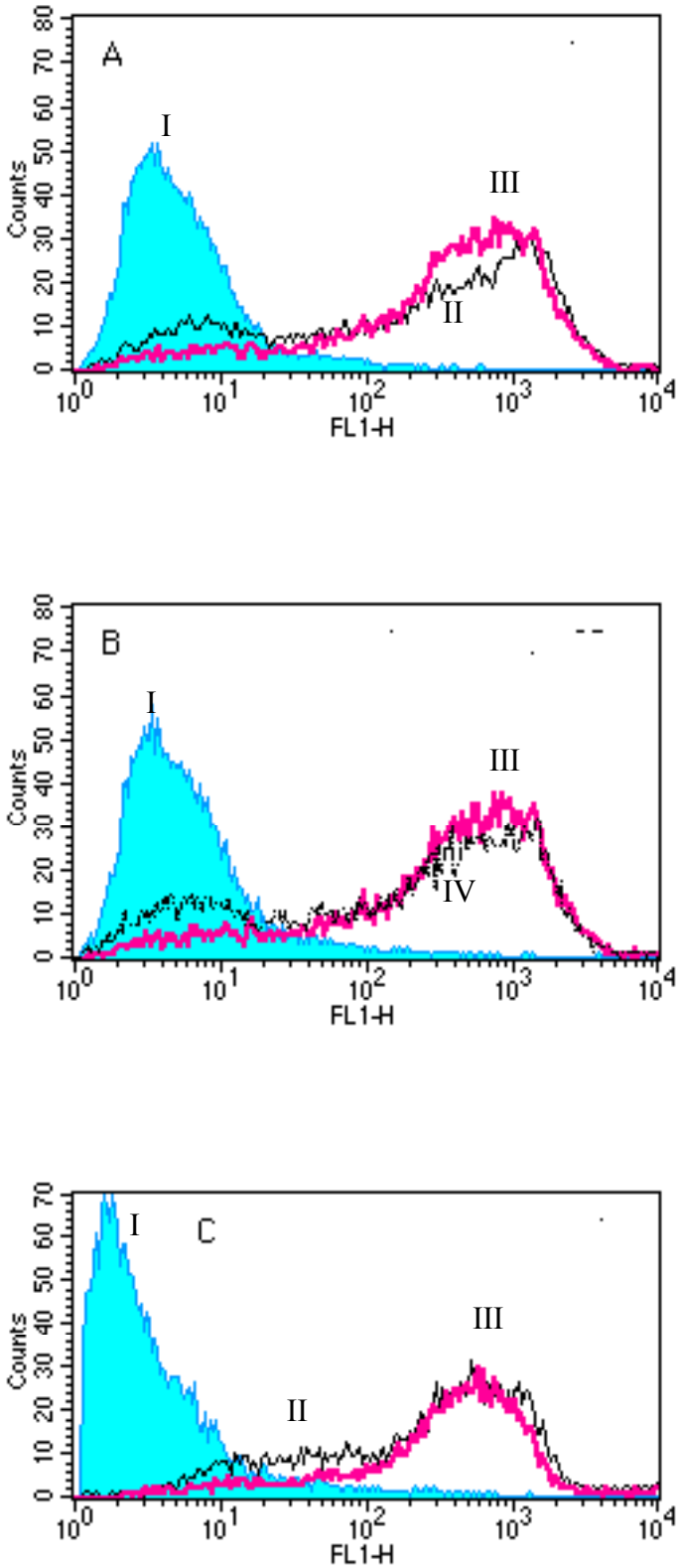


Figure 15. The effect of simvastatin on TNF-α-induced VCAM-1 expression on HUVEC (A), or on human saphenous vein EC (C), and the reversal of simvastatin effect by mevalonate on HUVEC (B). Confluent HUVEC were pretreated overnight with 1 μM simvastatin (A), or 1 μM simvastatin with 400 μM mevalonate (B). TNF-α (10 ng/ml) was added to the medium for 6 hours and VCAM-1 expression at the cell surface was measured by flow cytometry analysis. The same experiment was performed with human saphenous vein EC (C). I represents VCAM-1 expression in control HUVEC/saphenous vein; II represents VCAM-1 expression after TNF-α treatment; III represents TNF-α induced VCAM-1 expression after an overnight preincubation with simvastatin; and IV represents VCAM-1 expression after TNF-α and an overnight preincubation with simvastatin and mevalonate.

Dimitrova et al. J Thromb Haemost, in press 2003

Figure 16

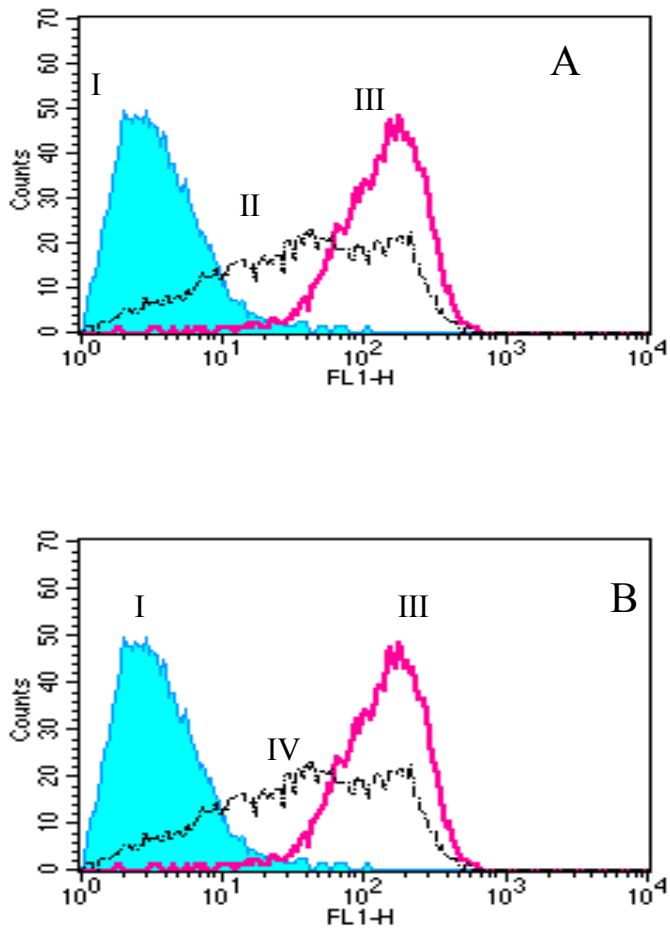


Figure 16. The effect of fluvastatin on TNF-α mediated E-selectin expression. Confluent HUVEC were pretreated overnight with 5 μM fluvastatin (A), 5 μM fluvastatin with 400 μM mevalonate (B). TNF-α (10 ng/ml) was added to the medium for 4 hours and surface E-selectin adhesion molecules were measured by flow cytometry analysis as described in the Methods section. I represents E-selectin expression in control HUVEC, II represents E-selectin expression after TNF-α, III- E-selectin expression of fluvastatin pretreated TNF-α stimulated cells, and IVrepresents E-selectin expression after overnight preincubation with fluvastatin + mevalonate and stimulation with TNF-α.

Dimitrova et al. J Thromb Haemost, in press 2003

Figure 17

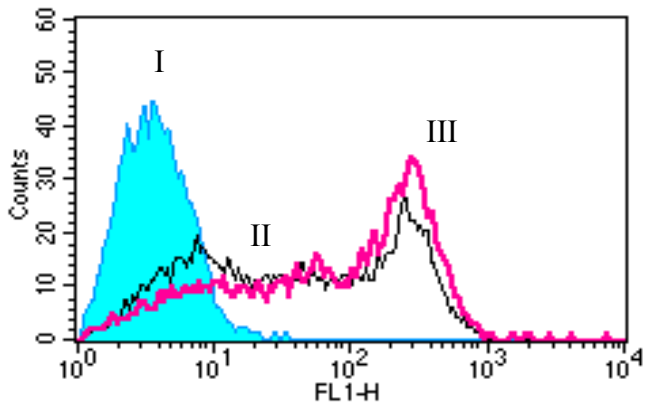


Figure 17. The effect of pravastatin on TNF-α mediated E-selectin expression. Confluent HUVEC were pretreated overnight with 15 μM pravastatin. TNF-α (10 ng/ml) was added to the medium for 4 hours and surface E-selectin adhesion molecules were measured by flow cytometry analysis as described in the Methods section. I represents E-selectin expression in control HUVEC, II represents E-selectin expression after TNF-α, III- E-selectin expression of pravastatin pretreated TNF-α stimulated cells

Figure 18

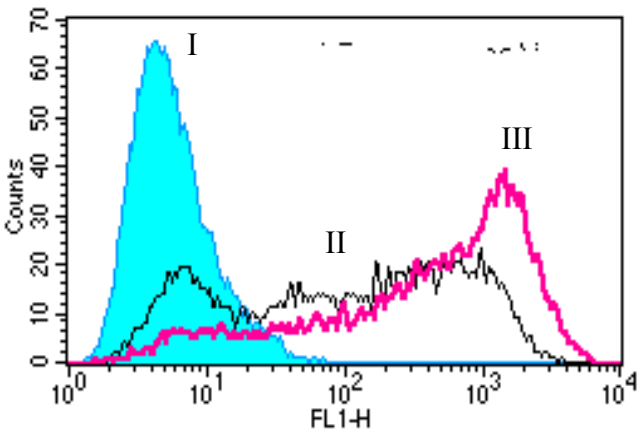


Figure 18. The effect of simvastatin on LPS mediated E-selectin expression. Confluent HUVEC were pretreated overnight with 2.5 μM simvastatin. LPS (20 ng/ml) was added to the medium for 4 hours and surface E-selectin adhesion molecules were measured by flow cytometry analysis. I represents E-selectin expression in control HUVEC, II represents E-selectin expression after LPS, III- E-selectin expression of simvastatin pretreated LPS stimulated cells.

Figure 19

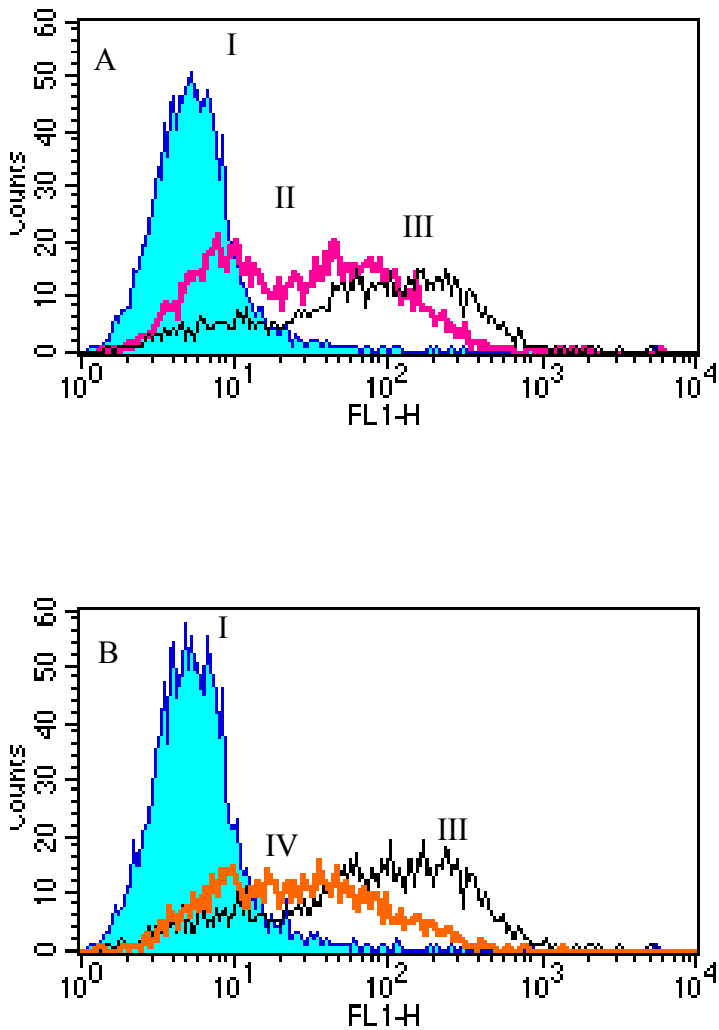
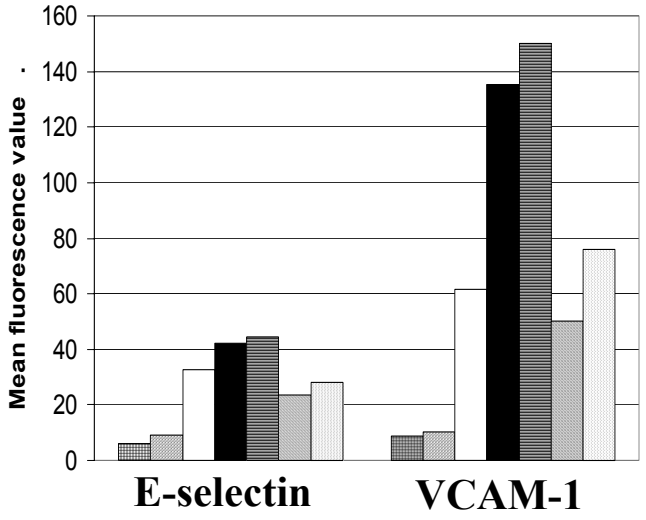


Figure 19. The effect of fluvastatin on APLA-induced VCAM-1 expression. Confluent HUVEC were pretreated overnight with 5 μM fluvastatin. Purified patient APLA (500 μg/ml) was added to the medium for 24 hours and surface VCAM-1adhesion molecules were measured by flow cytometry analysis. I represents VCAM-1 expression in control HUVEC, II represents VCAM-1 expression after APLA incubation, III represents VCAM-1 expression of fluvastatin pretreated APLA stimulated cells (the figure represents the results with APLA obtained from serum of patient 1).

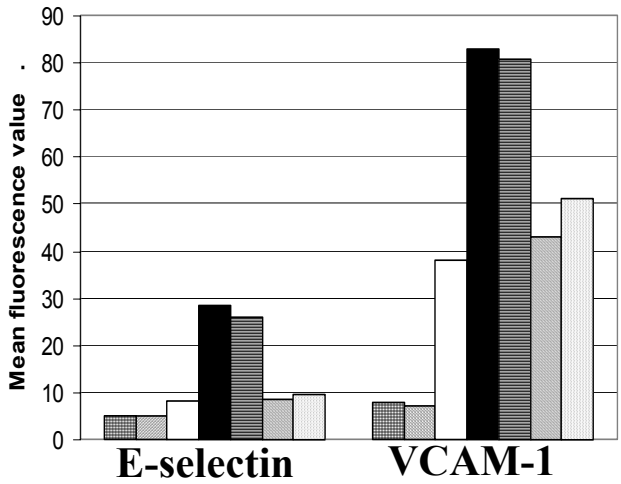
Figure 20

Patient 1



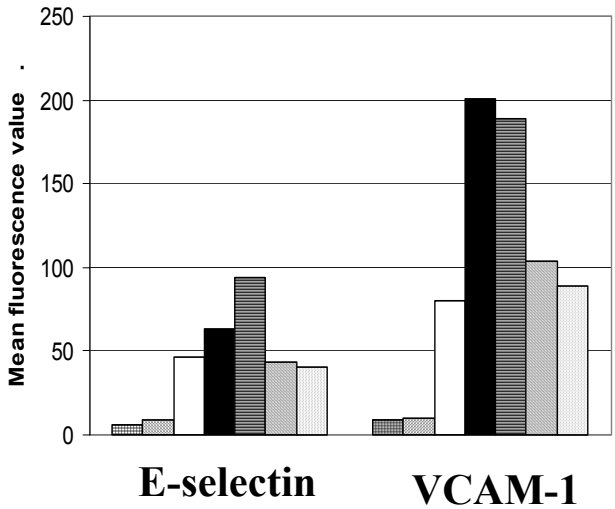
	E-selectin							VCAM-1						
CT IgG	–	+	–	–	–	–	–	–	+	–	–	–	–	–
APLA	–	–	+	+	+	+	+	–	–	+	+	+	+	+
5μM fluvastatin	–	–	–	+	–	s	+	–	–	–	+	–	+	+
10μM GGTI	–	–	–	–	+	–	–	–	–	–	–	+	–	–
400μM mevalonate	–	–	–	–	–	+	–	–	–	–	–	–	+	–
15μM GGPP	–	–	–	–	–	–	+	–	–	–	–	–	–	+

Patient 3



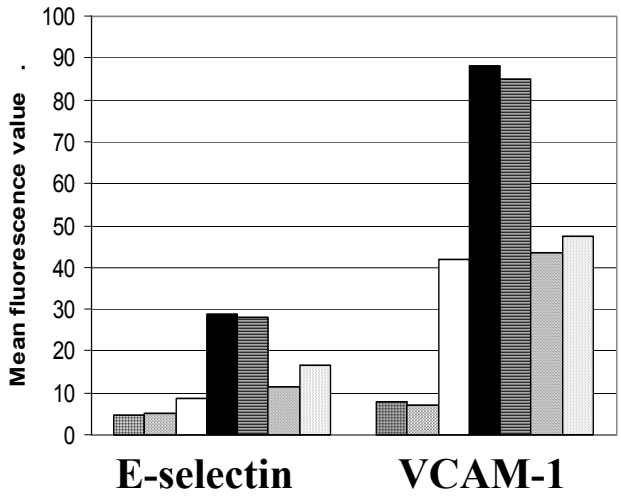
	E-selectin							VCAM-1						
CT IgG	–	+	–	–	–	–	–	–	+	–	–	–	–	–
APLA	–	–	+	+	+	+	+	–	–	+	+	+	+	+
5μM fluvastatin	–	–	–	+	–	s	+	–	–	–	+	–	+	+
10μM GGTI	–	–	–	–	+	–	–	–	–	–	–	+	–	–
400μM mevalonate	–	–	–	–	–	+	–	–	–	–	–	–	+	–
15μM GGPP	–	–	–	–	–	–	+	–	–	–	–	–	–	+

Patient 2



	E-selectin							VCAM-1						
CT IgG	–	+	–	–	–	–	–	–	+	–	–	–	–	–
APLA	–	–	+	+	+	+	+	–	–	+	+	+	+	+
5μM fluvastatin	–	–	–	+	–	s	+	–	–	–	+	–	+	+
10μM GGTI	–	–	–	–	+	–	–	–	–	–	–	+	–	–
400μM mevalonate	–	–	–	–	–	+	–	–	–	–	–	–	+	–
15μM GGPP	–	–	–	–	–	–	+	–	–	–	–	–	–	+

Patient 4



	E-selectin							VCAM-1						
CT IgG	–	+	–	–	–	–	–	–	+	–	–	–	–	–
APLA	–	–	+	+	+	+	+	–	–	+	+	+	+	+
5μM fluvastatin	–	–	–	+	–	+	+	–	–	–	+	–	+	+
10μM GGTI	–	–	–	–	+	–	–	–	–	–	–	+	–	–
400μM mevalonate	–	–	–	–	–	+	–	–	–	–	–	–	+	–
15μM GGPP	–	–	–	–	–	–	+	–	–	–	–	–	–	+

Figure 20

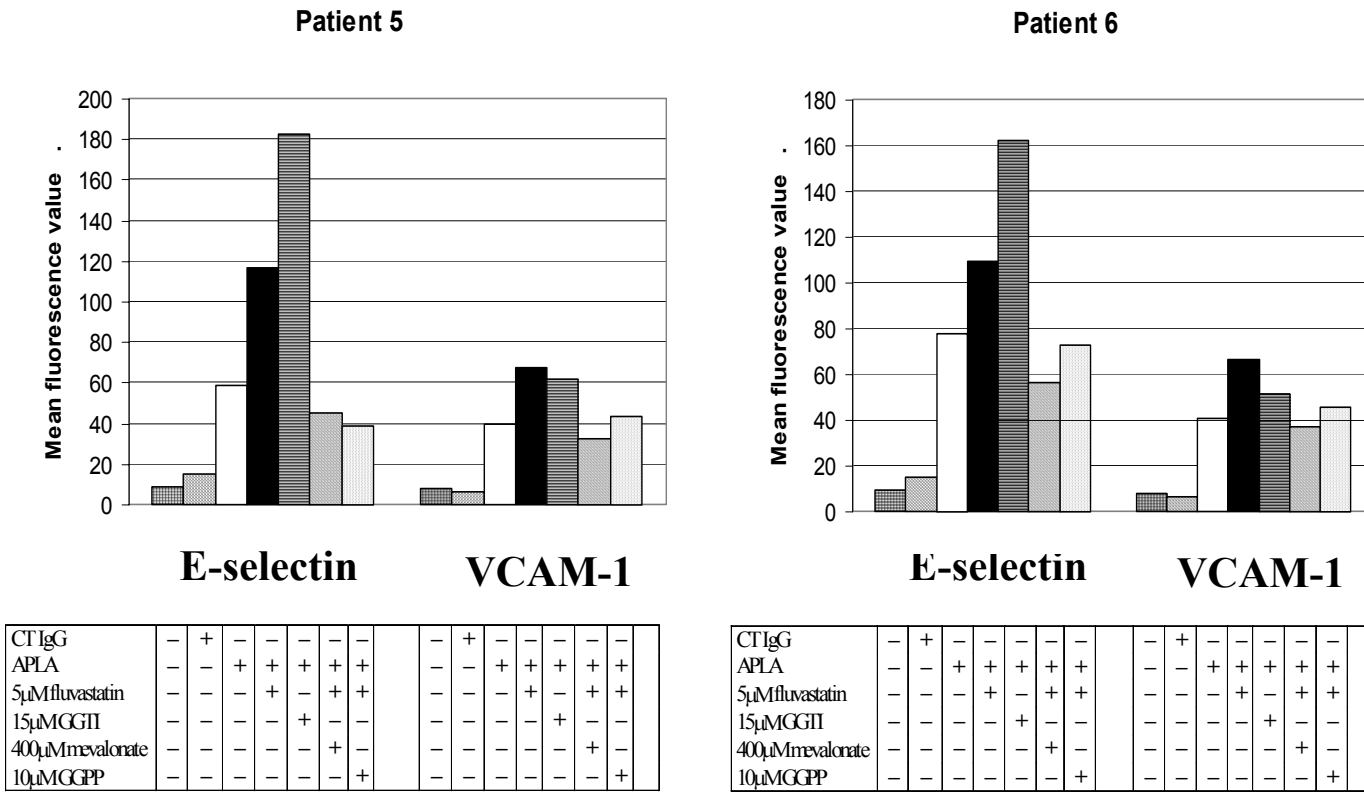


Figure 20. The effect of fluvastatin and GGTI-286 on APLA-induced VCAM-1 expression and its reversal by mevalonate and GGPP. Confluent HUVEC were pretreated overnight with 5 μM fluvastatin, or 5 μM fluvastatin with 400 μM mevalonate, or 5 μM fluvastatin with 15 μM GGPP, or 10 μM GGTI-286 . Purified patient APLA (500 μg/ml) were added to the medium for 24 hours and surface VCAM-1 adhesion molecules were measured by flow cytometry analysis.

Figure 21

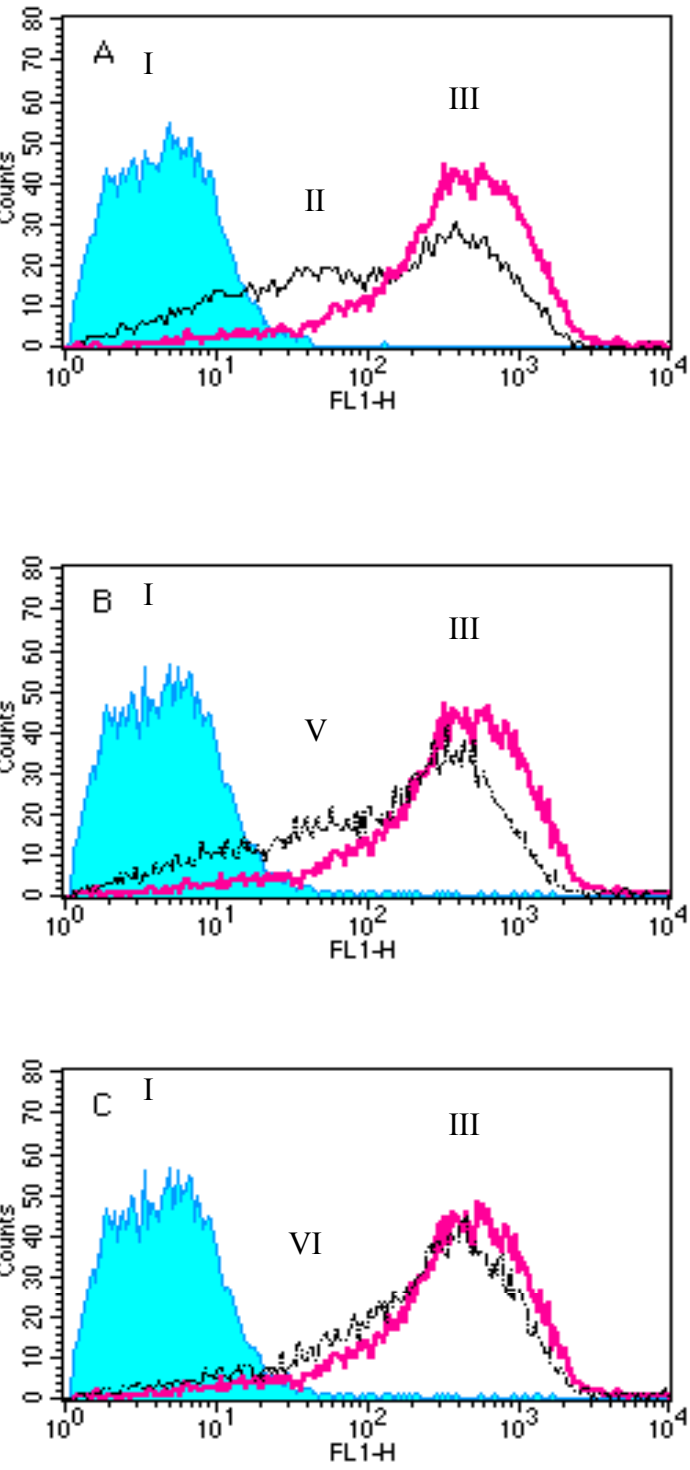


Figure 21. Inhibition by the isoprenoid intermediates farnesyl pyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) of the potentiating effect of simvastatin on TNF-α induced E-selectin expression. Fig. 21A shows the effect of simvastatin on TNF-α induced E-selectin expression, Fig. 21B shows the effect of GGPP on simvastatin pretreated and TNF-α stimulated HUVEC and Fig. 21C shows the effect of FPP on simvastatin pretreated and TNF-α stimulated HUVEC, compared to the effect of GGPP. Confluent HUVEC were pretreated overnight with 2.5 μM simvastatin alone, or in the presence of 15 μM GGPP or 15 μM FPP. Cells were incubated with TNF-α (10 ng/ml) for 4 hours and E-selectin expression was measured by flow cytometry analysis. I represents E-selectin expression on control HUVEC; II represents E-selectin expression after TNF-α stimulation; III represents expression of E-selectin on simvastatin pretreated TNF-α stimulated cells; V represents the TNF-α induced expression of E-selectin after an overnight preincubation with simvastatin + GGPP; and VI represents the TNF-α induced E-selectin expression after preincubation with simvastatin + FPP.

Dimitrova et al. J Thromb Haemost, in press 2003

Figure 22

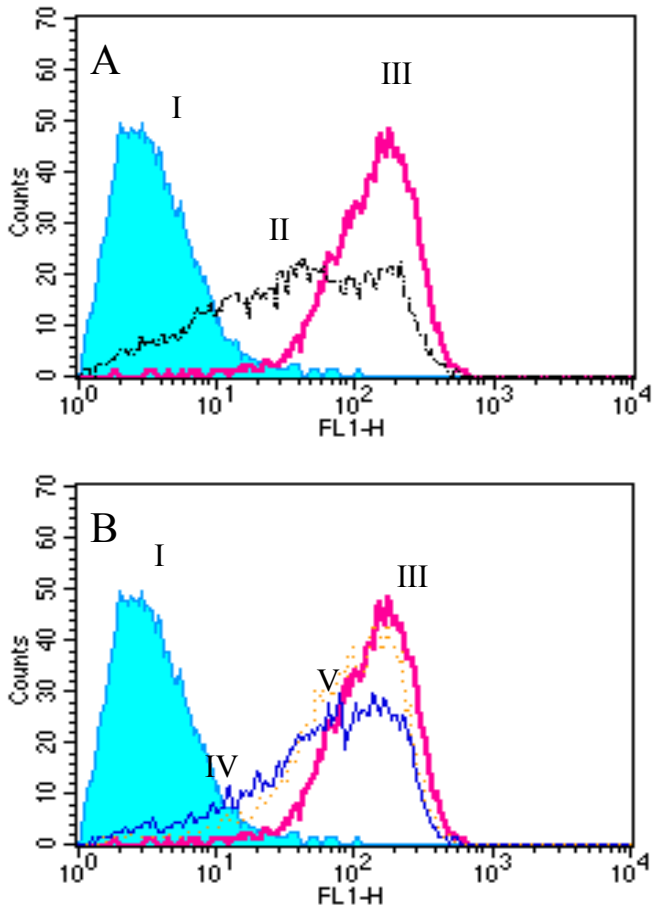


Figure 22. The effect of fluvastatin on TNF-α mediated E-selectin expression (A) and its reversal by GGPP or FPP (B). Confluent HUVEC were pretreated overnight with 5 μM fluvastatin (A), or 5 μM fluvastatin with 15 mM GGPP or 15 mM FPP (B). TNF-α (10 ng/ml) was added to the medium for 4 hours and E-selectin expression on the cell surface was measured by flow cytometry analysis as described in the Methods section. I represents E-selectin expression on control HUVEC; II represents E-selectin expression after TNF-α treatment; III represents the E-selectin expression of fluvastatin pretreated and TNF-α stimulated cells; IV represents the TNF-α induced expression of E-selectin after an overnight preincubation with fluvastatin + GGPP, and V represents TNF-α induced E-selectin expression after an overnight preincubation with fluvastatin + FPP.

Dimitrova et al. J Thromb Haemost, in press 2003

Figure 23

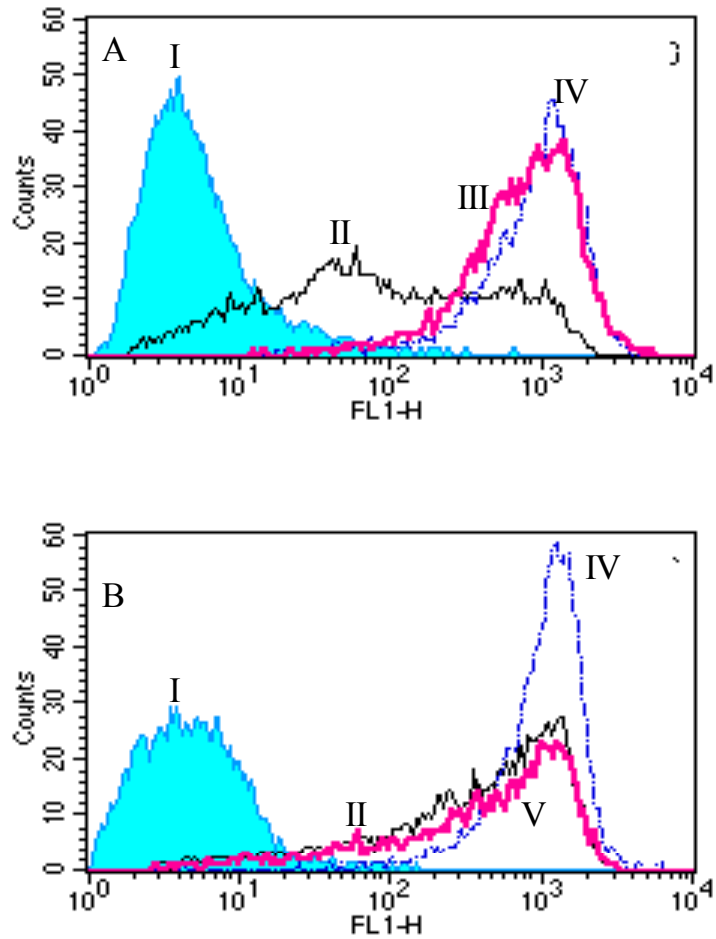


Figure 23. Effect of inhibitors of the farnesyltransferase inhibitor-277 (FTI-277) or the geranylgeranyltransferase inhibitor-286 (GGTI-286) on TNF-α induced adhesion molecules expression. Fig. 23A demonstrates the effect of GGTI-286 on TNF-α induced E-selectin expression, and Fig. 23B shows the effect of FTI-277 on TNF-α induced E-selectin expression, compared with the effect of GGTI-286. Confluent HUVEC were pretreated for 48 hours with 2.5 μM simvastatin, or with 10 μM GGTI-286, or with 2.5 μM FTI-277 added to the complete medium. The medium was changed and fresh inhibitors were added every 24 hours. Then, TNF-α (10 ng/ml) was added to the medium for 4 hours and E-selectin expression was measured by flow cytometry analysis. I represents E-selectin expression on control HUVEC; II represents E-selectin expression after TNF-α treatment; III represents E-selectin expression after overnight preincubation with simvastatin and consequent stimulation with TNF-α; IV represents TNF-α induced E-selectin expression after overnight preincubation with GGTI-286; and V represents TNF-α induced E-selectin after overnight preincubation with FTI-277.

Dimitrova et al. J Thromb Haemost, in press 2003

REFFERENCES

Aikawa M, Rabkin E, Sugiyama S, Voglic SJ, Fukumoto Y, Furukawa Y, Shiomi M, Schoen FJ, Libby P. An HMG-CoA reductase inhibitor, cerivastatin, suppresses growth of macrophages expressing matrix metalloproteinases and tissue factor in vivo and in vitro. Circulation 2001; 103: 276-283

Allal C, Favre G, Couderc B, Salicio S, Sixou S, Hamilton AD, Sebti SM, Lajoie-Mazenc I, Pradines A. RhoA prenylation is required for promotion of cell growth and transformation and cytoskeleton organization but not for induction of serum response element transcription. J Biol Chem 2000; 275: 31001-31008

Amengual O, Atsumi T, Khamashta MA, Hughes GR. The role of the tissue factor pathway in the hypercoagulable state in patients with the antiphospholipid syndrome. Thromb Haemost 1998; 79: 276-281

Amerongen GP, Delft S, Vermeer MA, Collard JG, van Hinsbergh VW. Activation of RhoA by thrombin in endothelial hyperpermeability: role of rho kinase and protein tyrosine kinases. Circ Res 2000; 87: 335-340

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

Amoroso A, Mitterhofer AP, Del Porto F, Garzia P, Ferri GM, Galluzzo S, Vadacca M, Caccavo D, Afeltra A. Antibodies to anionic phospholipids and anti-beta(2)-GPI: association with thrombosis and thrombocytopenia in systemic lupus erythematosus. Hum Immunol 2003; 64: 265-273

Andrews TC, Raby K, Barry J, Naimi CL, Allred E, Ganz P, Selwyn AP. Effects of cholesterol reduction on myocardial ischemia in patients with coronary artery disease. Circulation 1997; 95: 324-328

Arvieux J, Jacob MC, Roussel B, Bensa JC, Colomb MG. Neutrophil activation by anti-beta 2 glycoprotein I monoclonal antibodies via Fc gamma receptor II. J Leukoc Biol 1995; 57: 387-394

Asherson RA, Cervera R, Piette JC, Shoenfeld Y, Espinosa G, Petri MA, Lim E, Lau TC, Gurjal A, Jedryka-GoralA, Chwalinska-Sadowska H, Dibner RJ, Rojas-Rodriguez J, Garcia-Carrasco M, Grandone JT, Parke AL, Barbosa P, Vasconcelos C, Ramos-Casals M, Font J, Ingelmo M. Catastrophic antiphospholipid syndrome: clues to the pathogenesis from a series of 80 patients. Medicine (Baltimore) 2001; 80: 355-377

Asherson RA, Cervera R, Piette JC, Font J, Lie JT, Burcoglu A, Lim K, Munoz-Rodriguez FJ, Levy RA, Boue F, Rossert J, Ingelmo M. Catastrophic antiphospholipid syndrome. Clinical and laboratory features of 50 patients. Medicine (Baltimore) 1998; 77: 195-207

Atsumi T, Ieko M, Bertolaccini M Ichikawa K, Tsutsumi A, Matsuura E, Koike T. Association of autoantibodies against phosphatidylserine-prothrombin complexes with manifestation of the antiphospholipid syndrome and with the presence of lupus anticoagulant. Arthritis Rheum 2000; 43: 1982- 1993

Atsumi T, Khamashta MA, Ames PRJ, Ichikawa K, Koike T, Hughes GR. Effect of Beta2-glycoprotein I and human monoclonal anticardiolipin antibody on the protein S/C4b-binding protein system. Lupus 1997; 6: 358-364

Atsumi T, Khamashta MA, Haworth RS, Ichikawa K, Koike T, Hughes GR. Artherial disease and thrombosis in the antiphospholipid syndrome: a pathogenic role for endothelin 1. Arthritis Rheum 1998; 41: 800-807 (b)

Atsumi T, Khamashta MA, Amengual O, Donohoe S, Mackie I, Ichikawa K, Koike T, Hughes GR. Binding of anticardiolipin antibodies to protein C via beta2-glycoprotein I (beta2-GPI): a possible mechanism in the inhibitory effect of antiphospholipid antibodies on the protein C system. Clin Exp Immunol 1998; 112: 325-33

Backos M, Rai R, Baxter N, Chilcott IT, Cohen H, Regan L. Pregnancy complications in women with recurrent miscarriage associated with antiphospholipid antibodies treated with low dose aspirin and heparin. Br J Obstet Gynaecol 1999; 106: 102-107

Bakker-Arkema RG, Davidson MH, Goldstein RJ, Goldstein RJ, Davignon J, Isaacsohn JL, Weiss SR, Keilson LM, Brown WV, Miller VT, Shurzinske LJ, Black DM. Efficacy and safety of a new HMG-CoA reductase inhibitor, atorvastatin, in patients with hypertriglyceridemia. JAMA 1996; 275: 128-133

Barquinero J, Ordi-Ros J, Selva A, Perez-Peman P, Vilardell M, Khamashta M. Antibodies against platelet-activating factor in patients with antiphospholipid antibodies. Lupus 1994; 3: 55-58

Bernard C, Exquis B, Reber G, de Moerloose P. Determination of anti-cardiolipin and other antibodies in HIV-1-infected patients. J Acquir Immune Defic Syndr 1990; 3: 536-539

Bernot D, Benoliel AM, Peiretti F, Lopez S, Bonardo B, Bongrand P, Juhan-Vague I, Nalbone G. Effect of atorvastatin on adhesive phenotype of human endothelial cells activated by tumor necrosis factor alpha. J Cardiovasc Pharmacol 2003; 41: 316-324

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

Bick R. Antiphospholipid thrombosis syndromes. Clin Appl Thromb Hemost 2001; 7: 241-58

Blann AD, Gurney D, Hughes E, Buggins P, Silverman SH, Lip GY. Influence of pravastatin on lipoproteins, and on endothelial, platelet , and inflammatory markers in subjects with peripheral artery disease. Am J Cardiol 2001; 88: 89-92

Blank M, George J, Fishman P, Levy Y, Toder V, Savion S, Barak V, Koike T, Shoenfeld Y. Ciprofloxacin immunomodulation of experimental antiphospholipid syndrome associated with elevation of interleukin-3 and granulocyte-macrophage colony stimulating factor expression. Arthritis Rheum 1998; 41: 224-232

Blank M, Waisman A, Mozes E, Koike T, Shoenfeld Y. Characteristics and pathogenic role of anti-beta2-glycoprotein I single-chain Fv domains: induction of experimental antiphospholipid syndrome. Int Immunol 1999; 11: 1917-1926

Balasubramanian K, Chandra J, Schroit AJ. Immune clearance of phosphatidylserine - expressing cells: the role of beta2-glycoprotein I in macrophage recognition. J Biol Chem 1997; 272: 31113-31117

Bokarewa MI, Blombäck M, Egberg N, Rosen S. A new variant of interaction between phospholipid antibodies and the protein C system. Blood Coag Fibrinolysis 1994; 5: 37-41

Bombeli T, Schwartz B, Harlan J. Endothelial cells undergoing apoptosis become proadhesive for nonactivated platelets. Blood, 1998; 93: 3831-3838

Borrell M, Sala N, de Castellarnau C, Lopez S, Gari M, Fontcuberta J. Immunoglobulin fractions isolated from patients with antiphospholipid antibodies prevent the inactivation of factor Va by activated protein C on human endothelial cells. Thromb Haemost 1992; 68: 268-272

Bowie EJW, Thompson JH, Pascuzzi CA, Owen CA. Thrombosis in systemic lupus erythematosus despite circulating anticoagulants. J Lab Clin Med 1963; 62: 416-430

Branch DW, Rodgers GM. Induction of endothelial cell tissue factor activity by sera from patients with antiphospholipid syndrome: a possible mechanism of thrombosis. Am J Obstet Gynecol 1993; 168: 206-210

Branch DW, Silver R, Pierangeli S, van Leeuwen I, Harris EN. Antiphospholipid antibodies other than lupus anticoagulant and anticardiolipin antibodies in women with recurrent pregnancy loss, fertile controls, and antiphospholipid syndrome. Obstet Gynecol 1997; 89: 549-555

Branch DW. Antiphospholipid antibodies and reproductive outcome: the current state of affairs. J Reprod Immunol 1998; 38: 75-87

Brandt J, Triplett D, Aving B, Scharrer I. Scientific Subcommittee on Lupus Anticoagulants/ Phospholipid-Dependent Antibodies of the Scientific and Standartisation Committee of the ISTH. Thromb Haemost 1995; 74: 1185-1190

Brey RL, Escalante A. Neurological manifestations of antiphospholipid antibody syndrome. Lupus 1998; S2: S67-74

Bruce I, Clark-Soloninka CA, Spitzer KA, Gladman DD, Urowitz MB, Laskin CA. Prevalence of antibodies to anti-beta2-glycoprotein I in systemic lupus erythematosus and their association with antiphospholipid antibody syndrome criteria: a single center study and literature review. J Rheumatol 2000; 27: 2833-2837

Bruce IN, Gladman DD, Urowitz MB. Premature atherosclerosis in systemic lupus erythematosus. Rheum Dis Clin North Am 2000; 26: 257-278 (b)

Brunner HI, Chan WS, Ginsberg JS, Feldman BM. Longterm anticoagulation is preferable for patients with antiphospholipid antibody syndrome. Results of a decision analysis. J Rheumatol 2002; 29: 490-501

Campbell AL, Pierangeli SS, Wellhausen S, Harris EN. Comparison of the effects of anticardiolipin antibodies from patients with the antiphospholipid syndrome and with syphilis on platelet activation and aggregation. Thromb Haemost 1995; 73: 529-534

Carhuapoma JR, Mitsias P, Levine SR. Cerebral venous thrombosis and anticardiolipin antibodies. Stroke 1997; 28: 2363-2369

Carreras L, Forastiero R, Martinuzzo M. Interaction between antiphospholipid antibodies and eicosanoids. In Hughes Syndrome, Antiphospholipid Syndrome. Edited by Khamashta MA, London: Springer- Verlag 2000: 337-347

Carson CW, Comp PC, Rezaie AR, Esmon NL, Esmon CT. Antibodies to thrombomodulin are found in patients with lupus anticoagulant and unexplained thrombosis. J Rheumatol 2000; 27: 384-390

Case CC, Ballantyne CM. Statins and inflammatory markers. Curr Atherosclerosis Reports 2002; 4: 42-47

Cervera R, Piette JC, Font J, Khamashta MA, Shoenfeld Y, Camps MT, Jacobsen S, Lakos G, Tincani A,Kontopoulou-Griva I, Galeazzi M, Meroni PL, Derksen RH, de Groot PG, Gromnica-Ihle E, Baleva M, Mosca M,Bombardieri S, Houssiau F, Gris JC, Quere I, Hachulla E, Vasconcelos C, Roch B, Fernandez-Nebro A, Boffa MC, Hughes GR, Ingelmo M; Euro-Phospholipid Project Group. Antiphospholipid syndrome: clinical and immunologic manifestations and patterns of disease expression in a cohort of 1,000 patients. Arthritis Rheum 2002; 46: 1019-1027

Champley LW, Pattison NS, McKay EJ. Separation of lupus anticoagulants from anticardiolipin antibodies by ion- exchange and gel-filtration chromatography. Haemostasis 1991; 21: 25-29

Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM, Stern DM. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 1998; 91: 3527-3561

Clements JE, Montrelaro RC, Zink MC, Amedee AM, Miller S, Trichel AM, Jagerski B, Hauer D, Martin LN, Bohm RP, et al. Cross protective immune responses induced in rhesus macaques by immunization with attenuated macrophage-tropic simian immunodeficiency virus. J Virol 1995; 69: 2737-2744

Clyne LP, Brandt JT, Maas RL. The laboratory diagnosis of lupus anticoagulants. Arch Intern Med 1988; 148:1060-1063

Clyne LP, Yen Y, Kriz NS, Breitenstein MG. The lupus anticoagulant: high incidence of “negative ” mixing studies in human immunodeficiency virus-positive population. Arch Pathol Lab Med 1993; 117: 595-601

Cohen J, Bakimer R, Blank M, Valesini G, Shoenfeld Y. Pathogenic serum IgG anticardiolipin antibodies and the idiotypic network. Ann Rheum Dis 1993; 52: 742-748

Conley CL, Hartmann RC. A hemorrhagic disorder caused by circulating anticoagulant in patients with disseminated lupus erythematosus. J Clin Invest 1952; 31: 621-629

Constans J, Guerin V, Couchouron A Seigneur M, Ryman A, Blann AD, Amiral J, Amara A, Peuchant E, Moreau JF, Pellegrin I, Pellegrin JL, Fleury H, Leng B, Conri C. Autoantibodies directed against phospholipids or human beta 2-glycoprotein I in HIV-

seropositive patients: relationship with endothelial activation and antimalonic dialdehyde antibodies. Eur J Clin Invest 1998; 28: 115-122

Corsini A, Bellosta S, Baetta R, Fumagalli R, Paoletti R, Bernini F. New insights into the pharmacodynamic and pharmacokinetic properties of statins. Pharmacol Ther 1999; 84: 413-428

Cuadrado MJ, Lopez-Pedrera C, Khamashta MA, Camps MT, Tinahones F, Torres A, Hughes GR, Velasco F. Thrombosis in primary antiphospholipid syndrome: a pivotal role for monocyte tissue factor expression. Arthritis Rheum 1997; 40: 834-841

Dalhback B, Hildebrand B. Inherited resistance to activated protein C is corrected by anticoagulant cofactor activity found to be a property of factor V. Proc Natl Acad Sci USA 1994; 91: 1396-1400

Daniel JL, Dangelmaier C, Jin J, Kim YB, Kunapuli SP. Role of intracellular signaling events in ADP-induced platelet aggregation. Thromb Haemost 1999; 82: 1322–1326

de Groot PG, Horbach DA, Derksen RH. Protein C and other cofactors involved in the binding of antiphospholipid antibodies: relation to the pathogenesis of thrombosis. Lupus 1996; 5: 488-493

Del Papa N, Raschi E, catelli L, Khamashta MA, Ichikawa K, Tincani A, Balestrieri G, Meroni PL. Endothelial cells as a target for antiphospholipid antibodies: role of anti-Beta2-glycoprotein I antibodies. Am J Reprod Immunol 1997; 38: 212-217

Di Prima MA, Sorice M, Vullo V, Mastroianni CM, Amendolea MA, Masala C. Anticardiolipin antibody in the acquired immunodeficiency syndrome: a marker of Pneumocystis carinii infection? J Infect 1989; 18:100-101

Di Virgilio F, Chiozzi P, Ferrari D, Falzoni S, Sanz JM, Morelli A, Torboli M, Bolognesi G, Baricordi OR. Nucleotide receptors: an emerging family of regulatory molecules in blood cells. Blood 2001; 97: 587–600

Diri E, Cucurull E, Gharavi AE, Kapoor D, Mendez EA, Scopelitis E, Wilson WA. Antiphospholipid (Hugles') syndrome in African-Americans: IgA anti-CL and anti-beta2 glycoprotein-I is the most frequent isotype. Lupus 1999;8:263-268

Dobado-Berrios PM, Lopez-Pedrera C, Velasco F, Aguirre MA, Torres A, Cuadrado MJ. Increased levels of tissue factor mRNA in mononuclear blood cells of patients with primary antiphospholipid syndrome. Thromb Haemost 1999; 82: 1578-1582

Donohoe S, Kingdom JC, Mackie IJ. Affinity purified human antiphospholipid antibodies bind normal term placenta. Lupus 1999; 8: 525-531

Downs JR, Clearfield M, Weis S, Whitney E, Shapiro DR, Beere PA, Langendorfer A, Stein EA, Kruyer W, Gotto AM Jr. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. JAMA 1998; 279: 1615-1622

Dueymes M, Levy Y, Ziporen L, Jamin C, Piette JC, Shoenfeld Y, Youinou P. Do some antiphospholipid antibodies target endothelial cells? Ann Med Interne (Paris) 1996; 147 (S1): 22-23

Dunoyer-Geindre S, Kruithof EK, Galve-de Rochemonteix B, Rosnoblet C, Gruenberg J, Reber G, de Moerloose P. Localization of beta2-glycoprotein 1 in late endosomes of human endothelial cells. Thromb Haemost 2001; 85: 903-907

Dunoyer-Geindre S, de Moerloose P, Galve-de Rochemonteix B, Reber G, Kruithof EK. NFkappaB is an essential intermediate in the activation of endothelial cells by anti-beta(2)-glycoprotein 1 antibodies. Thromb Haemost 2002; 88: 851-857

Durrani OM, Gordon C, Murray PI. Primary anti-phospholipid antibody syndrome (APS): current concepts. Surv Ophthalmol 2002; 47: 215-238

Emeis JJ, van den Eijnden-Schrauwen Y, van den Hoogen CM de Priester W, Westmuckett A, Lupu F. An endothelial storage granule for tissue-type plasminogen activator. J Cell Biol 1997; 139: 245-256

Emmi L, Bergamini C, Spinelli A, Liotta F, Marchione T, Caldini A, Fanelli A, De Cristofaro MT, Dal Pozzo G. Possible pathogenetic role of activated platelets in the primary antiphospholipid syndrome involving the central nervous system. Ann N Y Acad Sci 1997; 823: 188-200

Erkan D, Yazici Y, Peterson MG, Sammaritano L, Lockshin MD. A cross-sectional study of clinical thrombotic risk factors and preventive treatments in antiphospholipid syndrome. Rheumatol 2002; 41: 924-929

Erwards CJ, Hart DJ, Spector TD. Oral statins and increased bone-mineral density in postmenopausal women. Lancet 2000; 355: 2218-2219

Escolar G, Font J, Reverter JC, Lopez-Soto A, Garrido M, Cervera R, Ingelmo M, Castillo R, Ordinas A. Plasma from systemic lupus erythematosus patients with antiphospholipid antibodies promotes platelet aggregation. Arterioscler Thromb 1992; 12: 196-200

Esmon CT. Thrombomodulin as model of molecular mechanisms that modulate protease specificity and function at the vessel surface. FASEB Journal 1995; 9: 946-955

Essig M, Nguyen G, Prie D, Escoubet B, Sraer JD, Friedlander G. 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors increase fibrinolytic activity in rat aortic endothelial cells. Role of geranylgeranylation and Rho proteins. Circ Res 1998; 83: 683-690

Eto M, Kozai T, Cosentino F, Joch H, Luscher TF. Statin prevents tissue factor expression in human endothelial cells: role of Rho/Rho-kinase and Akt pathways. Circulation 2002; 105: 1756-1759

Exner T, Hill P, Cleland J, Berry E. Studies on an unusual von Willebrand's variant--type IID. Aust N Z J Med 1990; 20: 553-557

Exner T. Conceptions and misconceptions in testing for lupus anticoagulants. J Autoimmun 2000; 15: 179-183

Faden D, Tincani A, Tanzi P, et al. Anti-beta 2 glycoprotein I antibodies in a general obstetric population: preliminary results on the prevalence and correlation with pregnancy outcome. Anti-beta2 glycoprotein I antibodies are associated with some obstetrical complications, mainly preeclampsia-eclampsia. Eur J Obstet Gynecol Reprod Biol 1997; 73: 37-42

Farnier M, Davignon J. Current and future treatment of hyperlipidemia: the role of statins. Am J Cardiol 1998; 82: 3J-10J

Feinstein DI, Rapaport SI. Acquired inhibitors of blood coagulation. Progress in Hemost and Thromb 1972; 1: 175-187

Feron O, Dessy C, Desager JP, Balligand JL. Hydroxy-methylglutaryl-coenzyme A reductase inhibition promotes endothelial nitric oxide synthase activation through a decrease in caveolin abundance. Circulation 2001; 103: 113-118

Ferro D, Basili S, Roccaforte S, Di Franco M, Cipollone F, Ciabattoni G, Davi G. Determinants of enhanced thromboxane biosynthesis in patients with systemic lupus erythematosus. Arthritis Rheum 1999; 42: 2689-2697

Finazzi G, Brancaccio V, Moia M, Ciaverella N, Mazzucconi MG, Schinco PC, Ruggeri M, Pogliani EM, Gamba G, Rossi E, Baudo F, Manotti C, D'Angelo A, Palareti G, De Stefano V, Berrettini M, Barbui T. Natural history and risk factors for thrombosis in 360 patients with antiphospholipid antibodies: a four-year prospective study from the Italian Registry. Am J Med 1996;100: 530-536

Fisher JE, Rogers MJ, Halasy JM, Luckman SP, Hughes DE, Masarachia PJ, Wesolowski G, Russell RG, Rodan GA, Reszka AA. Alendronate mechanism of action: geranylgeraniol, an intermediate in the mevalonate pathway, prevents inhibition of osteoclast formation, bone resorption, and kinase activation in vitro. Proc Natl Acad Sci USA 1999; 96: 133-138

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

Galli M, Confurius P, Maassen C, Hemker HC, de Baets MH, van Breda-Vriesman PJ, Barbui T, Zwaal RF, Bevers EM. Anticardiolipin antibodies(ACA) directed not to cardiolipin but to a plasma cofactor. Lancet 1990; 335: 1554-1559

Galli M, Daldossi M, Barbui T. Anti-glycoprotein Ib/IX and IIb/IIIa antibodies in patients with antiphospholipid antibodies. Thromb Haemost 1994; 71: 571-575

Galli M, Finazzi G, Bevers EM, Barbui T. Kaolin clotting time and dilute Russell's viper venom time between prothrombin-dependent and beta 2-glycoprotein I-dependent antiphospholipid antibodies. Blood 1995; 86: 617-623

Galli M. Thrombocytopenia in the antiphospholipid syndrome. Br J Haematol 1996; 93:1-5

Galli M. Different anticoagulant and immunological properties of anti-prothrombin antibodies in patients with antiphospholipid antibodies. Thromb Haemost 1997; 75: 721-724

Galli M, Barbui T. Prothrombin as a cofactor for antiphospholipids. Lupus 1998; 7: 37-40

Galli M. Which antiphospholipid antibodies should be measured in the antiphospholipid syndrome? Haemostasis 2000; 30 (S2): 57-62

Galve-de Rochemonteix B, Kobayashi T, Rosnoblet C, Lindsay M, Parton RG, Reber G, de Maistre E, Wahl D, Kruithof EK, Gruenberg J, de Moerloose P. Interaction of

antiphospholipid antibodies with late endosomes of human endothelial cells. Arterioscler Thromb Vasc Biol 2000; 20: 563-574

Gattorno M, Buoncompagni A, Molinari AC, Barbano GC, Morreale G, Stalla F, Picco P, Mori PG, Pistoia V. Antiphospholipid antibodies in paediatric systemic lupus erythematosus, juvenile chronic arthritis and overlap syndromes: SLE patients with both lupus anticoagulant and high-titre anticardiolipin antibodies are at risk for clinical manifestations related to the antiphospholipid syndrome. Br J Rheumatol 1995; 34: 873-881

Gauthier TW, Scalia R, Murohara T, Guo JP, Lefer AM. Nitric oxide protects against leukocyte-endothelium interactions in the early stages of hypercholesterolemia. Arterioscler Thromb Vasc Biol 1995; 15: 1652-1659

Gavaghan TP, Krilis SA, Daggard GE Baron DE, Hickie JB, Chesterman CN. Anticardiolipin antibodies and occlusion of coronary artery bypass grafts. Lancet 1987; ii:977-978

Gennari LC, Blanco AN, Alberto MF, Grosso SH, Peirano AA, Lazzari MA. Antiphospholipid antibodies impact the protein C (PC) pathway behavior. Am J Hematol 2002;71: 128-130

George J, Afek A, Gilburd B, Levy Y, Blank M, Kopolovic J, Harats D, Shoenfeld Y. Atherosclerosis in LDL-receptor knockout mice is accelerated by immunization with anticardiolipin antibodies. Lupus 1997; 6: 723-729

George J, Blank M, Levy Y, Meroni P, Damianovich M, Tincani A, Shoenfeld Y. Differential experimental antiphospholipid syndrome. Circulation 1998; 97: 900–906

George J, Gilburd B, Langevitz P, et al. Beta2 glycoprotein I containing immune-complexes in lupus patients: association with thrombocytopenia and lipoprotein (a) levels. Lupus 1999; 8: 116-120

Gharavi AE, Pierangeli SS. Origin of antiphospholipid antibodies: induction of aPL by viral peptides. Lupus 1998; 7: 52-54

Gharavi A, Pierangeli S, Levy R Levy RA, Harris EN. Mechanisms of pregnancy loss in antiphospholipid syndrome. Clin Obstet Gynecol 2001; 44: 11-19

Gleicher N, Harlow L, Zilberstein M. Regulatory effect of antiphospholipid antibodies on signal transduction: a possible model for autoantibody-induced reproductive failure. Am J Obstet Gynecol 1992; 167: 637-642

Godeau B, Piette JC, Fromont P, Intrator L, Schaeffer A, Bierling P. Specific antiplatelet glycoprotein autoantibodies are associated with the thrombocytopenia of primary antiphospholipid syndrome. Br J Haematol 1997; 98: 873-879

Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature 1990; 343: 425-430

Greaves M, Cohen H, MacHin SJ, Mackie I. Guidelines on the investigation and management of the antiphospholipid syndrome. Br J Haematol 2000; 109: 704-715

Grip O, Janciauskiene S, Lindgren S. Pravastatin down-regulates inflammatory mediators in human monocytes in vitro. Eur J Pharmacol 2000; 410: 83-92

Gryglewski RJ, Chlopicki S, Uracz W, Marcinkiewicz E. Significance of endothelial prostacyclin and nitric oxide in peripheral and pulmonary circulation. Med Sci Monit 2001; 7: 1-16

Guglielmone H, Fernandez EJ. Distribution of lupus anticoagulant and anticardiolipin antibody isotypes in a population with antiphospholipid syndrome. J Rheumatol 1999; 26: 86-90

Guidelines on oral anticoagulation: third edition. Br J Haematol 1998; 101: 374-387

Gushiken FC, Arnett FC, Thiagarajan P. Primary antiphospholipid antibody syndrome with mutations in the phospholipid binding domain of beta2-glycoprotein I. Am J Hematol 2000; 65: 160-165

Hackman A, Abe Y, Insull WJ, Pownall H, Smith L, Dunn K, Gotto AM Jr, Ballantyne CM. Levels of soluble cell adhesion molecules in patients with dyslipidemia. Circulation 1996; 93: 1334-1338

Hajjar KA. Celular receptors in the regulation of plasmin generation. Thromb Haemost 1995; 74: 294-301

Harats D, George J, Levy Y, Khamashta MA, Hughes GR, Shoenfeld Y. Atheroma: links with antiphospholipid antibodies, Hughes syndrome and lupus. QJM 1999; 92: 57-59

Harris EN, Gharavi AE, Boey ML, Patel BM, Mackworth-Young CG, Loizou S, Hughes GR. Anticardiolipin antibodies: detection by radioimmunoassay and association with thrombosis in systemic lupus erythematosus. Lancet 1983; 2: 1211-1214

Hebert PR, Gaziano JM, Chan KS, Hennekens CH. Cholesterol lowering with statin drugs, risk of stroke, and total mortality. An overview of randomized trials. JAMA 1997; 278: 313-321

Hernandez-Perera O, Perez-Sala D, Navarro-Antolin J, Sanchez-Pascuala R, Hernandez G, Diaz C, Lamas S. Effects of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, atorvastatin and simvastatin, on the expression of endothelin-1 and endothelial nitric oxide synthase in vascular endothelial cells. J Clin Invest 1998; 101: 2711-2719

Holers VM, Girardi G, Mo L, Guthridge JM, Molina H, Pierangeli SS, Espinola R, Xiaowei LE, Mao D, Vialpando CG, Salmon JE. Complement C3 activation is required for antiphospholipid antibody-induced fetal loss. J Exp Med 2002; 195: 211-220

Horbach DA. Lupus anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with systemic lupus erythematosus. Thromb Haemost 1996; 76: 916-924

Horkko S, Miller E, Branch DW, Palinski W, Witztum JL.The epitopes for some antiphospholipid antibodies are adducts of oxidized phospholipid and beta2 glycoprotein 1 (and other proteins). Proc Natl Acad Sci U S A 1997; 94: 10356-10361

Huber K, Christ G, Wojta J, Gulba D. Plasminogen activator inhibitor type-1 in cardiovascular disease. Thromb Res 2001; 103 (S1): 7-19

Ichihara K, Satoh K. Disparity between angiographic regression and clinical event rates with hydrophobic statins. The Lancet 2002; 359: 2195-2198

Ieko M, Sawada KI, Koike T, Notoya A, Mukai M, Kohno M, Wada N, Itoh T, Yoshioka N. The putative mechanism of thrombosis in antiphospholipid syndrome: impairement of the protein C and the fibrinolitic systems by monoclonal anticardiolipin antibodies. Semin Thromb Hemost 1999; 25: 503-507

Ieko M, Ichikawa K, Triplett DA, Matsuura E, Atsumi T, Sawada K, Koike T. Beta2-glycoprotein I is necessary to inhibit protein C activity by monoclonal anticardiolipin antibodies. Arthritis Rheum 1999; 42: 167-174 (b)

Ieko M, Ichikawa K, Atsumi T, Takeuchi R, Sawada KI, Yasukouchi T, Koike T. Effects of beta2-glycoprotein I and monoclonal anticardiolipin antibodies on extrinsic fibrinolysis. Semin Thromb Hemost 2000; 26: 85-90

Ikeda U, Takahashi M, Shimada K. Monocyte-endothelial cell interaction in atherogenesis and thrombosis. Clin Cardiol 1998; 21: 11-14

Ikematsu W, Luan FL, La Rosa L, Beltrami B, Nicoletti F, Buyon JP, Meroni PL, Balestrieri G, Casali P. Human anticardiolipin monoclonal antibodies cause placental necrosis and fetal loss in BALB/c mice. Arthritis Rheum 1998; 41: 1026-1039

International Consensus Statement on Preliminary Classification Criteria for Definite Antiphospholipid Syndrome. Arthritis Rheum 1999; 42: 1309-1311

Istvan ES, Deisenhofer J. The structure of the catalytic portion of human HMG-CoA reductase. Biochim Biophys Acta 2000; 1529: 9-18

Jaeger U, Kapiotis S, Pabinger I, Puchhammer E, Kyrle PA, Lechner K. Transient lupus anticoagulant associated with hypoprothrombinemia and factor XII deficiency following adenovirus infection. Ann Hemat 1993; 67: 95-99

Jaffe EA, Nachman RL, Becker CG, Minick CRCulture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973; 52: 2745-2756

Jilma B, Joukhadar C, Derhaschnig U, Rassoul F, Richter V, Wolzt M, Dorner GT, Petternel V, Wagner OF. Levels of adhesion molecules do not decrease after 3 months of statin therapy in moderate hypercholesterolaemia. Clin Sci 2003; 104: 189-193

Jick H, Zornberg GL, Jick SS, Seshadri S, Drachman DA. Statins and the risk of dementia. Lancet 2000; 356: 1627-1631

Jone DW, Nicholls PJ, Donohoe S, Gallimore MJ, Winter M. Antibodies to factor XII are distinct from antibodies to prothrombin in patients with the anti-phospholipid syndrome. Thromb Haemost 2002; 87: 426-430

Jones DW, Gallimor MJ, MacKie IJ, Harris SL, Winter M. Reduced factor XII levels in patients with the antiphospholipid syndrome are associated with antibodies to factor XII. Br J Haematol 2000; 110: 721-726

Joseph JE, Harrison P, Mackie IJ, Isenberg DA, Machin SJ. Increased circulating platelet-leucocyte complexes and platelet activation in patients with antiphospholipid syndrome, systemic lupus erythematosus and rheumatoid arthritis. Br J Haematol 2001; 115: 451-459

Kandiah DA, Sali A, Sheng Y Victoria EJ, Marquis DM, Coutts SM, Krilis SA. Current insights into the antiphospholipid syndrome: clinical, immunological, and molecular aspects. Adv Immunol 1998; 70: 507-63

Kaneider NC, Reinisch CM, Dunzendorfer S, Meierhofer C, Djanani A, Wiedermann CJ. Induction of apoptosis and inhibition of migration of inflammatory and vascular wall cells by cerivastatin. Atheroscl 2001; 158: 23-33

Kaneider NC, Egger P, Dunzendorfer S, Wiedermann CJ. Rho-GTPase-dependent platelet-neutrophil interaction affected by HMG-CoA reductase inhibition with altered adenosine nucleotide release and function. Arterioscler Thromb Vasc Biol 2002; 22: 1029-1035

Kearon C, Gent M, Hirsh J, Weitz J, Kovacs MJ, Anderson DR, Turpie AG, Green D, Ginsberg JS, Wells P, MacKinnon B, Julian JA. A comparison of three months of anticoagulation with extended anticoagulation for a first episode of idiopathic venous thromboembolism. N Eng J Med 1999; 340: 901-907

Ma K, Simantov R, Zhang JC, Silverstein R, Hajjar KA, McCrae KR. High affinity binding of Beta2-glycoprotein I to human endothelial cells is mediated by annexin II. J Biol Chem 2000; 20: 15541-15548

Khamashta MA, Cervera R, Asherson RA, Font J, Gil A, Coltart DJ, Vazquez JJ, Pare C, Ingelmo M, Oliver J. Association of antibodies against phospholipids with heart valve disease in systemic lupus erythematosus. Lancet 1990; 335: 1541-1544

Khamashta MA. Management of anti-phospholipid syndrome. CPD Rheumatology 1999; 1: 22-29

Kobayashi T, Stang E, Fang KS, de Moerloose P de Moerloose P, Parton RG, Gruenberg J. A lipid associated with the antiphospholipid syndrome regulates endosome structure and function. Nature 1998; 392: 193-197

Koh KK. Effects of statins on vascular wall: vasomotor function, inflammation, and plaque stability. Cardiovasc Res, 2000; 47: 648-657

Konstantopoulos K, Neelamegham S, Burns AR Hentzen E, Kansas GS, Snapp KR, Berg EL, Hellums JD, Smith CW, McIntire LV, Simon SI. Venous levels of shear support neutrophil-platelet adhesion and neutrophil aggregation in blood via P-selectin and beta2-integrin. Circulation 1998; 98: 873–882

Kornberg A, Blank M, Kaufman S, Shoenfeld Y. Induction of tissue factor-like activity in monocytes by anti-cardiolipin antibodies. J Immunol 1994; 153: 1328-1332

Kornberg A, Renaudineau Y, Blank M, Blank M, Youinou P, Shoenfeld Y. Anti-beta 2-glycoprotein I antibodies and anti-endothelial cell antibodies induce tissue factor in endothelial cells. Isr Med Assoc J 2000; 2S: 27-31

Kothe H, Dalhoff K, Rupp J, Muller A, Kreuzer J, Maass M, Katus HA. Hydroxymethylglutaryl coenzyme A reductase inhibitors modify the inflammatory

response of human macrophages and endothelial cells infected with Chlamydia pneumoniae. Circulation 2000; 101: 1760-1763

Krnic-Barrie S, O'Connor CR, Looney SW, Looney SW, Pierangeli SS, Harris EN. A retrospective review of 61 patients with antiphospholipid syndrome. Analysis of factors influencing recurrent thrombosis. Arch Intern Med 1997; 157: 2101-2108

Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa WC, Walsh K. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. Nat Med 2000; 6: 1004-1010

Kutteh, W. Antiphospholipid antibody-associated recurrent pregnancy loss: treatment with heparin and low-dose aspirin is superior to low-dose aspirin alone. Am J Obst Gynecol 1996; 174: 1584-1589

Kutteh WH. Antiphospholipid antibodies and reproduction. J Reprod Immunol 1997; 35: 151-171

Kwak B, Mulhaupt F, Myit S, Mach F. Statins as a newly recognized type of immunomodulator. Nature Med 2000; 6: 1399-1402

Kwak B, Mulhaupt F, Veillard N, Pelli G, Mach F. The HMG-CoA reductase inhibitor simvastatin inhibits IFN-gamma induced MHC class II expression in human vascular endothelial cells. Swiss Med Wkly 2001; 131: 41-46

La Rosa JC. Effects of statins on risk of coronary disease. A meta-analysis of randomised controlled trials. JAMA 1999; 282: 2340-2346

Lackner KJ, von Landenberg C, Barlage S, Schmitz G. Analysis of prothrombotic effects of two human monoclonal IgG antiphospholipid antibodies of apparently similar specificity. Thromb Haemost 2000; 83: 583-588

Lacos Get al. Isotype distribution and clinical relevance of anti-beta2-glycoprotein I: importance of IgA isotype. Clin Exp Immunol 1999; 117: 574-579

Lanir N, Zilberman M, Yron I, Tennenbaum G, Shechter Y, Brenner B. Reactivity patterns of antiphospholipid antibodies and endothelial cells: effect of antiendothelial antibodies on cell migration. J Lab Clin Med 1998; 131: 548-556

Larner J, Jane J, Laws E, Packer R, Packer R, Myers C, Shaffrey M. A phase I-II trial of lovastatin for anaplastic astrocytoma and glioblastoma multiforme. Am J Clin Oncol 1998; 21: 579-583

Laskin C, Bombardier C, Hannah M, Mandel FP, Ritchie JW, Farewell V, Farine D, Spitzer K, Fielding L, Soloninka CA, Yeung M. Prednisone and aspirin in women with autoantibodies and unexplained recurrent fetal loss. N Engl J Med 1997; 337: 148-153

Laszik Z, Mitro A, Taylor FB Ferrell G, Esmon CT. Human protein C receptor is resent primarily on endothelium of large blood vessels- implications for the control of the Protein C pathway. Circulation 1997; 96: 3633-3640

Laszik ZG, Zhou XJ, Ferrell GL, Silva FG, Esmon CT. Down-regulation of endothelial expression of endothelial cell protein C receptor and thrombomodulin in coronary atherosclerosis. Am J Pathol 2001; 159: 797-802

Laufs U, La Fata V, Plutzky J, Liao JK. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. Circulation 1998; 97: 1129-1135

Laufs U, Liao JK. Post-transcriptional regulation of endothelial nitric oxyde synthase mRNA stability by Rho GTPase. J Biol Chem 1998; 273: 24266-24271(b)

Laufs U, Liao JK. Targeting rho in cardiovascular disease. Circ Res 2000: 87; 526-528

Laufs U, Marra D, Node K, Liao JK. 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors attenuate smooth muscle cell proliferation by preventing rho GTPase-induced-down regulation of p27 (Kip1). J Biol Chem 1999; 274: 21926-21931

Lea AP, McTavish D. Atorvastatin- a review of its pharmacology and therapeutic potential an the menagement of hyperlipidaemias. Drugs 1997; 53: 828-847

Lefer AM, Scalia R, Lefer DJ. Vascular effects of HMG-CoA-reductase inhibitors (statins) unrelated to cholesterol lowering: new concepts for cardiovascular disease. Cardiovasc Res 2001; 49: 281-287

Levine SR, Brey RL, Sawaya KL, Salowich-Palm L, Kokkinos J, Kostrzema B, Perry M, Havstad S, Carey J. Recurrent stroke and thrombo-occlusive events in the antiphospholipid syndrome. Ann Neurology 1995; 38: 119-124

Levy RA, Avvad E, Oliveira J, Porto LC. Placental pathology in antiphospholipid syndrome. Lupus 1998; 7(S 2): 81-85

Levy RA, Gharavi AE, Sammaritano LR, Habina L, Lockshin MD. Fatty acid chain is a critical epitope for antiphospholipid antibody. J Clin Immunol 1990;10: 141-145

Lieby P, Soley A, Levallois et al. The clonal analysis of anticardiolipin antibodies in a single patient with primary antiphospholipid syndrome reveals an extreme antibody heterogeneity. Blood 2001; 97: 3820-3828

Lijnen P, Celis H, Fagard R, Staessen J, Amery A. LiInfluence of cholesterol lowering on plasma membrane lipids and cationic transport systems. J Hypertens 1994; 12: 59-64

Loizou S, McCrea JD, Rudge AC, Reynolds R, Boyle CC, Harris EN. Measurement of anti-cardiolipin antibodies by an ELISA: standartization and quantitation of results. Clin Exp Immunol 1985; 62: 738-745

Lupu C, Goodwin CA, Westmuckett AD, Emeis JJ, Scully MF, Kakkar VV, Lupu F. Tissue factor pathway inhibitor in endothelial cells colocalizes with glycolipid microdomains/caveolae. Arterioscl Thromb Vasc Biol 1997; 17: 2964-2974

Male C, Mitchell L, Julian J, Vegh P, Joshua P, Adams M, David M, Andrew ME. Acquired activated protein C resistance is associated with lupus anticoagulants and thrombotic events in pediatric patients with systemic lupus erythematosus. Blood 2001; 97: 844-849

Margaglione M, Giovanna A, Collaizzo D Cappucci G, del Popolo A, Brancaccio V, Ciampa A, Grandone E, Di Minno G. Coexistence of factor V Leiden and factor II A20210 mutations and recurrent venous thromboembolism. Thromb Haemost 1999; 82: 1583-1587

Martinuzzo M, Forastiero R, Adamczuk Y, Cerrato G, Carreras LO. Activated protein C resistance in patients with anti-beta 2 glycoprotein I antibodies. Blood Coagul Fibrinolysis 1996; 7: 702-704

Martinuzzo ME, Maclouf J, Carreras LO, Levy-Toledano S. Antiphospholipid antibodies enhance thrombin-induced platelet activation and thromboxane formation. Thromb Haemost 1993; 70: 667-671

Matalon ST, Shoenfeld Y, Blank M, Yacobi S, Blumenfeld Z, Ornoy A. The effects of IgG purified from women with SLE and associated pregnancy loss on rat embryos in culture. Am J Reprod Immunol 2002; 48: 296-304

Matozaki T, Nakanishi H, Takai Y. Small G-protein networks: Their crosstalk and signal cascades. Cellular Signalling 2000; 12: 515-524

Maxwell AJ, Tsao PS, Cooke JP. Modulation of the of nitric oxide synthase pathway in atherosclerosis. Experimental Physiology 1998; 83: 573-584

McNeill HP, Chesterman CN, Krilis SA. Immunology and clinical importance of antiphospholipid antibodies. Adv Immunol 1991; 49: 193-280

McTaggart F, Buckett L, Davidson R, Holdgate G, McCormick A, Schneck D, Smith G, Warwick M. Preclinical and clinical pharmacology of Rosuvastatin, a new 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor. Am J Cardiol 2001; 87: 28B-32B

Mengarelli A, Minotti C, Palumbo G, Arcieri P, Gentile G, Iori AP, Arcese W, Mandelli F, Avvisati G. High levels of antiphospholipid antibodies are associated with cytomegalovirus infection in unrelated bone marrow and cord blood allogenic stem cell transplantation. Br J Haematol 2000; 108: 126-131

Merill J. Which antiphospholipid antibody tests are more useful ? Rheum Dis Clin 2001; 27: 525-549

Meroni P, Raschi E, Camera M, Testoni C, Nicoletti F, Tincani A, Khamashta MA, Balestrieri G, Tremoli E, Hess DC. Endothelial activation by aPL: a potential pathogenetic mechanism for the clinical manifestations of the syndrome. J Autoimmun 2000; 15: 237-240

Meroni P, Riboldi P. Pathogenic mechanisms mediating antiphospholipid syndrome. Curr Opin Rheumatol 2001; 13: 377-382

Meroni PL, Raschi E, Testoni C, Tincani A, Balestrieri G. Antiphospholipid antibodies and the endothelium. Rheum Dis Clin North Am 2001; 27: 587-602 (b)

Meroni PL, Raschi E, Testoni C, Tincani A, Balestrieri G, Molteni R, Khamashta MA, Tremoli E, Camera M. Statins prevent endothelial cell activation induced by antiphospholipid (anti-beta2-glycoprotein I) antibodies: effect on the proadhesive and proinflammatory phenotype. Arthritis Rheum 2001; 44: 2870-2878 (c)

Merrill JT , Shen C, Gugnani M, Lahita RG, Mongey AB. High prevalence of antiphospholipid antibodies in patients taking procainamide. J Rheumatol 1997; 24:1083-1087

Mevorach D, Zhon JL, Song X, Elkon KB. Systemic exposure to irradiated apoptotic cells induces autoantibody production. J Exp Med 1998; 188: 387-392

Miyazaki H, Matsuoka H, Cooke JP, Usui M, Ueda S, Okuda S, Imaizumi T. Endogenous nitric oxide synthase inhibitor: a novel marker of atherosclerosis. Circulation 1999; 99: 1141-1146

Montalban J, Codina A, Ordi J, Vilardell M, Khamashta MA, Hughes GR. Antiphospholipid antibodies in cerebral ischemia. Stroke 1991; 22: 750-753

Mori T, Takeya H, Nishioka J, Gabazza EC, Suzuki K. beta 2-Glycoprotein I modulates the anticoagulant activity of activated protein C on the phospholipid surface. Thromb Haemost 1996; 75: 49-55

Muller C, Bockhorn AG, Klusmeier S, Kiehl M, Roeder C, Kalthoff H, Koch OM. Lovastatin inhibits proliferation of pancreatic cancer cell lines with mutant as well as with wild-type K-ras oncogene but has different effects on protein phosphorylation and induction of apoptosis. Int J Oncol 1998; 12: 717-723

Muller C, Kiehl MG, van de Loo J, Koch OM. Lovastatin induces p21WAF1/Cip1 in human vascular smooth muscle cells: influence on protein phosphorylation, cell cycle, induction of apoptosis, and growth inhibition. Int J Mol Med 1999; 1: 63-68

Munoz-Rodriguez FJ, Font J, Cervera R, Reverter JC, Tassies D, Espinosa G, Lopez-Soto A, Carmona F, Balasch J, Ordinas A, Ingelmo M. Clinical study and follow-up of 100 patients with the antiphospholipid syndrome. Semin Arthritis Rheum 1999; 29: 182-90

Nakamura N, Ban T, Yamaji K, Yoneda Y, Wada Y. Localization of the apoptosis-including activity of lupus anticoagulant in an annexin V-binding antibody subset. J Clin Invest, 1998; 101: 1951-1959

Nesher G, Ilany J, Rosenmann D, Abraham AS. Valvular dysfunction in antiphospholipid syndrome: prevalence, clinical features, and treatment. Sem Arthr Rheum 1997; 27: 27-35

Nilsson IM, Astedt B, Hedner U, Berezin D. Intrauterine death and circulating anticoagulant (“antithromboplastin”). Acta Med Scand 1975; 197: 153–159

Niu XF, Smith CW, Kubes P. Intracellular oxidative stress induced by nitric oxide synthesis inhibition increases endothelial cell adhesion to neutrophils. Circ Res 1994; 74: 1133-1140

Niwa S, Totsuka T, Hayashi S. Inhibitory effect of fluvastatin, an HMG-CoA reductase inihibitor, on the expression of adhesion molecules on human monocyte cell line. Int J Immunopharmacol 1996; 18: 669-675

Nojima J, Suehisa E, Akita N, Toku M, Fushimi R, Tada H, Kuratsune H, Machii T, Kitani T, Amino N. Risk of arterial thrombosis in patients with anticardiolipin antibodies and lupus anticoagulant. Br J Haematol 1997; 96: 447-50

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

Nojima J, Kuratsune H, Suehisa E, Futsukaichi Y, Yamanishi H, Machii T, Iwatani Y, Kanakura Y. Association between the prevalence of antibodies to beta(2)-glycoprotein I, prothrombin, protein C, protein S, and annexin V in patients with systemic lupus erythematosus and thrombotic and thrombocytopenic complications. Clin Chem 2001; 47: 1008-1015

Nojima J, Kuratsune H, Suehisa E, Kawasaki T, Machii T, Kitani T, Iwatani Y, Kanakura Y. Acquired activated protein C resistance is associated with the co-existence of anti-prothrombin antibodies and lupus anticoagulant activity in patients with systemic lupus erythematosus. Br J Haematol 2002; 118: 577-583

Notarbartolo A, Davi G, Averna M, Barbagallo CM, Ganci A, Giammarresi C, La Placa FP, Patrono C. Inhibition of thromboxane biosynthesis and platelet function by simvastatin in type IIa hypercholesterolemia. Arterioscler Thromb Vasc Biol 1995; 15: 247-251

O'Donnell VB, Freeman BA. Interactions between nitric oxide and lipid oxidation pathways: implications for vascular disease. Circ Res 2001; 88: 12-21

Olee T, Pierangeli SS, Handley HH, Le DT, Wei X, Lai CJ, En J, Novotny W, Harris EN, Woods VL Jr, Chen PP. A monoclonal IgG anticardiolipin antibody from a patient with the antiphospholipid syndrome is thrombogenic in mice. Proc Natl Acad Sci U S A 1996; 93: 8606-8611

Oosting J, Derksen RH, Bobbink IW, Hackeng TM, Bouma BN, de Groot PG. Antiphospholipid antibodies directed against a combination of phospholipids with prothrombin, protein C, or protein S: an explanation for their pathogenic mechanism? Blood 1993; 81: 2618-2625

Oosting JD, Preissner KT, Derksen RH, de Groot PG. Autoantibodies directed against the epidermal growth factor-like domains of thrombomodulin inhibit protein C activation in vitro. Br J Haematol 1993; 85: 761-768 (b)

Oshiro BT, Silver RM, Scott JR, Yu H, Branch DW. Antiphospholipid antibodies and fetal death. Obstet Gynecol 1996; 87: 489-493

Pahan K, Sheikh FG, Namboodiri AM, Singh I. Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages. J Clin Invest 1997; 100: 2671-2679

Peaceman AM, Rehnberg KA. The effect of immunoglobulin G fractions from patients with lupus anticoagulant on placental prostacyclin and thromboxane production. Am J Obstet Gynecol 1993; 169: 1403-1406

Pearson JD. Normal endothelial cell function. Lupus 2000; 9: 183-188

Pedersen TR. Statin trials and goals of cholesterol-lowering therapy after AMI. Am Heart J 1999; 138: 177-182

Pengo V, Biasiolo A, Brocco T, Tonetto S, Ruffatti A. Autoantibodies to phospholipid-binding plasma proteins in patients with thrombosis and phospholipid-reactive antibodies. Thromb Haemost 1996; 75: 721-724

Pengo V, Biasiolo A, Fior MG. Autoimmune antiphospholipid antibodies are directed against cryptic epitope expressed when beta2-glycoprotein I is bound to a suitable surface. Thromb Haemost 1995; 73: 29-34

Pengo V, Thiagarajan P, Shapiro SS. Immunological specificity and mechanisms of action of IgG lupus anticoagulants. Blood 1987; 70: 69-76

Petri M. Pathogenesis and treatment of antiphospholipid antibody syndrome. Med Clin North Am 1997; 81: 151-177

Petrovas , Vlachoyiannopoulos PG, Kordossis T, Moutsopoulos HM. Antiphospholipid antibodies in HIV infection and SLE with or without antiphospholipid syndrome: comparisons of phospholipid specificity, avidity and reactivity with beta2-GPI. J Autoimmun 1999; 13: 347-355

Pierangeli S, Harris EN. In vivo models of thrombosis for the antiphospholipid syndrome. Lupus 1996; 5: 451-455 (b)

Pierangeli S, Colden-Stanfield M, Liu X, Barker JH, Anderson GL, Harris EN. Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells in vitro and in vivo. Circulation 1999; 99:1997-2002

Pierangeli S, Liu X, Espinola R, Olee T, Zhu M, Harris NE, Chen PP. Functional analyses of patient-derived IgG monoclonal anticardiolipin antibodies using in vivo thrombosis and in vivo microcirculation models. Thromb Haemost 2000; 84: 388-395

Pierangeli S, Gharavi A, Harris N. Testing for antiphospholipid antibodies: problems and solutions. Clin Obstet Gynecol 2001; 44: 48-57

Pittoni V, Isenberg D. Apoptosis and antiphospholipid antibodies. Semin Arthritis Rheum 1998; 28:163-178

Potzsch B, Kawamura H, Preissner KT, Schmidt M, Seelig C, Muller-Berghaus G. Acquired protein C dysfunction but not decreased activity of thrombomodulin is a possible marker of thrombophilia in patients with lupus anticoagulant. J Lab Clin Med 1995; 125: 56-65

Prandoni P, Lensing, A, Cogo A, Cuppini S, Villalta S, Carta M, Cattelan AM, Polistena P, Bernardi E, Prins MH. The long-term clinical course of acute deep venous thrombosis. Ann Int Med1996; 125: 1-7

Prandoni P, Lensing, A, Prins M, Bagatella P, Scudeller A, Girolami A. Which is the outcome of post-thrombotic syndrome? Thromb Haemost 1999; 82: 1358

Pruefer D, Scalia R, Lefer AM. Simvastatin inhibits leukocyte-endothelial cell interactions and protects against inflammatory processes in normocholesterolemic rats. Arterioscler Thromb Vasc Biol 1999; 19: 2894-2900

Radomski MW, Rees DD, Dutra A, Moncada S. S-nitroso-glutathione inhibits platelet activation in vitro and in vivo. Br J Pharmacol 1992; 107: 745-749

Rai R, Cohen H, Dave M, Regan L. Regan L. Randomised controlled trial of aspirin and aspirin plus heparin in pregnant women with recurrent miscarriage associated with phospholipid antibodies (or antiphospholipid antibodies). BMJ 1997; 314: 253-257

Rand J, Wu XX, Andree HA, Ross JB, Rusinova E, Gascon-Lema MG, Calandri C, Harpel PC. Antiphospholipid antibodies accelerate plasma coagulation by inhibiting annexin V binding to phospholipids: a “lupus procoagulant” phenomenon. Blood, 1998; 92: 1652-1660

Rand J, Wu XX. Antibody-mediated disruption of the annexin-V antithrombotic shield: a new mechanism for thrombosis in the antiphospholipid syndrome. Thromb Haemost 1999; 82: 649-655

Rand JH, Wu XX, Andree HAM, Lockwood CJ, Guller S, Scher J, Harpel PC. Pregnancy loss in the antiphospholipid-antibody syndrome: a possible thrombogenic mechanism. N Engl J Med 1997; 337: 154-160

Rand JH, Wu XX, Guller S, Scher J, Andree HA, Lockwood CJ. Antiphospholipid immunoglobulin G antibodies reduce annexin-V levels on syncytiotrophoblast apical membranes and in culture media of placental villi. Am J Obstet Gynecol 1997; 177: 918-923

Rand JH. Molecular pathogenesis of the antiphospholipid syndrome. Circ Res 2002; 11: 29-37

Rand JH. The pathogenic role of annexin-V in the antiphospholipid syndrome. Curr Rheumatol Rep 2000; 3: 246-251

Rasmussen L, Hansen P, Nabipour M, Olesen P, Kristiansen MT, Ledet T. Diverse effects of inhibition of 3-hydroxy-3-methylglutaryl-CoA reductaseon the expression of VCAM-1 and E-selectin in endothelial cells. J Biochem 2001; 360: 363-370

Rauch J, Janoff AS. Phospholipid in hexagonal II phase is immunogenic: evidence for immunorecognition of nonbilayer lipid phases in vivo. Proc Natl Acad Sci USA 1990; 87: 4112-4114

Rauch U, Osende JI, Chesebro JH,et al. Statins and cardiovascular diseases: the multiple effects of lipid-lowering therapy by statins. Atherosclerosis 2000; 153: 181-189.

Reber G, Roisin JP, Migaud M, de Moerloose P. Comparison of a platelet neutralization procedure with a hexagonal phase phospholipid neutralization assay as confirmatory tests for the presence of lupus anticoagulants. Thromb Res 1994; 73: 131-135

Reber G, Arvieux J, Comby E, Degenne D, de Moerloose P, Sanmarco M, Potron G. Multicenter evaluation of nine commercial kits for the quantitation of anticardiolipin antibodies. The Working Group on Methodologies in Haemostasis from the GEHT (Groupe d'Etudes sur l'Hemostase et la Thrombose). Thromb Haemost 1995; 73: 444-552

Reber G, Schousboe I, Tincani A, Sanmarco M, Kveder T, de Moerloose P, Boffa MC, Arvieux J. Inter-laboratory variability of anti-beta2-glycoprotein I measurement. A collaborative study in the frame of the European Forum on Antiphospholipid Antibodies Standardization Group. Thromb Haemost 2002; 88: 66-73

Reverter JC, Tassies D. Mechanisms of thrombosis in the antiphospholipid syndrome: binding to platelets. In Hughes Syndrome, Antiphospholipid syndrome. Edited by Khamashta MA. London: Springer-Verlag; 2000: 290-298

Robbins DL, Leung S, Miller-Blair DJ, Ziboh V. Effect of anticardiolipin/beta2-glycoprotein I complexes on production of thromboxane A2 by platelets from patients with the antiphospholipid syndrome. J Rheumatol 1998; 25: 51-56

Robert A. Two different incubation times for the activated partial thromboplastin time (APTT): a new criterion for diagnosis of lupus anticoagulant. Thromb Haemost 1994; 71: 220-224

Romano M, Mezzetti A, Marulli C, Ciabattoni G, Febo F, Di Ienno S, Roccaforte S, Vigneri S, Nubile G, Milani M, Davi G. Fluvastatin reduces soluble P-selectin and ICAM-1 levels in hypercholesterolemic patients: role of nitric oxide. J Invest Med 2000; 48: 183–189

Rosnoblet C, Vischer UM, Gerard RD, Irminger JC, Halban PA, Kruithof EK. Storage of tissue-type plasminogen activator in Weibel-Palade bodies of human endothelial cells. Arterioscler Thromb Vasc Biol 1999; 19: 1796-1803

Rosenson RS, Tangney CC, Casey LC. Inhibition of proinflammatory cytokine production by pravastatin. Lancet 1999; 353: 983-984

Roubey RA. Tissue factor pathway and the antiphospholipid syndrome. J Autoimmun 2000; 15: 217-220

Roubey RA, Eisenberg RA, Harper MF, Winfield JB. Anticardiolipin antibodies recognize beta2-glycoprotein I in the absence of phospholipid. J Immunol 1995; 154: 954-960

Rovere P, Sabbadini MG, Vallinoto C, Fascio U, Recigno M, Crosti M, Ricciardi-Castagnoli P, Balestrieri G, Tincani A, Manfredi AA. Dendritic cell presentation of antigens from apoptotic cells in a proinflammatory context: role of opsonizing anti-beta2-glycoprotein I antibodies. Arthritis Reum 1999; 42: 1412-1420

Rubanyi GM, Romero JC, Vanhoutte PM. Flow-induced release of endothelium derived relaxing factor. American Journal of Physiology 1986; 250:145-149

Sadeghi MM, Collinge M, Pardi R, Bender JR. Simvastatin modulates cytokine-mediated endothelial cell adhesion molecule induction: involvement of an inhibitory G protein. J Immunol 2000; 165: 2712-2718

Sammaritano LR, Gharavi AE, Lockshin MD. Antiphospholipid antibody syndrome: immunologic and clinical aspects. Semin Arthritis Rheum 1990; 81: 81-96

Sammaritano LR, Ng S, Sobel R, Lo SK, Simantov R, Furie R, Kaell A, Silverstein R, Salmon JE. Anticardiolipin IgG subclasses: association of IgG2 with arterial and/or venous thrombosis. Arthritis Rheum 1997; 40: 1998-2006

Sanghera DK, Nestlerode CS, Ferrell RE, Kamboh MI. Chimpanzee apolipoprotein H (beta2-glycoprotein I): report on the gene structure, a common polymorphism, and a high prevalence of antiphospholipid antibodies. Hum Genet 2001; 109: 63-72

Sanson BJ, Lensing AW, Prins MH, Ginsberg JS, Barkagan ZS, Lavenne-Pardonge E, Brenner B, Dulitzky M, Nielsen JD, Boda Z, Turi S, Mac Gillavry MR, Hamulyak K, Theunissen IM, Hunt BJ, Buller HR.Safety of low-molecular-weight heparin in pregnancy: a systematic review. Thromb Haemost 1999; 81: 668-672

Sardo MA, Castaldo M, Cinquegrani M, Bonaiuto M, Maesano A, Schepis F, Zema MC, Campo GM, Squadrito F,Saitta A. Effects of simvastatin treatment on sICAM-1 and sE-selectin levels in hypercholesterolemic subjects. Atherosclerosis 2001; 155: 143-147

Scandinavian Simvastatin Survival Study Group. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: Scandinavian Survival Study (4S). Lancet 1994; 344: 1383-1389

Schmidt A, Goepfert C, Feitsma K, Buddecke E. Lovastatin-stimulated superinduction of E-selectin, ICAM-1 and VCAM-1 in TNF-α activated human vascular endothelial cells. Atherosclerosis 2002; 164: 57-64

Schulman S, Svenungsson E, Granqvist S. Anticardiolipin antibodies predict early recurrence of thromboembolism and death among patients with venous thromboembolism following anticoagulant therapy. Am J Med 1998; 104: 332-338

Schulman S, Svenungsson E, Granqvist S. Anticardiolipin antibodies predict early recurrence of thromboembolism and death among patients with venous thromboembolism following anticoagulant therapy. Duration of Anticoagulation Study Group. Am J Med 1998;104: 332-338

Seeger H, Mueck AO, Lippert TH. Fluvastatin increases prostacyclin and decreases endothelin production by human umbilical vein endothelial cells. Int J Clin Pharmacol Ther 2000; 38: 270-272

Shapiro SS, Thiagarajan P. Lupus anticoagulants. Progress in Haemost and Thromb 1982; 6: 263-285

Shechter Y, Tal Y, Greenberg A, Brenner B. Platelet activation in patients with antiphospholipid syndrome. Blood Coagul Fibrinolysis 1998; 9: 653-657

Sheng Y, Kandiah DA, Krilis SA. Beta2-Glycoprotein I : Target antigen for 'antiphospholipid' antibodies. Immunological and molecular aspects. Lupus1998; 7 (S2): 5-9

Sheng Y, Reddel SW, Herzog H, Wang YX Wang YX, Brighton T, France MP, Robertson SA, Krilis SA. Impared thrombin generation in beta2-glycoprotein I null mice. J Biol Chem 2001; 276: 13817-13821

Shepherd J, Blauw G, Murphy MB, Bollen EL, Buckley BM, Cobbe SM, Ford I, Gaw A, Hyland M, Jukema JW, Kamper AM, Macfarlane PW, Meinders AE, Norrie J, Packard CJ, Perry IJ, Stott DJ, Sweeney BJ, Twomey C,Westendorp RG; PROSPER study group. PROspective Study of Pravastatin in the Elderly at Risk. on behalf of the PROSPER study group. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. Lancet 2002;360:1623-1630

Shi W, Chong BH, Hogg PJ, Chesterman CN. Anticatrdiolipin antibodies block the inhibition by beta2-glycoprotein I of the factor Xa generating activity of platelets. Thromb Haemost 1993; 70: 342-345

Shibata S, Harpel PC, Gharavi A, Rand J, Fillit H. Autoantibodies to heparin from patients with antiphospholipid antibody syndrome inhibit formation of antithrombin III-thrombin complexes. Blood 1994; 83: 2532-2540

Silver RM, Porter TF, van Leeuween I, Jeng G, Scott JR, Branch DW. Anticardiolipin antibodies: Clinical consequences of “low titers”. Obstet Gynecol 1996; 87: 494-500

Singh A, Immunogenesis of the antiphospholipid antibody syndrome: an update. Curr Opinion in Neurol and Hypertension 2001; 10: 355-358

Sletnes KE, Smith P, Abdelnoor M, Arnesen H, Wisloff F. Antiphospholipid antibodies after myocardial infarction and their relation to mortality, reinfarction, and non-haemorrhagic stroke. Lancet 1992; 339: 451-453

Smirnov MD, Triplett DT, Comp PC, Esmon NL, Esmon CT. On the role of phosphatidylethanolamine in the inhibition of activated protein C activity by antiphospholipid antibodies. J Clin Invest 1995, 95: 309-316

Sposito AC, Chapman MJ. Statin therapy in acute coronary syndromes: mechanistic insight into clinical benefit. Arterioscler Thromb Vasc Biol 2002; 22: 1524-1534

StenfloJ. Structure and function of protein C. Semin Thromb Haemost 1884; 10: 109-121

Sugi T, McIntyre JA. Certain autoantibodies to phosphatidylethanolamine (aPE) recognize factor XI and prekallikrein independently or in addition to the kininogens. J Autoimmun 2001; 17: 207-214

Sugiyama M, Kodama T, Konishi K, Abe K, Asami S, Oikawa S. Compactin and simvastatin, but not pravastatin, induce bone morphogenetic protein-2 in human osteosarcoma cells. Biochem Biophys Res Commun 2000; 271: 688-692

Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. Arterioscler Thromb Vasc Biol 2001; 2: 1712-1719

Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. Arterioscler Thromb Vasc Biol 2001; 21: 1712-1719

Teupser D, Bruegel M, Stein O, Stein Y, Thiery J. HMG-CoA reductase inhibitors reduce adhesion of human monocytes to endothelial cells. Biochem Biophys Res Commun 2001; 289: 838-844

Thiagarajan P, Pengo V, Shapiro SS. The use of dilute Russell viper venom time for the diagnosis of lupus anticoagulants. Blood 1986; 68: 869-874

Thiagarajan P, Shapiro SS, DeMarco L. Monoclonal immunoglobulin M coagulation inhibitor with phospholipid specificity: mechanism of a lupus anticoagulant. J Clin Invest 1980; 66: 397-405

Thrombosis prevention trial: randomised trial of low-intensity oral anticoagulation with warfarin and low-dose aspirin in the primary prevention of ischaemic heart disease in men at increased risk. The Medical Research Council's General Practice Research Framework. Lancet 1998; 351: 233-241

Tincani A, Spatola L, Cinquini M, Meroni P, Balestrieri G, Shoenfeld Y. Animal models of antiphospholipid syndrome. Rev Rhum Engl 1998; 65: 614-618

Tokita S, Arai M, Yamamoto N, Katagiri Y, Tanoue K, Igarashi K, Umeda M, Inoue K. Specific cross-reaction of IgG antiphospholipid antibody with platelet glycoprotein IIIa. Thromb Haemost 1996; 75:168-174

Triplett DA, Asherson RA. Pathophysiology of the catastrophic antiphospholipid syndrome (CAPS). Am J Hematol 2000; 65: 154-159

Triplett DA, Brandt JT, Kaczor D, Schaeffer J. Laboratory diagnosis of lupus inhibitors: a comparison of the tissue thromboplastin inhibition procedure with a new platelet neutralization procedure. Am J Clin Pathol 1983; 79: 678-682

Triplett DA. Protean manifestations of the antiphospholipid syndrome. Thromb Haemost 1995; 74: 329-337

Tzavara V, Vlachoyiannopoulos PG, Kordossis T, Galaris D, Travlou A, Dafni U, Moutsopoulos HM. Evidence for non-adaptive immune response in HIV infection. Eur J Clin Invest 1997; 27: 846-849

Urbich C, Dernbach E, Zeiher AM, Dimmeler S. Double-edged role of statins in angiogenesis signaling. Circ Res 2002; 90: 737-744

Uzui H, Harpf A, Liu M, Doherty TM, Shukla A, Chai NN, Tripathi PV, Jovinge S, Wilkin DJ, Asotra K, Shah PK, Rajavashisth TB. Increased expression of membrane type 3-matrix metalloproteinase in human atherosclerotic plaque: role of activated macrophages and inflammatory cytokines. Circulation 2002;106: 3024-3030

Vaarala O, Puurunen M, Manttari M, Manninen V, Aho K, Palosuo T. Antibodies to prothrombin imply a risk of myocardial infarction in middle-aged men. Thromb Haemost 1996; 75: 456-460

Van Aelst L, D'Souza-Schorey C. Rho GTPases and signaling nerworks. Genes Dev 1997; 11: 2295-2322

Vazquez-Mellado J, Lorente L, Richaud-Patin Alarcon-Segovia D. Exposure of anionic phospholipids upon platelet activation permits binding of Beta2-glycoprotein I and though it that of IgG antiphospholipid antibodies. Studies in platelets from patients with antiphospholipid syndrome and normal subjects. J Autoimmun 1994; 7: 335-348

Vega GL, Grundy SM. Effect of statins on metabolism of apo-B-containing lipoproteins in hypertriglyceridemic men. Am J Cardiol 1998; 81: 36B-42B

Vianna J, Munther A, Khamashta J, Font J, Cervera R, Lopez-Soto A, Tolosa C, Franz J, Selva A, Ingelmo M,et al. Comparison of the primary and secondary antiphospholipid syndrome: a Europian Multicenter Study of 114 patients. Am J Med 1994; 96: 3-9

Vila P, Hernandez MC Lopez-Fernandez MF Batlle J. Prevalence, follow-up and clinical significance of the anticardiolipin antibodiesin normal subjects. Thormb Haemost 1994; 72: 209-213

Visvanathan S, Geczy CL., Harmer JA, McNeil HP. Monocyte tissue factor induction by activation of Beta2-glycoprotein I-specific T lymphocytes is associated with thrombosis and fetal lossin patients with antiphospholipid antibodies. J Immunol 2000; 165: 2258-2262

Visvanathan S, McNeil HP. Cellular immunity to beta 2-glycoprotein-1 in patients with the antiphospholipid syndrome. J Immunol 1999; 162: 6919-6925

Vogel JJ, Reber G, de Moerloose P. Laboratory and clinical features in systemic lupus erythematosus patients with or without anticardiolipin antibodies. Thromb Res 1991; 62: 545-556

Vogt E, Ng AK, Rote NS. Antiphosphatidylserine antibody removes annexin Vand facilitates the binding of prothrombin at the surface of choriocarcinoma model of trophoblast differentiation. Am J Obstet Gynecol 1997; 177: 964-972

Wagner AH, Gebauer M, Guldenzoph B, Hecker M. 3-hydroxy-3-methylglutaryl coenzyme A reductase-independent inhibition of CD40 expression by atorvastatin in human endothelial cells. Arterioscler Thromb Vasc Biol 2002; 22: 1784-1789

Wahl DG, Bounameaux H, De Moerloose P, Sarasin FP. Prophylactic antithrombotic therapy for patients with systemic lupus erythematosus with or without antiphospholipid antibodies: do the benefits outweigh the risks? A decision analysis. Arch Intern Med 2000; 160: 2042– 2048

Walenga JM, Michal K, Hoppensteadt D, Wood JJ, Robinson JA, Bick RL. Vascular damage correlates between heparin-induced thrombocytopenia and the antiphospholipid syndrome. Clin Appl Thromb Hemost 1999; 5: (S 1): S76-84

Weitz-Schmidt G, Welzenbach K, Brinkmann V, Kamata T, Kallen J, Bruns C, Cottens S, Takada Y, Hommel U. Statins selectively inhibit leukocyte function antigen-1 by binding to a novel regulatory integrin site. Nat Med 2001; 7: 687-692

Weis M, Heeschen C, Glassford AJ Cooke JP. Statins have biphasic effects on angiogenesis. Circulation 2002;105: 739-745

Weitz-Schmidt G. Statins as anti-inflammatory agents. Trends Pharmacol Sci 2002; 23: 482-486

Werner N, Nickenig G, Laufs U. Pleiotropic effects of HMG-CoA reductase inhibitors. Basic Res Cardiol 2002; 97: 105-116

Werner N, Nickenig G, Laufs U. Pleiotropic effects of HMG-CoA reductase inhibitors. Basic Res Cardiol 2002; 97: 105-116

Wiener MH, Burke M, Fried M, Yust I. Thromboagglutination by anticardiolipin antibody complex in the antiphospholipid syndrome: a possible mechanism of immune-mediated thrombosis. Thromb Res 2001; 103: 193-199

Wiesbauer F, Kaun C, Zorn G, Maurer G, Huber K, Wojta J. HMG CoA reductase inhibitors affect the fibrinolytic system of human vascular cells in vitro: a comparative study using different statins. Br J Pharmacol 2002; 135: 284-292

Wilson WA, Gharavi AE, Koike T, Lockshin MD, Branch DW, Piette JC, Brey R, Derksen R, Harris EN, Hughes GR, Triplett DA, Khamashta MA. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. Arthritis Rheum 1999; 42: 1309-1311

Working Group on Haemostasis of the Sociéte Française De Biologie Clinique. Comparison of a standardized procedure with current laboratory practices for the detection of lupus anticoagulant in France. Thromb Haemost 1993; 70: 781-786

Zangari M, Lockwood CJ, Scher J, Rand JH. Protrombin activation fragment(F1.2) is increased in pregnant patients with antiphospholipid antibodies. Thromb Res 1997; 85: 177-183

Zimmerman H, Pearson JD. Extracellular methabolism of nucleotides and adenosine in the cardiovascular system. In Burnstock G, Dobson JG, Liang BT et al. Cardiovascular Biology of Purines, Boston: Kluwer, 1998; 342-358