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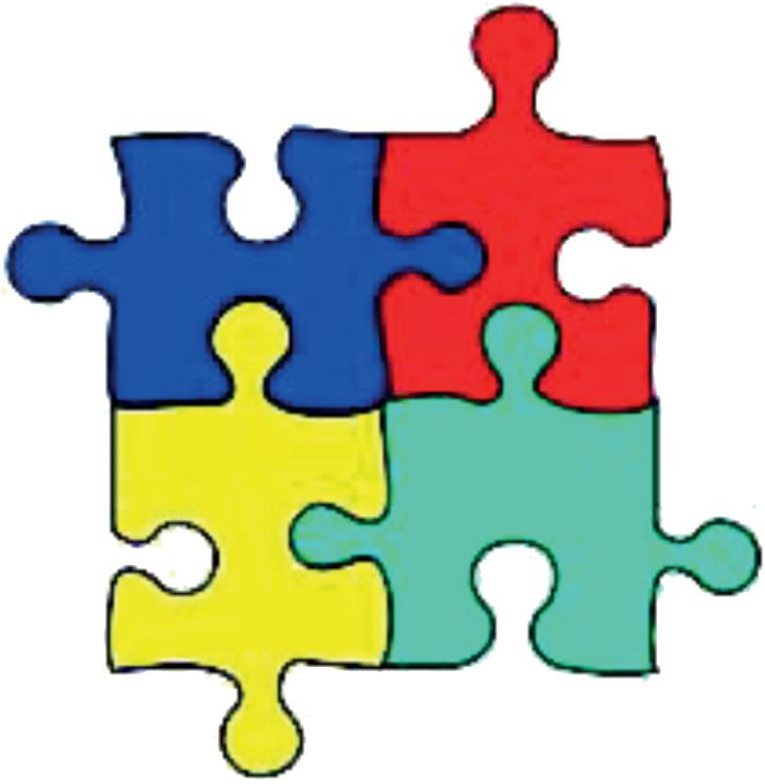
THROMBOTIC AND CARDIAC DISEASE IN THE ANTIPHOSPHOLIPID SYNDROME

Giorgia Grosso



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Thrombotic and Cardiac Disease in the Antiphospholipid Syndrome

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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I have come that they may have life,

and have it to the full

(John 10:10)

ABSTRACT

The antiphospholipid syndrome (APS) is a highly heterogeneous disease that presents with obstetric complications and/or thromboembolic events that can hit any side of the vascular tree in an unpredictable way. The common trait among all patients affected by the syndrome is the persistent presence of the antiphospholipid antibodies (aPL) and/or a prolonged coagulation time in vitro, the lupus anticoagulant (LA) test.

The pathogenesis of APS has not yet been clearly defined. Many molecules are involved in the maintenance of the equilibrium called hemostasis, interacting and cross-reacting in an incredibly perfect orchestrated balance that somehow, in APS, is broken. The aim of this thesis has been to try to shed some light on the complex mechanisms behind APS, by focusing on some of the many pieces of the puzzle that characterize the disease. The complement, coagulation and fibrinolytic systems have been the subjects of this fascinating journey, and two proteins in particular have been on focus: C4b-binding protein (C4BP) and Thrombin activatable fibrinolysis inhibitor (TAFI). These are both regulators of complement activation that at the same time play a role in coagulation. Moreover, the link between myocardial infarction (MI) and aPL/LA has been explored.

Paper I: TAFI and its activated form, TAFIa, were studied in patients affected by mainly primary APS (i.e. without other autoimmune diseases) and compared with a healthy control group. Moreover, C5a, a marker of complement activation, and markers of fibrinolysis were investigated and correlated with TAFI and TAFIa. Both TAFI and TAFIa are significantly higher in APS compared to controls. TAFIa is increased in APS patients affected by arterial thrombosis compared to other clinical manifestations, independently of traditional cardiovascular risk factors. TAFI is positively correlated with C5a, confirming its increase upon inflammation. TAFIa positively correlates with thrombomodulin, marker of endothelial damage/activation. The values of the clot lysis time (CLT) and of the permeability coefficient confirm impaired fibrinolysis in APS, with clots more resistant to lysis.

Paper II: we investigated the prevalence of aPL in a large and well-characterized cohort of patients after 6-10 weeks from a first MI, and compared it with age, gender and region matched controls. We demonstrated ten times higher prevalence of aPL of the IgG isotype in patients versus controls, independently of traditional cardiovascular risk factors, suggesting that IgG aPL positivity may be considered a potential risk factor for MI in the general population. No significant differences were observed for IgM and IgA isotypes.

Paper III: C4BP was investigated in a large cohort of patients affected by systemic lupus erythematosus (SLE), in primary APS and in controls. C4BP is lower in patients persistently positive for aPL and in patients treated with warfarin. C4BP correlates with markers of complement activation. Both persistent aPL positivity and warfarin are associated with C4BP reduction, and by means of a mediation analysis we were able to assess the relative contribution of these two variables: aPL have a direct reducing effect on C4BP of 11%, while warfarin contributes to 9% of the observed reduction.

Paper IV: After the results of paper III, we decided to study the effect of warfarin on complement and C4BP in the general population, comparing it with the direct oral anticoagulants (DOACs), during and after treatment discontinuation. Warfarin, as opposed to DOACs, is associated with increased markers of complement activation, which persist for at least three weeks after withdrawal. Higher C4BP levels characterize the patients after warfarin discontinuation, as a rebound effect. DOACs have no effect on complement, but, in contrast, we demonstrate that warfarin is associated with complement activation, partly but probably not only through inhibition of C4BP. This study is of relevance in the context of APS, since different anticoagulant mechanisms have been subjects of debate in recent years.

In conclusion, as also Ames stated regarding paper I[1], this thesis has tried to insert other linking pieces in the puzzle of APS. We confirm the presence of impaired fibrinolysis and complement activation in APS, and we have opened the path for a new era of research in the syndrome, where also the treatment with anticoagulants has to be considered for its potential impact on complement activation. Moreover, although causality could not be proven, we demonstrate that the prevalence of aPL after myocardial infarction in the general population is considerable and higher than generally anticipated.

LIST OF SCIENTIFIC PAPERS

I. **Thrombin activatable fibrinolysis inhibitor (TAFI) - A possible link between coagulation and complement activation in the antiphospholipid syndrome (APS)**
GIORGIA GROSSO, Anna Vikerfors, Barry Woodhams, Mariette Adam, Katarina Bremme, Margareta Holmström, Anna Ågren, Anna Eelde, Maria Bruzelius, Elisabet Svenungsson, Aleksandra Antovic
Thrombosis Research 2017, vol. 158:168-173

II. **Antiphospholipid antibodies in patients with Myocardial Infarction**
GIORGIA GROSSO, Natalie Sippl, Barbro Kjellström, Khaled Amara, Ulf de Faire, Kerstin Elvin, Bertil Lindahl, Per Näsman, Lars Rydén, Anna Norhammar, Elisabet Svenungsson
Annals of Internal Medicine 2019, vol. 170(4):277-280

III. **The complex relationship between C4b-Binding Protein, warfarin and antiphospholipid antibodies**
GIORGIA GROSSO, Kerstin Sandholm, Aleksandra Antovic, Iva Gunnarsson, Agneta Zickert, Anna Vikerfors, Lennart Truedsson, Maria Bruzelius, Bo Nilsson, Kristina Nilsson-Ekdahl, Elisabet Svenungsson
Thrombosis and Haemostasis, 2021

IV. **Anticoagulants and complement**
Kerstin Sandholm*, Manal Ibrahim-Kosta*, **GIORGIA GROSSO**, Aleksandra Antovic, Maria Hårdstedt, Camilla Mohlin, Oskar Eriksson, Elisabet Svenungsson, Bo Nilsson, Pierre-Emmanuel Morange, Maria Bruzelius, Kristina Nilsson-Ekdahl
Manuscript

* = equal contribution

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LIST OF ABBREVIATIONS

Anti-β_2GPI	Anti-β_2 glycoprotein I Antibodies
aCL	Anti-Cardiolipin Antibodies
ACS	Acute Coronary Syndrome
AI	Auto-Immune diseases
APC	Activated Protein C
aPL	Antiphospholipid Antibodies
APS	Antiphospholipid Syndrome
aPS/PT	anti-prothrombin/phosphatidylserine Antibodies
aPT	Anti-prothrombin Antibodies
aPTT	activated Partial Thromboplastin Time
AT	Antithrombin
AUC	Area Under the Curve
β_2GPI	β_2 glycoprotein I
BMI	Body Mass Index
C1INH	C1 inhibitor
CABG	Coronary Artery Bypass Graft
CAD	Coronary Artery Disease
CAPS	Catastrophic Antiphospholipid Syndrome
CCP	Complement Control Protein
CFH	Complement Factor H
CFI	Complement Factor I
CHD	Coronary Heart Disease
CI	Confidence Interval
CLT	Clot Lysis Time
CR	Complement Receptor
CRP	C-Reactive protein
Crry	C3 convertase inhibitor complement receptor 1 related gene/protein y
CV	Cardio-Vascular
C4BP	C4b-binding protein
DAF	Decay Accelerating Factor

DIC	Disseminated Intravascular Coagulation
DOAC	Direct Oral Anticoagulants
dRVVT	dilute Russell's Viper Venom Time
DVT	Deep Venous Thrombosis
EC	Endothelial cell
ELISA	Enzyme-linked Immunosorbent Assay
EMP	Endothelial-derived Microparticle
EPCR	Endothelial Protein C Receptor
ESR	Erythtrocyte Sedimentation Rate
ETP	Endogenous Thrombin Potential
GAPSS	Global Anti-Phospholipid Syndrome Score
HCQ	Hydroxychloroquine
HELLP	Hemolysis-Elevated Liver enzymes-Low Platelets
HIT	Heparin-Induced Thrombocytopenia
HMWK	High Molecular Weight Kininogen
ICAM-1	Intercellular Adhesion Molecule 1
IHD	Ischemic Heart Disease
IS	Ischemic Stroke
ISTH	International Society on Thrombosis and Haemostasis
LA	Lupus Anticoagulant
LDA	Low-Dose Aspirin
LMWH	Low Molecular Weight Heparin
LRP8	Low-density lipoprotein receptor-related protein 8
mAb	Monoclonal antibody
MASP-2	Mannose Associated Serine Protease 2
MCP	Membrane Cofactor Protein
MetS	Metabolic Syndrome
MI	Myocardial Infarction
MoMP	Monocyte-derived Microparticles
MP	Microparticle
MyD88	Myeloid differentiation factor 88

NET	Neutrophil Extracellular Trap
NFkB	Nuclear factor-κB
NSTEMI	Non ST-Elevation Myocardial Infarction
ODU	Optical Density Units
OR	Odds Ratio
oxLDL	Oxidized Low Density Lipoprotein
p38MAPK	P38 Mitogen-Activated Protein Kinase
PAI-1	Plasminogen Activator Inhibitor-1
pAPS	Primary Antiphospholipid Syndrome
PCI	Protein C Inhibitor
PE	Pulmonary Embolism
PF4	Platelet Factor 4
PLT	Platelet
PMP	Platelet-derived Microparticle
PS	Protein S
RA	Rheumatoid Arthritis
ROS	Reactive Oxygen Species
sAPS	Secondary Antiphospholipid Syndrome
SD	Standard Deviation
SEM	Scanning Electron Microscopy
SLE	Systemic Lupus Erythematosus
SLEDAI	SLE Disease Activity
SSC	Scientific and Standardization Committee
STEMI	ST-Elevation Myocardial Infarction
TAFI	Thrombin Activatable Fibrinolysis Inhibitor
TAFIa	Activated form of TAFI
TAT	Thrombin-Antithrombin
TCC	Terminal Complement Complex
TFPI	Tissue Factor Pathway Inhibitor
TG	Thrombin Generation
TLR	Toll-Like Receptor

TM	Thrombomodulin
TP	Triple Positive
tPA	tissue Plasminogen Activator
TTP	Thrombotic Thrombocytopenic Purpura
TX	Thromboxane
UA	Unstable Angina
UFH	Unfractioned Heparin
VCAM-1	Vascular cell adhesion protein 1
VDRL	Venereal Disease Research Laboratory
VTE	Venous Thrombo-Embolism
VWF	Von Willebrand Factor
WR	Wasserman reaction

1 INTRODUCTION

1.1 The Antiphospholipid Syndrome

The Antiphospholipid syndrome (APS) is defined by arterial, venous, small vessel thrombosis and/or obstetric morbidity together with confirmed positive tests for autoantibodies of the IgG and IgM isotypes targeting β_2 glycoprotein I (β_2GPI), cardiolipin (CL) or positivity in the functional lupus anticoagulant test (LA)[2]. APS is also associated with other "non-criteria" features, such as thrombocytopenia, heart valve disease[3], neurological, cutaneous, renal disorders, ocular problems and diffuse alveolar hemorrhage[4-6]. Primary (p) APS refers to the development of the syndrome independently of other autoimmune disorders, whereas secondary (s) APS is defined by the concomitant presence of other rheumatic diseases, most frequently Systemic Lupus Erythematosus (SLE).

By definition, the disease is diagnosed when a thrombotic or obstetric event has already occurred, so the major therapeutic challenge is to avoid further events (secondary prevention). In case of SLE or in the presence of non-criteria manifestations, primary prevention can also be an issue. Therefore it is important to be able to identify those patients that are at increased risk for future thrombotic or obstetric events, so that closer monitoring and earlier treatment can be applied.

1.2 History and classification criteria

The first detection of the antiphospholipid antibodies (aPL) occurred by chance, when August Paul von Wassermann developed a first test for the detection of syphilis, based on complement fixation, in 1906. *Spirocheta pallida* had been identified just the previous year, and there were no available direct microbiological tests at the moment. He had the idea of detecting complement activation in serum of patients affected by the infection, in response to the formation of antigen-antibody complexes, after introducing the antigen. The antigen used was initially derived from the causative agent *Treponema Pallidum*, later replaced by bovine tissues with similar results. In 1941 cardiolipin was identified as antigen. Cardiolipin is a phospholipid extracted from the heart or muscles of bovines. Patients with antibodies for syphilis tested positive for the so-called "Wasserman test", but it soon became clear that also some patients not affected by the infection were positive for the Wasserman reaction (WR), or for the later employed Venereal Disease Research Laboratory (VDRL) test for syphilis. Later on, it was demonstrated that these patients had antibodies against cardiolipin, but no syphilis infection.

In 1952 Moore and Mohr tried to understand and explain the reasons behind false positive serologic test results. They identified autoimmune diseases such as SLE, Rheumatoid arthritis and Sjögren's syndrome as conditions associated with a persistent (> 6 months) false positivity for the syphilis tests. In the same year Conley and Hartman reported two cases of hemorrhagic disorders associated with SLE and the presence of a so-called "circulating anticoagulant". Subsequent research in the fifties demonstrated an association between false positive WR tests and patients affected by SLE who had prolonged clotting times in vitro, which were not corrected by the addition of healthy donor plasma.

In 1963 Bowie et al identified the paradoxical association between thrombotic events and the circulating anticoagulant. In 1972, Feinstein and Rapaport coined the name "Lupus Anticoagulant (LA)" to define this phenomenon of prolonged clotting time in phospholipid-dependent assays. In the early 70s, Feinstein and Schleider observed that, by adding phospholipids to the in vitro clotting mixtures, the clotting time tended to normalize, and vice versa, by reducing the phospholipid content, the effect of LA was potentiated.

In 1983 Harris et al[7] described the first radioimmunoassay, then ELISA (see also 1.7.3), for the detection of anticardiolipin antibodies (aCL), with a 200 to 400-fold higher sensitivity than the precipitation method of the VDRL. In the same year, Hughes et al[8] wrote about the strong correlation between LA and aCL with thrombosis, spontaneous abortions and neurological abnormalities, especially (but not only) in patients affected by SLE.

In 1986 Hughes, Harris and Gharavi gave the first definition of "The Anticardiolipin Syndrome"[9], coined to link thrombosis and other typical clinical manifestations with the presence of aCL/LA.

Another major advance took place in 1990, when β_2GPI was discovered to be the main "cofactor" for the binding of aPL to cardiolipin on ELISA plates[10-12].

Two sets of classification criteria have been developed to define APS, referred to as the Sapporo criteria (1999)[13] and the Sydney criteria (Miyakis 2006)[2]. These criteria also define cut-offs and laboratory techniques to correctly identify the antibodies[7,14-18]. Therefore, when comparing actual with previous studies, it is important to take into account which set of classification criteria is used and also the methods employed to measure aPL/LA in the study of interest.

The main upgrades brought by the more recent Sydney criteria are the following:

- the stratification of APS patients according to the presence or absence of other risk factors for thrombosis/CV disease, together with older age (>55 men, >65 women) to avoid misclassification bias (more than 50% of APS patients have such risk factors)

- the addition of anti-β_2GPI IgG/IgM
- the introduction of a clear, quantifiable threshold for positivity at 99th percentile or >40 GPL/MPL (see 1.7.3), instead of moderate-high
- the sub-classification based on positivity for multiple vs single aPL and for which isotype/antigen
- the introduction of a temporal interval between aPL determination and clinical event that should be at least 12 weeks but no more than 5 years
- the prolongation of the interval to confirm persistence presence of aPL to 12 instead of 6 weeks, in order to increase specificity
- the introduction of clearer definitions of other features associated with APS, although these are still considered "non-criteria features" because of insufficient evidence of association with aPL

1.3 Epidemiology

The exact prevalence and incidence of APS are still unknown, but a recent estimate by Duarte-García et al[19] has been 50 cases (95% CI: 42-589) per 100 000 persons and 2.1 new cases (95% CI: 1.4-2.8) per 100 000 persons/year respectively, with no significant difference between men and women.

aPL are present in 30-40% of SLE patients and they are associated with thrombosis in circa one third of SLE-aPL+ (10-15% of total SLE)[20].

Information on epidemiologic characteristics of the disease have been described in specific disease cohorts, such as SLE patients[21] or through the Euro-Phospholipid project by Cervera et al[22-26], an observational study including 1000 APS patients from 13 European countries. This project could take place thanks to the efforts of the European Forum on Antiphospholipid Antibodies, a network of centers devoted to perform international collaborative studies on aPL. Therefore, since data have been gathered mainly by rheumatologists from a highly selected cohort, overestimations are possible. Data collection started in 1999, followed by two follow-up studies after 5 and 10 years, the last one published in 2015. The female-to-male ratio among patients with primary APS was 5, which decreased to 3.5 after excluding patients with SLE, and to 1.0 after excluding obstetric APS. The most frequent clinical manifestations at inclusion were DVT (38.9%), thrombocytopenia (29.6%), stroke (19.8%), pulmonary embolism (14.1%). During a 10-year follow-up, thrombocytopenia (8.7%) and stroke (5.3%) occurred most commonly. Early pregnancy loss was the most common obstetric manifestation (35.4%).

Regarding the mortality rate, 9.3% patients died in 10 years of follow-up, divided into 5.3% in the initial 5 years and 4% in the following 5 years follow-up. The unadjusted Standardized Mortality Ratio (SMR) for the total cohort, defined as the observed number of deaths over those expected from the general population of the studied area, was 1.8 (95% CI 1.5 to 2.1). The major causes of death during the 10-year period of observation were bacterial infections (21.5%), myocardial infarctions (MI) (13.9%), malignancies (13.9%), strokes (11.8%) and hemorrhages (10.7%).

The overall frequency of mortality of APS patients reported by Duarte-Garcia et al[19] in the period 2000-2015 was not notably different from that observed in the general population belonging to the same geographic region (SMR 1.6, 95% CI 0.74-3.05). Nevertheless, they reported a lower 5 and 10-year survival rate compared to Cervera et al[25], probably attributed to the fact that their inception cohort was older.

1.4 Catastrophic Antiphospholipid Syndrome (CAPS)

Catastrophic APS (CAPS) was described in 1992 by Asherson[27] as a life-threatening form characterized by rapidly occurring thrombosis, often affecting the microcirculation, in three or more organs, in the presence of aPL[28-31]. The preliminary classification criteria were issued in the occasion of the 10th international congress on aPL held in Taormina in 2002. Definite CAPS is defined when all the following four criteria are met: 1. Evidence of involvement of three or more organs, systems and/or tissues, 2. Development of manifestations in less than a week or simultaneously, 3. Confirmation by histopathology of small vessel occlusion in at least one organ or tissue, 4. Laboratory confirmation of the presence of aPL/LA. Probable CAPS is considered if only two targets are affected, if small vessel occlusion cannot be confirmed by histopathology, or if the third event develops in more than a week but less than a month despite anticoagulation, or if a patient is aPL+ but has never been tested before. CAPS belongs to the spectrum of the Thrombotic Michroangiopatic Syndromes, together with Thrombotic Thrombocytopenic Purpura (TTP), Disseminated Intravascular Coagulation (DIC), Heparin-Induced Thrombocytopenia (HIT), Hemolysis-Elevated Liver enzymes-Low Platelets (HELLP) and the newly discovered Covid-19 related thrombotic disorder. It affects ca 0.8% of APS cases and it has a very high mortality, because of multiorgan failure. It is important to underline that the mortality rate has decreased during the last decade (from 50% to 37%)[32], because of treatment improvements. This could be achieved thanks to the efforts of the European Forum on aPL that, in 2000, decided to start to collect data on CAPS cases from different European centers,

given the rarity of the syndrome and the difficulty to gather representative data from a single center. This gave life to the CAPS Registry, an international registry of patients with catastrophic APS. From this registry we have learnt that many CAPS patients have precipitating factors. Such factors were identified in ca 65% of cases, and the most commonly reported are infections (49%), surgery (17%), cancer (16%). The organs most often involved are the kidney (73%), the lungs (66%) in the form of acute respiratory distress syndrome (ARDS) or pulmonary embolism (PE), and the central nervous system (56%) where stroke and encephalopathy are common.

1.5 Pathogenesis

Both environmental and genetic factors contribute to susceptibility to the syndrome.

As far as **genetic** predisposition is concerned, an association between the HLA system and aPL production has been reported in different studies[33-35]. HLA-DRB1*07, HLA-DRB1*04, HLA-DRB1*13, HLA-DQB1*0302 polymorphisms are some examples of HLA class II alleles associated with aPL.

The HLA system plays a role in antigen presentation from antigen presenting cells to effector T-cells, that in turn activate B-cells to produce antibodies directed against those antigens, for example β_2GPI. Other polymorphisms potentially associated with APS have been found in target antigens, coagulation factors, or complement inhibitory proteins[36-39]. The gene encoding for β2GPI is located on chromosome 17q23 and some polymorphisms of single nucleotides have been associated with anti-β_2GPI production, the most popular being at position 247, confirmed by different studies[40-43], but not by all[44]. This single nucleotide change results in one amino acid substitution, the expression of Valine rather than Leucine, in domain V of the protein. This and other polymorphisms have been suggested to increase the antigenicity of the protein, leading to antibody production[45].

Environmental factors that have been associated with APS are infections[46], previous smoking[47], oxidative stress[48], trauma, vaccinations, drugs, increased apoptosis, e.g. during elevated cell turnover as in cancer, or in conditions of decreased clearance as in SLE.

Infections can trigger B-cells to produce aPL through molecular mimicry, a cross-reaction between epitopes of infectious agents and β_2GPI[49,50]. Some aPL can also be considered natural autoantibodies, defined as immunoglobulins present in the absence of exogenous antigen stimulation, that play a role in the innate immune system. aPL are known to increase transiently during infections[51] and may become pathogenic in case of adverse conditions, e.g. oxidative stress[45]. Transient aPL, i.e. aPL not confirmed by a second determination, are

generally not believed to be associated with thrombosis and consequently they are not considered a reason for anticoagulation. However, no studies have assessed the risk of thrombosis during the presence of transient antibodies[52]. This assumption should therefore be questioned as e.g. suggested by de Groot and Urbanus[53].

Apoptotic cells express phosphatidylserine on the outer membrane leaflet, so that phospholipid-binding proteins can bind. In case of excess of apoptosis or dysregulation in clearance of apoptotic cells, aPL production may be induced by an excess of antigen[54,55]. A mechanism can be due to maturation of antigen-presenting cells (dendritic cells) that stimulate T-helper cells, followed by subsequent B-cells antibody production against phospholipid-binding proteins[56]. Moreover, aPL may promote Fc receptor-dependent phagocytosis, which may have pro-inflammatory sequelae[57], among which is the activation of complement via the classical pathway.

The possible **antigenic targets** for aPL are[58,59]:

- the main antigens: β_2GPI (also in complex with oxLDL), phospholipids (e.g. vimentin, phosphatidylethanolamine, phosphatidylserine, cardiolipin, phosphatidylinositol)
- natural anticoagulants: protein C, protein S (also in complex with C4BP), annexin II and V[60], tissue factor pathway inhibitor (TFPI), antithrombin (AT)
- proteins of the fibrinolytic system: tissue plasminogen activator (tPA), plasmin
- coagulation factors: fXII, fXI, fVII, fV, high-molecular weight kininogen (HMWK), prothrombin (fII), thrombin (fIIa)

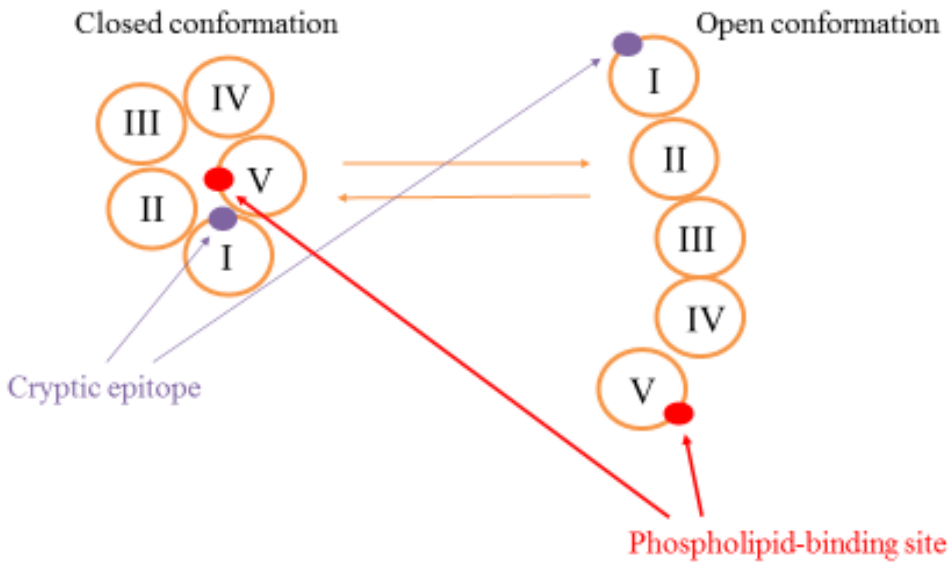
Some of these proteins are serine proteases that share epitopes with β_2GPI in their catalytic domains.

Beta$_2$ glycoprotein I (β_2GPI) is by many believed to be the predominant and specific antigenic target in APS[61]. The β_2GPI molecule is a single chain, highly glycosylated glycoprotein of 54 kDa molecular weight. It circulates in plasma at a concentration of 200μg/ml, of which 40% is bound to lipoproteins. It is produced by the liver, trophoblasts, endothelial cells, monocytes and platelets. It consists of 326 amino acids, organized into five domains, also called Short Consensus Repeats (SCR) or Complement Control Protein (CCP) modules. Domain V is made of 80 amino acids and it is found at the carboxyl-terminal, whereas domain I to IV are composed of 60 amino acids each and contain the amino-terminal portion of the glycoprotein. Domain V is an atypical SCR that contains a lysine-rich sequence, positively charged, that facilitates the binding of β_2GPI to anionic phospholipids, and it also binds to heparin[62], lipoproteins

and activated platelets with high affinity, through charge interactions. The affinity of β_2GPI to phospholipid surfaces is low, but increases by 100-fold in the presence of anti-β_2GPI. β_2GPI belongs to the Complement Control Protein (CCP) family, therefore it has both anticoagulant and complement regulatory functions (see section 1.10.4), and can assume different conformations[63] depending on pH, temperature, oxidative stress. It circulates in plasma in a closed, ring-like conformation, through the binding of Arginine (Arg)[39] and Arg[43] in domain I to Lysine (Lys)[305] and Lys[317] in domain V. When domain V is instead associated to negatively charged phospholipids, β_2GPI assumes a fish-hook like conformation, thereby exposing the cryptic epitope (Arg[39] and Arg[43]) on domain I[64] (figure 1). When two molecules of β_2GPI are close to each other in the open conformation, they attract aPL, that bind and dimerize the protein, fixing it in an active conformation, with increased immunogenicity[65].

Figure 1 – β_2 Glycoprotein I

Model of the conversion of β_2GPI from closed and circular on the left, into an open, hockey-stick like conformation on the right, assumed after binding to phospholipids. The latin numerals indicate the different domains, or CCPs/SCR. Note that the antibody-binding site (the cryptic epitope) is not accessible to the autoantibodies in the circular conformation.



Cell activation: it has been demonstrated that aPL are able to activate endothelial cells (ECs), monocytes, platelets (PLTs), and neutrophils, through intracellular signaling[20,66-70]. aPL, after binding to their target receptors, different for different cells, are transported to the nucleus via endosomal uptake, with different endocytic routes according to the cell type[71]. Once there, the complexes aPL-receptor are able to induce protein synthesis through other mediators in the intracellular signaling pathways, such as the adapter molecule Myeloid differentiation factor 88 (MyD88)[72], with subsequent activation and translocation of Nuclear factor-κB (NFkB) from cytoplasm to the nucleus (the same signaling molecules implicated in lipopolysaccharides or IL-1 cascade in endothelial cells) or through the activation of p38 mitogen-activated protein kinase (p38MAPK)[73], with subsequent phosphorylation of cytosolic phospholipase A2 (mainly in platelets)[74]. The activated cells express pro-coagulant/pro-inflammatory phenotypes that contribute to create a milieu prone to thrombosis.

Endothelial cells express Annexin II, a membrane-bound receptor for tissue plasminogen activator (tPA) and plasminogen, that lacks a transmembrane domain to intracellular pathway signaling. Annexin II has pro-fibrinolytic properties because it enhances tPA-dependent conversion of plasminogen into active plasmin at the endothelial cell surface, even in the absence of fibrin[75]. β_2GPI in its monomeric form can bind to Annexin II[76], targeting ECs for activation by aPL[77]. Other receptors that have been reported to act as mediators in β_2GPI-induced endothelial cell activation are Toll-like receptors (TLR) 2[78] and 4[79], pro-thrombotic and pro-inflammatory molecules that usually bind to microbial structures, but can also bind to β_2GPI.

Once activated, ECs express Tissue Factor (TF), pro-inflammatory cytokines, and adhesion molecules, such as Intercellular Adhesion Molecule 1 (ICAM-1), Vascular cell adhesion protein 1 (VCAM-1) and E-selectin[80].

Monocytes, once activated**,** mostly via Toll-like receptors 2 and 8, increase TF production, expression and release in plasma[81]. TF is an inducible glycoprotein that triggers the extrinsic coagulation pathway, through binding with fVII on the cell surface. A study by Cuadrado et al[82] demonstrated, by flow cytometry, that patients with primary (p)APS, especially those who are IgG aCL positive (+), have higher levels of TF expressed on the surface of monocytes, and also in plasma, compared to aPL-carriers, patients affected by thrombosis lacking aPL and healthy controls. pAPS patients also express more TF-related pro-coagulant activity on intact cells and cell lysates, as

showed by a chromogenic assay. No difference related to anticoagulant treatment was recorded. These results were confirmed by other groups[83].

Platelets (PLTs) are activated by the binding of aPL to phosphatidylserine and Low-Density Lipoprotein (LDL) receptor-related protein 8 (LRP8), also called Apolipoprotein E Receptor 2' (ApoER2'). β_2GPI directly binds to Glycoprotein Ibα (GPIbα)[84], a receptor for von Willebrand factor (VWF), among others[85], making it less available for platelet aggregation. Anti-β_2GPI, through competitively binding to β_2GPI, are supposed to activate platelets also through the increased availability of GPIbα for VWF, that derives from less β_2GPI binding to GPIbα[86]. Once activated, PLTs increase expression of glycoprotein IIb/IIIa (GPIIb/IIIa) and Thromboxane (TX) A2, the major eicosanoid produced by platelets with potent pro-aggregant and vasoconstrictor activities[87].

Anti-β_2GPI can activate **neutrophils** through TLR4 and reactive oxygen species formation. Activated neutrophils play a role in both inflammation and coagulation through NETosis (Neutrophil Extracellular Traps)[88]. NETs are a network of extracellular fibers released by neutrophils with the aim of catching microorganisms, preventing them from spreading. They are made of nuclear and granular components like chromatin, histones, dsDNA, Myeloperoxidase (MPO), Neutrophil Elastase (NE), and other microbicidal enzymes[89]. NETs bind and activate platelets, TF and fVII, and inactivate TFPI, accelerating thrombus formation[90]. They also inhibit fibrinolysis.

Activated ECs, PLTs and monocyte release **microparticles** (**MPs**). MPs are small vesicles (0.1-1μm), membrane-coated, released by exocytosis, that express anionic phospholipids (mainly phosphatidylserine), together with other surface antigens from the cell of origin. This phospholipid overexposure supports the assembly of clotting enzyme complexes with subsequent thrombin generation and increased thrombotic risk[91-95], as well as binding of β_2GPI[96,97]. A recent study by our group[97] reports that β_2GPI expressing-MPs are less frequent in the subset of aPL+ as compared to aPL negative (-) SLE patients. The hypothesis is that anti-β_2GPI bind to β_2GPI on the surface of MPs, thus hiding them from macrophage recognition and clearance. Many studies show that MPs are increased in APS/aPL+ subjects as compared to controls, in particular Endothelial-derived MPs (EMPs) and Monocyte-derived MPs (MoMPs)[94,95,98,99]. Numerous circulating MPs are also a possible source of autoantigens that can trigger production of autoantibodies. Another pro-coagulant mechanism involves platelet-derived MPs (PMPs): because of the abundance of fVa receptors on

their membranes, they provide a large catalytic surface for the prothrombinase reaction[96]. Other studies show the possible regulatory functions of MPs[93], e.g. when they mostly express Tissue Factor Pathway Inhibitor (TFPI), Endothelial cell Protein C receptor (EPCR), or Thrombomodulin (TM), or when PMPs bind to free protein S, stimulating APC binding, thus accelerating fVa inactivation[100]. The balance between pro-and anti-coagulant properties of MPs could be impaired by the presence of aPL, e.g. by binding to free protein S or to β_2GPI-phosphatidylserine on MP surfaces.

Dysregulation in hemostasis and inflammation represent the key mechanisms that foreshadow thrombosis. They depend on three enzymatic cascades that share close similarities in structure and function and relate tightly to each other by several crossover points: coagulation, fibrinolysis and complement[1,101-106] (see also 1.10.1).

Coagulation is enhanced by aPL-mediated platelet activation and increased TF production (see above). Moreover, aPL have been shown to inhibit natural anticoagulants such as protein S, protein C, annexin V[60], antithrombin and TFPI, among others.

Fibrinolysis may be impaired by interaction of aPL with annexin II and β_2GPI that both bind tPA on endothelial cells and promote fibrinolysis[107]. Other postulated mechanisms to explain hypofibrinolysis in APS consist in the increased activity of plasminogen activator inhibitor type 1 (PAI-1)[108], TAFIa, the presence of antibodies specifically directed against tPA[108], plasminogen and plasmin[109].

Complement is triggered mainly through the classical pathway initiated by antibody-antigen binding. It can activate ECs either directly through sC5b-9, or indirectly by C5a receptor. C5a can also activate neutrophils, with consequent NET and TF release[110]. Complement boosts inflammation, it enhances the release of reactive oxidants, proteolytic enzymes, pro-inflammatory cytokines. It induces trophoblast injury in pregnancy and can lead to fibrin deposition and thrombosis, by interacting with the coagulation system.

Unresolved questions, hypothesis regarding pathogenic aspects of APS: The activation of cells and systems that are ubiquitous in the human body and the presence of aPL that circulate systemically may explain why APS is characterized by thrombosis in almost every vessel of the body, in both arteries and veins, large vessels and microcirculation, as well as in the placenta. Nevertheless, it is yet not explained exactly how the systemic presence of aPL results in local thrombosis, and biomarkers that are able to predict the specific clinical phenotype of each and every patient have not been discovered yet.

It is known that aPL are an heterogeneous group of auto-antibodies with different sub-specificities[111] and this might contribute to tilt the balance preferentially towards one clinical manifestation rather than another. Local factors are also important, like the endothelium. In fact, hemostasis derives from a finely tuned balance between pro- and anti-coagulant forces and the endothelium plays a key role in keeping this balance. The endothelium is an organ itself, highly heterogeneous, a metabolically active interface between blood and tissues. It responds with a different distribution of receptors and cell-specific signaling pathways to local factors, differently expressed in time and space throughout the vascular tree[112,113], e.g. the extracellular milieu or even hemodynamic forces, namely the shear-stress and the cyclic strain, via mechanoreceptors. It is responsible for vasomotor tone through interactions with vascular smooth muscle cells and pericytes. It acts on vessel permeability, regulating the paracellular and transcellular exchange of fluids, ions and macromolecules between tissues and blood, as well as leukocyte adhesion and trafficking, recruiting them to areas of tissue damage via adhesion molecules and release of cytokines. It is crucial in regulating inflammation and hemostasis[114]. "If the arteries and veins of the body represent the interstate highways, and the capillaries the neighborhood streets, the endothelium may be thought of as the city sidewalk, brimming with life, commerce, and activity" (cit from Aird)[113].
Another research question in the pathogenesis of this disease is why the clinical phenotypes are so heterogeneous, i.e. why some individuals, despite being positive for aPL, remain asymptomatic, whereas others are hit by CAPS, the most severe form of the disease.
The "second hit" hypothesis[66] has been suggested to explain this clinical observation: a first hit, represented by the presence of aPL, alters the milieu in favor of a thrombophilic state, but a second hit that alters endothelial integrity e.g. infections, surgery, oxidative stress, complement activation, is necessary to trigger thrombus formation.
APS clinical manifestations derive from a systemic imbalance that meets a local specific imbalance, as expressed by the old Virchow's triad: a hypercoagulable state that interacts with a local stasis of flow or loss of vascular integrity for whatever reason, leading to thrombosis.

1.6 Obstetric Antiphospholipid Syndrome

According to Miyakis classification criteria[2], obstetric APS is defined by the same laboratory criteria as thrombotic APS, together with the presence of a history of three early consecutive miscarriages (< 10 weeks of gestation), and/or one miscarriage or stillbirth after 10 weeks of gestation, and/or one intra-uterine growth restriction or a premature birth before 34 weeks of gestation due to preeclampsia, eclampsia or placental insufficiency. Placental insufficiency is defined by abnormal or non-reassuring fetal surveillance test, abnormal Doppler flow velocimetry waveform analysis suggestive of fetal hypoxemia, oligohydramnios, and postnatal birth weight less than the 10^{th} percentile for the gestational age.

In order to be classified as APS, maternal anatomic or hormonal abnormalities, and paternal and maternal chromosomal causes of miscarriage should be excluded, and the fetus should be morphologically normal at ultrasound or by direct examination.

Obstetric APS may be characterized by different pathogenic mechanisms than thrombotic APS[115]. In mice models, the injection of aPL IgG can cause fetal damage without the requirement of a second hit, provided that the complement system is intact[116]. In humans, a very recently published study by Pregnolato et al[117] enlightens the association of even low titers aPL with APS obstetric complications, boosting the concept that thrombotic and obstetric APS may constitute distinct clinical entities. The biological explanation can be that β_2GPI is not expressed on resting vascular endothelium, whereas it is abundant on trophoblasts[20,118,119] even under physiological conditions. Moreover, thrombosis is not the main feature of obstetric APS[120], although present. Some mechanisms leading to thrombosis consist in the ability of aPL to disrupt the annexin V anticoagulant shield on trophoblast monolayers[60]. Nevertheless, defective placentation and inflammation are considered the major causes of pregnancy morbidity in APS, leading to impaired feto-maternal blood exchange. This is mediated by the expression of a decidual inflammatory phenotype and trophoblast damage. Experimental studies show that anti-β_2GPI can trigger placental inflammation through TLR4/MyD88 signaling pathway in trophoblasts[121]. This leads to the inhibition of trophoblasts proliferation and invasiveness, defective Human Chorionic Gonadotropin release and increased apoptosis.

Complement activation is also involved in placental inflammation (see section 1.10). It is still an open question if the predominant mechanism leading to complement activation is to be attributed to a dysregulation of complement inhibitory proteins or to an excess of complement activation products induced by aPL[122]. In fact, complement activation products are detected in normal placentas as well, but to a less extent and without associated tissue

damage, probably because of highly expressed complement regulatory proteins on cytotrophoblasts in healthy pregnancies.

1.7 Associations between thrombosis and aPL/LA

1.7.1 Prevalence of thrombosis in aPL/LA positive patients

A clear answer to the question of the prevalence of thrombotic events in aPL/LA positive patients and which aPL are the best predictor for thrombosis is not easy to give. Studies so far have been very heterogeneous in terms of:

- study design and enrollment/exclusion criteria
- isotype of aPL considered
- cut-offs and laboratory methods
- temporal interval between confirmation of aPL/LA tests and between aPL/LA determination and thrombotic event
- APS classification criteria (Sapporo or Sydney)
- type (first/recurrent) and site (arterial/venous/microthrombosis) of thrombotic event
- documentation/definition of thrombosis
- associations with other autoimmune diseases (primary/secondary APS)
- consideration of comorbidities or presence of other thrombotic/CV risk factors

As far as study design is concerned, cross-sectional and case-control studies give information on the strength of association between the two variables of interest (aPL/LA and thrombotic events) but they cannot establish causality, because it is not known if aPL/LA were present before the event or if they became positive as a response to it. Moreover, the choice of controls should be from the same source population as the cases, in order to reduce bias. Longitudinal studies are better in this sense, because they can attempt to establish causality, since they provide a clear temporal sequence between aPL/LA determination and thrombosis. Still, unless measured quite often and not just twice as recommended, we cannot know if aPL are permanent, transient, if and why their titers fluctuate over time and what are the effects of that. It is still debated if transient aPL can be pathogenic[123].

A meta-analysis by Galli et al[124,125], ranging from 1988-2000, concluded that positivity in the LA test is the strongest predictor of thrombosis, irrespective of the presence of SLE, or type and site of thrombotic event. Antibodies of the IgG isotype at higher titers are also more strongly associated with thrombosis, as compared to IgM. Some studies included in this review were longitudinal[126-130]. They showed higher rates of recurrent venous thromboembolism and sometimes even higher mortality rates[129] after cessation of

anticoagulant treatments, in patients who were IgG aCL+ as compared to aPL negative patients affected by previous thrombosis.

There is in general a consensus on the fact that triple positivity (TP), defined as the concomitant repeated presence of LA, aCL IgG or IgM and anti-β_2GPI IgG or IgM, is a marker of higher risk for thrombosis[131,132]. A previous study by Pengo et al[131], using the Sapporo criteria for the definition of APS, established an OR of 33.3 (95%CI 7.0-157.6) of thrombotic events in TP versus non-TP, adjusted for age, gender, other autoimmune diseases including SLE, and established risk factors for venous and arterial thromboembolism. A more recent prospective study[133] showed a cumulative incidence of thromboembolism in TPs carriers of 37.1% (19.9-54.3%) after 10 years of follow-up. The thrombotic events were equally distributed between the arterial and venous circulation. The annual rate of first thrombotic event was calculated as 5.3% in TP carriers as compared to 1.4% in single aPL positives carriers[134] and to 0.4% in the aPL negative general population in the age span 35 to 55 years[135]. TP also present a higher rate of recurrences, even under anticoagulant therapy, as well as worse pregnancy outcomes[136]. TP has the highest specificity and positive predictive value for APS diagnosis[137].

LA has been considered so far a stronger predictor of thrombosis than the specific aPL tests in SLE[124,125,138], but according to Pengo et al[139] it should be present together with at least one other aPL positivity to be considered associated with thrombosis. Also in the setting of the Warfarin in the Antiphospholipid Syndrome (WAPS) study[140], a prospective, multicenter, international study with the aim to compare standard vs high-intensity warfarin, an increased risk of prospective thrombosis was reported for LA+ patients who also belonged to the upper tertile of IgG anti-β_2GPI and anti-annexin V titers.

Anti-β_2GPI are considered to have the highest sensitivity and negative predictive value for the diagnosis of APS[137]. They seem to be associated with thrombosis, in particular when coexistent with LA+. An explanation for this has been attempted by some research groups (see section on non-criteria aPL on anti-domain I β_2GPI). Other data have shown that the presence of anti-β_2GPI and aCL without LA positivity has a modest association with thrombosis and low recurrence rate, although a prospective study by Neville et al[141] in 2009 concluded that anti-β_2GPI, in the concomitant absence of LA positivity, predict venous thromboembolism with HR 5.8 (1.4-24.1).

Overall, the positivity of just one aPL is by some not even considered associated with thrombosis[131,142]. Therefore, as compared to single test, the analysis of a complete aPL antibody profile is clearly superior in the quantification of thrombotic risk in APS patients.

Non-criteria aPL: In recent years, research has been directed towards trying to find some antigen sub-specificities or aPL isotypes that may increase the risk prediction for thrombosis, in order to better tailor treatment choices regarding type, intensity and duration of anticoagulation in the future. Some examples are anti-domain I β_2GPI[143-147], anti-prothrombin/phosphatidylserine antibodies (aPS/PT)[148-153], anti-prothrombin (aPT), IgA aPL[154] or other non-criteria aPL[111].

Anti-domain I β_2GPI IgG[144,145,155] are considered by some the pathogenic antibodies. They recognize the epitope Gly^{40}-Arg^{43} in the domain I in the NH2 terminal region of the molecule. It has been postulated that when anti-β_2GPI and aCL tests are both positive, the anti-β_2GPI assay may identify domain I. That's because the anti-domain I β_2GPI recognize the epitope just when β_2GPI is in its open conformation, thus bound to cardiolipin in solid-phase. Conversely, when anti-β_2GPI test is positive in the absence of aCL positivity, the assay is probably detecting antibodies against other domains, because domain I is hidden when β_2GPI is in circular conformation. Anti-domain I β_2GPI IgG are also believed to generate LA activity, as they are more frequently detected when also LA test is positive. They are highly associated with thromboembolism. Conversely, the non-domain I anti-β_2GPI IgG (anti-domain IV/V) are considered less or non-pathogenic because of their lack of association with thrombotic events, and they are more often found in isolated anti-β_2GPI positivity[145,156,157].

aPS/PT are antibodies that recognize the complex phosphatidylserine/prothrombin as antigen. Their prevalence is estimated as 27-51% in pAPS, 47-53% in sAPS, 10-33% in APS-negative SLE[158]. A study in pAPS from 2017[159] identified aPS/PT as strongly associated, in both titers and prevalence, with severe thrombotic and obstetric complications, as well as thrombotic microangiopathy and triple positivity. aPS/PT seem to correlate with the presence of other aPL and especially LA, raising the hypothesis that the test may be a surrogate for LA activity[160]. aPS/PT are also associated with other non-criteria APS features, such as thrombocytopenia or hemolytic anemia, although in lower titers and prevalence compared to thrombotic APS[158]. Quadruple positivity (the concurrent presence of LA, aCL, anti-β_2GPI and aPS/PT) seems to confer an even higher risk (30-fold) for thrombosis than triple positivity[161,162].

Another relevant aspect concerns which subclasses of IgG isotype[163] present more association with thrombosis in APS and how the subclass distribution changes over time and disease activity. The subclass response is influenced by the environment (cytokines), the chronicity of antigen stimulation, and genetics. Generally speaking, IgG_1 and IgG_3 are activated by protein antigens mainly through T-cells, whereas IgG_2 response is mostly activated by carbohydrate antigens. IgG_2 and IgG_4 are considered weaker in activating complement via the classical pathway[59].

In the study by Samarkos et al[163], IgG_2 seem to be more prevalent in anti-β_2GPI, whereas IgG_2 and IgG_3 are more frequently elevated in aCL, although IgG_1 aCL is the predominant subclass in terms of mean percentage levels.

Further studies on the association between different aPL and thrombosis are needed.

1.7.2 Prevalence of aPL/LA in thromboembolism

Despite the difficulties in drawing definite conclusions, Andreoli et al, on behalf of the APS Alliance For Clinical Trials and International Networking (APS ACTION), tried to assess the frequency of aPL+ in the general population affected by thromboembolism, by reviewing 120 papers published between 1984-2011[164,165]. The overall percentages (IQR) of prevalence of aPL+ that have been collected are the following: 13.5% (6.8-23.3) in stroke, 11% (4-23) in MI, 6% (2-13) in pregnancy morbidity and 9.5% (5.3-15.8) in DVT.

Nevertheless, the authors pointed out several limitations:

- 60% of papers were published before 2000
- all three aPL tests (anti-β_2GPI, aCL and LA) were performed in just 11% of the papers, since the majority were published in early years of APS research, when anti-β_2GPI had not been yet recognized and were not even part of the old criteria
- there was a lack of standardization of aPL tests: low titers aCL (<20 units) were considered positive in 36% of papers, anti-β_2GPI had an heterogeneous cut-off and only 20% of the studies confirmed aPL+ by repeating testing
- circa 50% of the studies had a retrospective study design
- concomitant thrombotic risk factors were statistically evaluated in 45% of stroke studies, 43% of DVT papers and in 36% MI studies. Thus, it is not possible to correctly infer associations when the results are not adjusted for other thrombotic risk factors

1.7.3 Measurements of aPL/LA

Since APS diagnosis depends strongly on the presence of aPL/LA, it is of outmost importance that they are correctly and consistently identified. With this purpose, the International society of thrombosis and hemostasis (ISTH)/Scientific and Standardization Committee (SCC) published updated guidelines for the laboratory identification of aPL/LA in 1991, 1995[166], 2009[167], 2020[168,169].

Lupus anticoagulant (LA)

Three steps are necessary to identify LA:

1. The **screening** test, with a phospholipid-dependent assay such as dilute Russell's viper venom time (dRVVT) or a sensitive activated Partial Thromboplastin Time (aPTT) with silica as activator and low phospholipid concentration. Samples should be collected in sodium citrate 9:1 and subsequently double centrifugated at 2000-2500g for 15min at room temperature to obtain platelet-poor plasma, with a minimal number of residual platelets $<10^7$/ml. The test is suspected to be positive for LA when the clotting time is prolonged (>99th percentile of healthy donors).
2. The **mixing** test, by adding plasma 1:1 from a pool of at least 40 adult healthy donors. If the clotting time normalizes, it indicates a natural or acquired deficiency of coagulation factors. If it is still prolonged, it strengthens the suspicion of LA positivity[170].
3. The **confirmation** step consists in adding phospholipid-containing reagents to reach a state of excess of phoshospholipids. If the clotting time now normalizes, it confirms LA positivity[171].

More than one screening test should be performed in order to increase the sensitivity of the assay. dRVVT and LA-sensitive aPTT are recommended. dRVVT is based on a viper venom that directly activates fX. aPTT tests can be more or less sensitive to LA, depending on the phospholipid content that triggers coagulation. The 2009 guidelines added the recommendation of testing for LA when there is a reasonable suspicion of APS as cause of thrombosis (high pre-test probability). In fact, the assays for LA determination have low specificity with consequent higher risk of false-positive results. False-negative results are also possible, for example when platelets, being a source of phospholipids, are not completely removed in the process of freeze-thawing[172].

Both false-negative and false-positive LA results can occur when testing patients on anticoagulant therapy and separate recommendations were written last year in order to minimize errors and guide LA detection in this setting[169]. False positive or negative results

can also occur during pregnancy. The recommendation in this case is to wait at least 6 weeks post-partum, ideally 3 months before performing the test.

aCL and anti-β_2GPI

The first aCL test was established in 1983 by Harris et al[7] as a radioimmunoassay using cardiolipin as antigen, diluting serum samples with a mixture of gelatin/phosphate buffered saline and radiolabeled antibodies anti-human IgG or IgM. Later, fetal calf serum or adult bovine serum or plasma were added to the buffer/diluent to increase the optical density readings of bound aCL and reduce unspecific binding, therefore increasing the specificity of the assay. Afterwards, anti-human IgG/IgM antibodies were enzyme-labelled instead of radio-labelled.

In the '90s it was discovered that the reason why these diluents give a better signal is that they contain other antigens that, in turn, bind to cardiolipin-coated wells. Examples of these are plasma protein β_2GPI, which binds to cardiolipin with high affinity, but also lipopolysaccharide binding protein (LBP), C4b-binding protein (C4BP), and thrombin-modified antithrombin (AT)[173], making it more and more evident that aPL are an heterogeneous group of antibodies that bind to different antigens.

Enzyme-linked immunosorbent assays (ELISA) utilize cardiolipin or other anionic phospholipid in solid-phase, but most aPL need β_2GPI in order to bind to cardiolipin[10]. The reason why fluid-phase immunoassays are insensitive to aPL is because anti-β_2GPI do not recognize the glycoprotein in its circular conformation. Instead, solid-phase are prepared using negatively charged plates that can bind human β_2GPI.

When β_2GPI was discovered as the main antigen of aPL, solid-phase ELISA assays were developed to specifically detect anti-β_2GPI. Some discrepancies unfortunately exist between different assays. Some examples are:

- the type of plates
- the concentration and orientation of the protein on the plates
- the source of β_2GPI and its purity: not all human anti-β2GPI bind to animal-derived β_2GPI and antibodies against bovine proteins can be found in human blood

At first, the results of the tests were given as "positive" or "negative", without quantification. When it was noticed that patients with higher titers of antibodies, expressed as Optical Density Units (ODU), had a stronger association with thrombosis/obstetric complications, it became important to define also the level of positivity. Due to the high intra- and inter-laboratory variability, it was challenging but necessary to reach a standardization of the assays used for aCL testing[16,18,174,175]. In 2010, at the 13th International Congress on aPL, a

task force of researchers discussed critical questions related to standardization of aPL testing, providing international recommendations for pre-analytical (e.g. obtaining and preparing the samples), analytical and post-analytical issues[18]. Cut offs for aPL were established as the 99th percentile value of a reference population representative of the target population being tested. Between the 95th (mean ± 2SD) and 99th percentile (mean ± 3SD), results are considered indeterminate and should be repeated. For aCL, also >40GPL/MPL was established as cut-off for positivity, considering that one GPL/MPL arbitrary unit is defined as the cardiolipin-binding activity of 1μg/ml of affinity-purified IgG or IgM aCL. The coefficient of variation (CV) should be <10%, max 20%. The last guidelines were published in 2020[168,169,176].

Apart from repeating the tests after at least 12 weeks according to classification criteria, it is not known if and how often these tests should be repeated during the course of the disease, and not even if the risk of thrombosis decreases after negative results. An old prospective study by Out et al[177] showed fluctuations in LA/aPL positivity in SLE patients, followed-up for a median of 26 months, mostly for IgG aCL (49%) and IgM aCL (30%), whereas LA was more stable (23%). These fluctuations were influenced partly by disease activity (especially for IgG aCL) and prednisone use (for LA). The PROMISSE study reported a modest decrease of aPL/LA during pregnancy that returns to baseline by 3 months post-partum[178]. A more recent prospective study on SLE shows that aCL tend to normalize in 40-50% of patients, depending on the isotype (more normalization for IgA and IgM than for IgG aCL), whereas this was true for LA in just 20%. Seventy-six percent of patients who became LA negative and 60% of those who had a normalization of IgG aCL, reacquired LA/IgG aCL within 5 years, in comparison with 37% and 17% in case of IgM and IgA aCL, respectively[179].

1.8 Thrombin Activatable Fibrinolysis inhibitor (TAFI) and fibrinolysis in APS

1.8.1 Characteristics of the clot in APS

Fibrin is the main component of the clot. It is a highly extensible fiber, that can be stretched up to 3.2-fold before breaking, and up to 4.3-fold when stabilized by cross-linking with fXIIIa. Clots are made of networks of polymerized fibrin monomers, which structure is characterized by different fibrin thickness, branching angles, quality and quantity of fibrinogen, pore size[180]. They are best visualized by Scanning Electron Microscopy (SEM). Both genetic (EuroCLOT study[181]) and environmental factors such as oxidative stress contribute to the characteristics of the clot, as well as local variables such as fibrinogen,

thrombin, fibrinolysis inhibitors concentration, the binding properties of tissue plasminogen activator (tPA) and plasminogen.

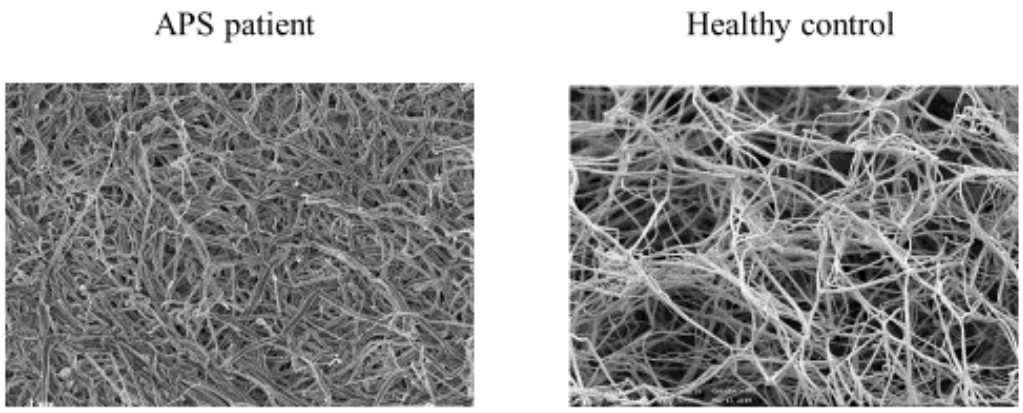
The Darcy constant Ks and Clot lysis time (CLT) can be used to numerically quantify the permeability of the clot (see also section 3.2.1). When Ks is low and/or CLT is prolonged, it indicates a less permeable clot, tightly packed, composed by thinner, shorter and more numerous fibrin strands that are difficult to lyse. Conversely, when Ks is high and CLT short, it means that the clot is highly permeable, easy to lyse, composed by thicker fibers that may lead to bleeding. These parameters are usually inversely correlated with thrombin and fibrinogen concentration.

For a description of the fibrinolytic process, see section 1.10.1.

Alteration of fibrinolysis/fibrin structure associates with thrombosis in the general population[182-185]. The Darcy constant Ks has been shown to be reduced by 25% in acute thrombosis and advanced atherosclerosis, by 10-15% in subjects >65 years old and in obese patients (BMI > 35 kg/m^2), by 15-20% in subjects with positive family history of thrombosis. It is increased by statins, antithrombotic agents and anticoagulants. Tighter fibrin clots, denser structures with decreased permeability and increased stiffness have been demonstrated in patients affected by venous thromboembolism (DVT[186] and PE), arterial thromboembolism (MI and stroke[187]) and even metabolic syndrome. The metabolic syndrome is one of the main risk factor for CV diseases, defined by waist circumference >94 cm in men or >80 cm in women plus any 2 of: triglyceride >1.7 mmol/L or specific treatment, HDL <1.03 mmol/L in men or <1.29 mmol/L in women or specific treatment, systolic blood pressure >130 mm Hg or diastolic blood pressure >85 mm Hg or specific treatment, fasting glucose >5.6 mmol/L or diabetes mellitus. It has been shown that clot permeability decreases proportional to the increasing presence of metabolic syndrome variables[181].

The clot properties of APS patients are different not only from healthy controls, but also from non-APS patients affected by thrombosis (figure 2). The fibrin network in APS clots is even more compact, less permeable and poorly lysable[188,189]. It contains more complement fragments C5-C9 (especially higher in triple positives and in LA+), immunoglobulins, proteins involved in platelets and neutrophils activation, NETosis and inflammation[190].

Figure 2 – Scanning Electron Microscopy (SEM) illustrating the difference in clot structure between patients affected by APS and healthy controls



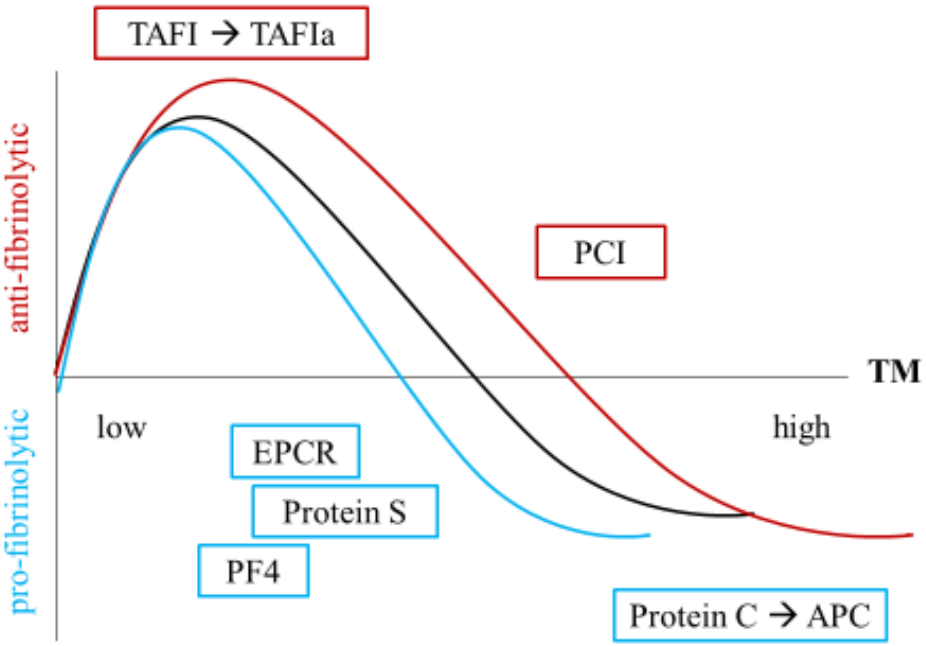
1.8.2 TAFI

TAFI is a carboxypeptidase B-like zymogen of 58kDa that downregulates fibrinolysis after activation. Its gene is located on chromosome 13q14 and spans 11 exons. It is produced mostly by hepatocytes, macrophages and megakaryocytes and it has a variable concentration in plasma, varying between 4 and 15 μg/ml[191]. Twenty-five percent of this variability can be explained by genetic polymorphisms, the rest by environmental factors[192]. TAFI can be measured in plasma with an antigenic method (sandwich ELISA) or by activity (functional) assay[191]. TAFI is mostly released in plasma as an acute-phase reactant. Its activated form, TAFIa, is a 35kDa highly unstable enzyme with a half-life of ca 8-10 minutes at 37°C, 2 hours at room temperature, whereas it is stable at 0°C. Because of this instability, it is challenging to measure[191,193]. TAFIa cleaves carboxyl-terminal lysine and arginine residues from partially degraded fibrin, limiting tPA and plasminogen binding and subsequent plasmin generation, also promoting the inhibition of plasmin by α2-antiplasmin. TAFI is activated during coagulation, with the aim of protecting and stabilizing the newly formed clot. It has been shown in vitro to be triggered by trypsin, plasmin and thrombin, and enhanced by 1250-fold by the thrombin-thrombomodulin complex (IIa-TM)[192]. Thrombomodulin (TM) is an integral membrane glycoprotein made of 557 amino acids organized in five distinct domains, with a molecular weight of 70-100kDa, mainly expressed on the luminal surface of endothelial cells, but it is also found in circulation as a shorter soluble variant. Its plasma concentration is ca 10ng/ml and it increases in conditions of endothelial dysfunction/activation, e.g. during Acute Respiratory Distress Syndrome (ARDS), DIC, hypertension[194] or in case of liver or renal failure[195]. TM has also anticoagulant properties: it inhibits thrombin's pro-coagulant functions and works as a cofactor for thrombin in the

activation of anticoagulant protein C. Thrombin has both pro-coagulant and anti-coagulant functions depending on its concentration, the so called "thrombin paradox": anticoagulant at lower concentrations, pro-coagulant at higher concentrations. Also the complex thrombin-TM has a dual function on fibrinolysis: anti-fibrinolytic at low TM concentrations, pro-fibrinolytic at higher TM concentrations. This phenomenon has been called the "TM paradox" (figure 3). It was shown by observing a prolonged clot lysis time (CLT) at lower TM concentration that was reduced with increasing TM concentrations. The reduction of the CLT was less pronounced after the addition of a protein C or protein S inhibitor[196]. The explanation is that the complex thrombin-TM activates protein C at higher TM concentrations in vitro in normal plasma (ca 10nM), whereas the same complex preferentially activates TAFI at low TM concentration (ca 1nM). In fact, activated PC (APC) inhibits TAFI activation through the downregulation of thrombin generation in a sort of negative feedback loop. Together with protein S, APC inactivates fVa and fVIIIa that are needed for thrombin activation. To confirm these findings, Mosnier et al[196] used also plasma from a patient homozygous for factor V^{Leiden}, showing that, in this case, the activation of TAFI is not decreased for increasing TM concentrations, as expected. In fact, since the mutated fVa is resistant to APC cleavage (see also section 1.10.5), the activity of the prothrombinase complex is unopposed, leading to increased thrombin generation, able to activate more TAFIa for increasing TM concentrations. They also demonstrated the same properties of TM on cultured human microvascular endothelial cells. Vessel size and location are also determinants of local TM expression on endothelial cells. Small arteries such as those in the brain or in the coronary circulation have lower concentrations of TM compared with veins. Thus, in the vessels where TM concentration is lower, high levels of TAFI may downregulate fibrinolysis and reduce the capacity to remove fibrin clots. Other factors are involved in the regulation of TAFI activation and can steer the balance more towards TAFI or protein C activation independently from TM concentration. For example, protein C inhibitor (PCI) and coagulation factors fXa and fXIa tend to limit protein C activation even at higher TM concentration, shifting the curve towards more activation of TAFI, whereas others, such as protein S, endothelial protein C receptor (EPCR) or platelet factor-4 (PF4), activate less TAFI at lower TM concentration, favoring protein C activation[197] (figure 3).

Figure 3 – The thrombomodulin (TM) paradox

The black curve indicates pro- and anti-fibrinolytic effects mediated by TM, depending on its concentration. Stimulation of TAFI activation by low concentrations of TM is responsible for anti-fibrinolytic effects (red color), whereas stimulation of protein C activation at higher concentrations of TM is responsible for pro-fibrinolytic effects (light blue color). Cofactors that stimulate protein C activation, APC anticoagulant and pro-fibrinolytic activity are illustrated in light blue (curve shifts to the left), whereas those that enhance TAFI activation and its anti-fibrinolytic effects are colored in red (curve shifts to the right).



Apart from its anti-fibrinolytic properties, TAFIa is also anti-inflammatory because it inactivates, via cleavage of the COOH-terminal arginine residues, the two most powerful anaphylatoxins derived from complement activation, C3a and C5a, as well as bradykinin (a vasodilator) and osteopontin (promoter of chemotaxis)[192,193,197-201]. Also TM has anti-complement properties by enhancing factor I degradation of C3b[114,201,202,1].

TAFI has been studied in relation to venous[203-205] and arterial[206-210] thrombosis, as well as in SLE[211], RA[212] and Behcet disease[213-215], with controversial results. The most relevant studies in this context are reported in table 1.

Table 1 – TAFI in non-APS

Author, Journal, Year	What	Patients	Controls	Findings
Van Tilburg, Blood 2000[205]	TAFI (electroimmunoassay)	474 first DVT, enrolled 6 months after anticoagulant discontinuation (unless indefinitely)	474 age and sex-matched healthy	Increase of TAFI with age in healthy women. TAFI >90th percentile in 14% DVT vs 9% controls, but same mean. OR of DVT 1.7 (95%CI 1.1-2.5) TAFI > vs < 90th. Impact of anticoagulants not stated
Santamaria, Stroke 2003[209]	TAFIa (functional assay)	114 ischemic stroke (IS) < 80 years old, mean age 56, 45% women	150 age and sex-matched healthy, 55% women	Higher in IS vs controls (113.7% vs 102.6%, p <.05). Adjusted OR 5.7 (95%CI 2.3-14.1) for IS when TAFIa >120%. No difference in LA and aCL between groups. No correlation between TAFIa and CV risk factors, which were all more prevalent in IS vs controls
Eichinger, Blood 2004[203]	TAFI (ELISA)	600 first spontaneous VTE prospectively (mean follow-up 45 months), enrolled 4 weeks after anticoagulant discontinuation, 56% women	-	High TAFI (>75th percentile) correlated with recurrence 2 years after anticoagulation (14.5% recurrence with high TAFI vs 6.8% with low TAFI, p=.006)
Donnmez, Thrombosis Research 2005[214]	TAFI (ELISA), CRP	105 Behcet disease (BD), 61% men, mean age 36, all negative aPL	53 age and sex matched healthy	Higher in BD vs controls (91.1 vs 14.3, p<.001), no difference in subgroups with/without thrombosis. No correlation between TAFI and CRP

Ringwald, Thrombosis Research 2007[211]	TAFI (ELISA)	67 SLE, 15% men, mean age 40, 27% (18/67) past thrombosis: 9/18 (50%) arterial, 5/18 (28%) venous, 4/18 (22%) both. 23/67 (34%) LA+	67 healthy matched for age, sex, hormone therapy, smoking	Not significantly higher in SLE vs controls (75.7 vs 66.9, p=0.07), no difference according to previous thrombosis/LA
Malyszko, Polskie Archiwum Medycyny Wewnetrznej 2008[207]	TAFI (antigenic method), TAFIa (chromogenic method), TM (ELISA)	72 essential hypertension: 27/72 untreated, 13/72 ACE-I, 32/72 β-blocker. All non-smokers	-	TAFI weakly correlated with diastolic BP in untreated patients (r=0.27) and patients on β-blocker (r=0.25), with systolic BP in all (r=0.27)
Ricart, British journal of haematology 2008[215]	TAFI (ELISA), TAFIa (STA-Stachrom TAFI)	79 BD: 22/79 previous VTE, 3/79 stroke and angina. Samples collected >6 months after thrombosis	84 healthy	TAFI (p=.013) and TAFIa (p<.001) higher in BD vs controls. BD have higher CV risk factors. TAFIa remains higher after adjustment. TAFIa higher in previous thrombosis vs non-thrombosis (p=.024)
Tregouet, J Thromb Haemost. 2009[210]	TAFI, TAFIa/TAFIai (ELISA)	1668 CAD mean follow-up 2.3 yrs. 56/1668 CV deaths and 35/1668 non-fatal CV events. Nobody on anticoagulants	-	TAFIa/TAFIai HR for CV death 1.69 (95%CI 1.07-2.67) adjusted for traditional CV risk factors, CRP, markers of thrombin generation and fibrinolysis
De Bruijne, J Thromb Haemost. 2009[206]	TAFI, TAFIa, TAFIai (in house ELISA + functional assay)	327 patients: 218/327 first CHD, 109/327 IS/TIA, 46% men, mean age 43 years	322 healthy	TAFIai higher vs controls (145.1 vs 137.5, p=.02). TAFI higher in CHD vs controls (109.4 vs 102.8, p=.02)
Meltzer, Haematologica 2009[208]	TAFIa (chromogenic)	554 first MI men < 70yrs, mean age 56	643 healthy, mean age 57	Lowest quartile of TAFIa associated with first MI (OR 2.8, 95%CI 1.9-4.0; adjusted OR 3.4, 95%CI 2.3-5.1). Positive correlations of TAFIa with cholesterol, triglycerides and CRP

Peters, Annals of Rheumatic diseases 2009[212]	TAFI (in-house activity-based assay)	21 RA with CRP >10mg/L, 33% men, mean age 63	21 age and sex-matched RA with normal CRP <10mg/L	Higher (147% vs 114%, p<.01) in active RA, non-significant difference after adjustment for ESR and DAS28
Meltzer, Blood 2010[204]	TAFIa (chromogenic, in house), PAI-1, plasminogen	770 first DVT (62%), first PE (28%), or both (10%), enrolled 3 months after anticoagulant discontinuation (unless indefinitely), mean age 49, 45% men	743 frequency matched for age, sex and geographical area	PAI-1 and TAFIa positively associated with CLT and VTE, plasminogen inversely associated with CLT. Prolonged CLT associated with VTE
Donnmez, Thrombosis Research 2010[213]	TAFIa (ELISA), TM	89 BD, 50% men, mean age 40, 18/89 VTE	86 healthy, 42% men, mean age 38	TAFIa lower in BD (13.5 vs 26.8), TM higher (3.2 vs 2.6). No difference of both TAFIa and TM according to VTE. Positively correlated TAFIa-TM in healthy (r=0.37), negative in BD (r= -0.51)

1.8.3 TAFI in APS

TAFI antigen has been studied in relation to aPL/LA first in 2007 in LA positive patients affected by SLE[211] (see table 1), and no difference in antigen levels were found according to LA positivity or previous thrombosis.

The studies on TAFI specifically in APS are presented in table 2, and they were mainly in relation to pregnancy, obstetric complications and CLT[216-218]. The only previous study that measured also the activated form of TAFI (TAFIa) in non-pregnant APS patients, classified according to Sapporo criteria, was performed in Japan, by Ieko et al[219] in 2010. They noticed that APS patients have higher levels of both TAFI and TAFIa compared to healthy controls, but lower than patients with other autoimmune diseases (including SLE, rheumatoid arthritis, Sjögren, Systemic Sclerosis), and no APS. There was no difference between pAPS and sAPS in both TAFI and TAFIa levels, but after comparing SLE patients with sAPS vs non sAPS, TAFIa was higher in the second group. TAFIa was lower in aPS/PT+ and LA+, as compared to negative. They also showed that purified monoclonal IgG aPL inversely correlate with TAFIa in vitro, in a concentration dependent manner. The authors suggested that aPL may affect the thrombin-TM complex by binding to phospholipid-binding proteins, in particular β_2GPI that has been shown to inhibit protein C activation in vitro[220]. In this way, although lacking definitive proof of a causal relationship, aPL could steer the thrombin-TM action away from TAFI activation. Nevertheless, previous research has demonstrated the inhibitory effects of aPL on protein C activation[221,222].

Table 2 - TAFI in APS

Author, Journal, Year	What	Patients	Controls	Findings
Martinez-Zamora, American journal of Reproductive Immunology 2009[217]	TAFI (ELISA)	67 pregnant pAPS (Miyakis)	66 pregnant non APS	Prolonged baseline CLT in APS vs controls. No difference in CLT or TAFI for thrombosis/obstetrical outcome. Progressive increase of TAFI and CLT in pregnancy, normalizing after delivery
Ieko, International Journal of Hematology 2010[219]	TAFI (ELISA), TAFIa (chromogenic), aPS/PT	68 APS (Sapporo criteria), mean age 46, 90% women: 27/68 pAPS, 41/68 sAPS, 39/41 SLE sAPS	66 healthy, mean age 46, 62% women, 46 other autoimmune diseases (AI), mean age 42, 83% women, 27/46 SLE non sAPS (2/27 aCL+)	TAFI higher in APS vs controls (p<.001), higher in AI vs APS (p=.024) and controls (p<.001), no difference in pAPS vs sAPS. TAFIa higher in APS and AI vs controls (p<.001 for both), higher in AI vs APS (p>.001), higher in AI vs sAPS (p<.001), no difference in pAPS vs sAPS. TAFIa higher in SLE non sAPS vs SLE sAPS, TAFI same. TAFIa lower in LA+ and aPS/PT+, same TAFI. Purified monoclonal IgG aPL inversely correlated with TAFIa**,** concentration dependent
Martinez-Zamora, American journal of Reproductive Immunology 2010[218]	TAFI (ELISA)	82 pregnant with severe pre-eclampsia (PE), 10 APS with pre-eclampsia	76 APS pregnant, 89 healthy pregnant	CLT > in PE, APS-PE and APS vs controls, longer in PE ad APS-PE vs APS. TAFI higher in PE and APS-PE vs APS and controls
Martinez-Zamora, Fertility and Sterility 2010[216]	TAFI (ELISA)	63 APS previous >3 spontaneous abortions <10th week, mean age 33	119 non APS previous >3 spontaneous abortions <10th week; 64 age matched healthy	TAFI higher in abortion non APS, CLT prolonged in APS and abortions non APS vs controls

1.9 Myocardial Infarction in APS

Myocardial Infarction (MI) is not one of the most common manifestations in APS. In the Euro-Phospholipid project study[22-25] it was the presenting clinical feature in 2.8% of patients, with a cumulative frequency of 5.5% registered from symptom onset until enrollment and 1.9% was the reported incidence during 10 years follow up. In the retrospective, population-based study of Duarte-Garcia, no MI were registered[19]. Taking it from another perspective, in patients affected by MI, the prevalence of aPL/LA has been estimated to be around 11%, as outlined before[55].

1.9.1 Traditional cardiovascular risk factors

Traditional cardiovascular (CV) risk factors should be considered in the evaluation of APS patients, especially regarding the risk of arterial events[223,224].

The SCORE risk estimation system was created in 2003 by the European Society of Cardiology (ESC) in order to estimate the risk of a fatal MI within 10 years through charts easily applicable in the clinical practice[223]. The need for new scoring systems derived by the fact that the only available risk estimator until that time was calculated from the Framingham cohort[224] and it became clear that it was not correctly applicable to the European population, because of a lower incidence of coronary heart disease as compared to the United States. The SCORE system estimates the total burden of CV risk of an individual, considering the interaction between total cholesterol (or total cholesterol/HDL ratio), age, sex, smoking status and systolic blood pressure. The charts are differentiated in low and high risk European countries, based on the baseline cause-specific mortality. The estimation doesn't include non-fatal CV events, because their definition is not unanimous across different studies and the availability of data is restricted. Diabetes is not included in the chart because of lack of unanimous data, but men with diabetes have approximately a two-fold and women a four-fold risk compared to that given by the charts. Diabetes-related factors, such as type and duration of diabetes, glycemic control, presence of retinopathy, microalbuminuria and proteinuria are considered in separate, specific CV risk calculations tailored for diabetic patients. Similarly, patients with inflammatory joint disorders are considered to have a higher CV risk than the general population, similar to, or even higher, than diabetes[225]. In order to take this into account, the more recent EULAR recommendations advocate the inclusion of a multiplication factor of 1.5 in the CV risk prediction models for all patients affected by rheumatoid arthritis. In the overarching principles of the same document, the authors also state that the higher CV risk may also apply to other inflammatory joint diseases[226].

Also patients affected by APS are at increased risk of thromboembolism compared to the general population. The Global Anti-Phospholipid Syndrome Score (GAPSS)[227] is an attempt to take into consideration some clinical and laboratory APS-related factors that are believed to increase the risk of thrombosis in any vessel or pregnancy complications. The variables included in the score are a combination of some conventional CV risk factors (hyperlipidaemia and hypertension), together with criteria and non-criteria aPL (IgG/IgM aCL, IgG/IgM anti-β_2GPI, LA and IgG/IgM aPS/PT)[228,229]. It remains to be assessed whether novel biomarkers, such as anti-domain I β_2GPI, could further refine APS risk prediction.

1.9.2 Atherosclerosis and Autoimmunity

Atherosclerosis is the main cause of coronary arteries occlusions and carotid artery thromboembolism, leading to myocardial infarction and stroke, respectively. Subclinical inflammation and endothelial dysfunction are involved in its pathogenesis. Many studies over the recent years have hypothesized a link between autoantibodies and autoimmune-mediated accelerated atherosclerosis[230,231], in particular IgG anti-oxLDL/β_2GPI[232-237]. Oxidized LDL (OxLDL) are low density lipoproteins that have been oxidized in their lipid and apo-B components by lipid peroxidation. This process is believed to take place mostly in the subendothelium and it represents one of the earliest pro-atherogenic events. It has been demonstrated that β_2GPI is abundantly present within the atherosclerotic lesions, both intra- and extracellularly. In the intima, OxLDL form a complex with β_2GPI, which constitute a target for anti-oxLDL/β_2GPI[238]. IgG anti-oxLDL/β_2GPI have been shown to enhance the uptake of oxLDL by macrophages via scavenger receptors[239] and to promote the formation of macrophage-derived foam cells[237] and the development of fatty streaks in the arterial wall. Macrophages then produce matrix-degrading proteases and TF that eventually lead to plaque rupture and thrombus formation[240]. IgG anti-oxLDL/β_2GPI have been found in the circulation of patients with APS/SLE[241] and some studies have shown a positive correlation with arterial thrombosis[242].

1.9.3 aPL/LA in patients with Ischemic Heart Disease

The literature on Myocardial Infarction (MI) and positivity for aPL/LA is large and controversial (table 3), due to limited sample size, selected populations and/or non-standardized/old methods/variable cut offs for antibody determination. Therefore it is not easy to compare different studies and give a unanimous answer to the question of the association between aPL/LA and Ischemic Heart Disease.

In 1986 the pioneer study of Hamsten et al[243] observed an increased prevalence of patients with elevated aCL titers in a highly selected series of young patients after a MI. Furthermore, high titers of these antibodies correlated with recurrent venous or arterial thrombosis during 36-64 months of follow-up. No difference was reported between aCL+ and aCL- patients regarding CV risk factors or angiographic findings. In the following years, other studies demonstrated associations between aCL and ischemic heart disease/MI, whereas in others the frequency of elevated aCL antibodies was not particularly higher in patients than in control subjects. The majority of the studies did not report a prognostic role for aPL on subsequent cardiovascular complications[244-254], though some did[242,255,256]. Many studies were performed on survivors of MI or in patients with established coronary heart disease (CHD), sometimes measuring aPL very close to the time of the event. Others, like Vaarala et al[253] and Wu et al[242], were able to investigate the association between aCL and the risk of MI prospectively and longitudinally, collecting samples in initially healthy subjects in the context of primary prevention studies, with long follow-ups. Some studies, like Meroni et al[257], focused on particular antigenic specificities of aPL (anti-β_2GPI) in a highly selected population (premenopausal women). Others, like Davies and Hunt[258], focused their research on young adults hit by a first MI with normal coronary angiograms and no signs of atherosclerosis. They showed an unexpectedly high prevalence of LA positivity, which steered treatment towards anticoagulation, which turned out to be efficacious in terms of lack of recurrences during follow-up. Also Urbanus et al[259], in a case-control study, found a significant difference in LA positivity between MI patients and healthy subjects, but not in other specific aPL, after adjustment for traditional CV risk factors.

Table 3 – MI and APS/aPL

Author, Journal, Year	What	Patients	Controls	When	Findings
Hamsten, The Lancet 1986[243]	aCL IgG (Harris cut offs 5 GPL)	62 MI, 90% men, < 45 years, mean age 41 years	-	3, 12, 36 months post MI	21% (13/62) positives in 2 occasions 11% (7/62) positives in all three occasions. No difference in risk factors and angiographic findings. 61% (8/13) in aCL+ re-thrombosis vs 25% (12/49) in aCL- at 36-64 months follow-up
Morton, The Lancet 1986[256]	aCL IgG, IgM	83 CABG < 70 years old, 83% men, mean age 55, LDA/placebo. 55% (46/83) had prior MI	-	2 days before operation, 7-10 days postoperation, 3 months, 11-12.5 months	Association between preoperative aCL, occlusions and number of occluded anastomosis. No difference in preoperative aCL+ between prior MI or not. Occlusion rate showed a trend: the higher aCL titers, the more occlusions. Postoperative aCL+ higher in prior MI vs not. 20% (17/83) aCL+ preoperative, 82% (68/83) raised aCL in 12 months follow-up
Klemp, Clin exp Immunol 1988[248]	aCL IgG, IgM	86 IHD, 63% men, mean age 61: 21% non ECG changes, 38% ECG changes, 41% MI	124 healthy mean age 31, 62 RA mean age 49, 20 tuberculosis mean age 31	1-5 days after admission	Higher aCL in IHD vs other groups. No differences within IHD, no association with complications
Mattila, Clin Immun and immunopat 1989[260]	aCL solid-phase ELISA	40 MI, all males < 50 years old, mean age 45	41 general population mean age 39 and 30 chronic CHD mean age 41 (19/30 had previous MI)	At admission, after 4 weeks, 3 months (patients only)	18% higher increase in IgG, 26% IgA, 43% IgM between 0 and 4 weeks compared to controls. Slightly decreased at 3 months
De Caterina, Am J Cardiology 1989[245]	aCL IgG and IgM, cut off 10 U	119 chest pain: 75/119 stable CAD, 29/119 unstable CAD, 15/119 non ischemic chest pain (mean age and male/female non reported)	83 healthy, 72% men	At recruitment	IgG aCL >1U in 42% (50/119) vs 53% (43/83) in controls and 43% (45/104) in CAD. Titers: 2.67 vs 2.62 and 2.74 CAD. IgM >1U in 5%. Never >10U! No association with recurrences at 2 years follow-up

Eber, Klin Wochenschr 1990[247]	aCL IgG and IgM, cut offs 6 MPL, 10 GPL	74 men, mean age 60, chest pain: 16/74 CAD mean age 67, 34/74 CAD + prior MI mean age 59, 14/74 MI mean age 61, 10/74 without CAD mean age 61	-	After coronary angiography	No differences between the groups, no association with severity/chronicity of CAD
Sletnes, Thromb Research 1990[251]	aCL 97.5th percentile, anticephalin	49 MI, 71% men, median age 67 years. 28% (14/49) had previous MI. 2/14 aCL+, 4/14 anticephalin+	-	At admittance, after 1 week, 6 weeks, 9 months	6% (3/49) aCL+, 16% anticephalin+ constant in the 4 measurements apart from 4% (2/49) that became IgG + > 6 weeks
Sletnes, The Lancet 1992[252]	aCL IgG and IgM, cut off 97.5th percentile, 5 GPL, 3.6 MPL, anticephalin	597 MI <75 years, 70% men, mean age 62, 1983-86 warfarin	158 healthy and with cataract, mean age 47 years	6-180 days post MI, mean 28 days	6% (37/594) vs 4% controls (ns) aCL+, 13% anticephalin+ MI vs 4% controls. After adjustment for traditional CV risk factors, no association with mortality or recurrence at 3 years follow-up
Cortellaro, The Lancet 1992[244]	IgG aCL and LA	62 MI < 45 years old (mean age and male/female non reported)	-	3 months after MI	13% (8/62) aPL+. No association with complications at 24 months follow-up
Phadke, Br heart J 1993[249]	aCL IgG and IgM. Cut offs 5 GPL, 3 MPL, low 5-15, 3-6, respectively	307 MI < 60 years old, 80% men, median age 53, and 160 unstable angina, 62% men, median age 52	Age and sex matched healthy	Day 1 and 5, 5 weeks, 5 months, 12 months after	No difference and no association with complications at follow-up
Raghavan, J Clin Pathol 1993[250]	aCL IgG cut off 10 GPL, IgM cut off 5 MPL (ELISA)	111 suspected MI, 75% men. Confirmed MI in 83/111 patients, median age 61 years: 16% with previous MI, 36% previous angina.	28/111 IHD and unstable angina, median age 63: 57% with previous MI, 75% previous angina	On admission, after 24h, 5-7 days, 6-8 weeks	18% (20/111) aCL+, 60% male, older. 8% (9/111) aCL+ consistently: 2/9 IgM+, 7/9 IgG+. No differences between MI and IHD, between prior MI or not, no association with complications
Edwards, The Lancet 1993[261]	aCL IgG and IgM cut off 25 GPL/MPL	249: 159/249 MI, 87% men, mean age 58 years, 90/249 unstable angina, 71% men, mean age 56 years	299 healthy, 50% men, mean age 47 years	At admission	No difference. No aCL + < 45 years

Yilmaz, Clin Cardiol 1994[254]	aCL IgG and IgM (ELISA), cut offs 6 MPL, 11 GPL	76 CHD, 85% men, mean age 42: 32 MI, 22 CHD and prior MI, 22 CHD	22 healthy, 54% men, mean age 27	3, 14, 90 days after MI, the other groups just once	47% (36/76) IgG aCL+, 12 GPL vs 7 GPL in controls (low levels). No difference in IgM. No correlation with age, risk factors, morbidity, mortality, severity, complications at 22 months follow-up
Diaz, Cardiology 1994[246]	aCL >25% absorbance cut off (solid-phase ELISA)	22 unstable angina, 95.5% males, mean age 60, 27.3% MI	-	< 48h	36.4% (8/22) aCL+, <50 years more aCL+. No differences in CV risk factors, severity of disease, recurrences, outcome, complexity, thrombus localizations
Adler, Lupus 1995[262]	aCL, anti-phosphatidylserine, anti-phosphatidylethanolamine, anti-phosphatidylcholine (ELISA)	102 MI, 78% men	102 healthy, 10 APS	< 4h and after 3 months	aPL higher in <50 years old vs >50. At least one aPL high in 7% of MI, 6% aCL+, all men <50. Normalized at 3 months. No association with traditional CV risk factors.
Vaarala, Circulation 1995[253]	aCL IgG (ELISA)	Dyslipidemic (LDL≥5.2mmol/L) men, Gemfibrozil/placebo, 133 MI (107 non-fatal MI, 26 cardiac deaths) mean age 49	133 non-MI matched for treatment and geography, mean age 47	At entry in the cohort (1981-82), max 5 years before MI	The highest quartile of aCL titers had an adjusted OR for MI of 2.0 (95% CI 1.1-3.5). aCL correlated to anti-oxLDL (r=0.40). No association with mortality. IgG aCL 0.41 optical density units (ODU) in MI vs 0.36 ODU in controls, higher in smokers
Zuckerman, Am J Med 1996[263]	aCL IgG and IgM, cut offs 10 GPL and 8 MPL	124 MI < 65yrs, 72.5% men, mean age 55	76 chest pain, 76% men, 62% IHD, 16% prior MI, 6.5% prior thrombosis, matched for age and CV risk factors	Admission < 24h, 3 months	14% (17/124) aCL+ vs 3% (2/76) in controls, IgG in 9.6%, remained in all but 1 after 3 months. Higher rate of thrombosis and re-MI at 19 months follow-up in aCL+ vs aCL- (41% vs 4% thrombosis, 35% vs 10% re-MI)
Wu, Arterioscl, Thrombosis, Vasc Biology 1997[242]	aCL IgG, IgA, IgM	Healthy < 50 years men. 119 developed MI between 50-70 years old: 80/119 non-fatal, 39/119 fatal	138 non-MI up to 70 years	At enrollment (before MI), cohort (1970-72). Follow-up for 20 years	Raised aCL IgG and IgA correlated with incidence of MI and MI-related mortality 10-20 years later. Adjusted OR aCL IgG 1.49 (95%CI 1.11-1.99), IgA 1.48 (95%CI 1.06-2.06). More CV risk factors in MI vs non-MI. Trend higher titers in fatal MI - nonfatal MI – controls

Bili, Circulation 2000[255]	aCL and anti-β_2GPI IgG, IgM (ELISA). Cut offs 23 GPL (=23µg/ml) and 11 MPL for aCL, 20 SGU and SMU for anti-β2GPI	1150 MI, 75% men, 48% > 60 years old, 19% had a previous MI	-	Acute before discharge, mean 10.9 days after MI	Higher aCL IgG (HR 1.63 adjusted, Cox, p=0.01) and lower aCL IgM (HR 1.76 adjusted, p=0.02) independent risk for recurrent cardiac events (fatal or not, n=131) at mean 24.6 months follow-up. Higher recurrences in high IgG aCL and low IgM aCL (p<0.001). Dichotomized in quartiles, higher >12.5GPL, >4.1MPL! r=0.50, p<0.005 between IgM aCL and IgM anti-β_2GPI
Dropinski, Med Sci Monit 2003[264]	aCL IgG, IgM, cut offs 10 GPL and 20 MPL	50 male <50 years old survivors of MI, mean age 45	50 healthy, mean age 44	> 6 months	24% (12/50) aCL+ in MI survivors vs 6% (3/50) in controls, crude: all other traditional CV risk factors differed between groups. Mean IgG 9.2 vs 7.7, IgM 18.5 vs 12.1. correlation aPL with IMT r=0.31
Veres, Lupus 2004[265]	aCL and anti-β_2GPI IgG, IgA, IgM and LA. Cut offs 22 GPL, 16 MPL for aCL, 15 SGU, 43 SAU, 34 SMU for anti-β_2GPI	111 ACS, 59% men, mean age 59: 38/111 Unstable Angina (UA), 26/111 NSTEMI, 47/111 STEMI	50 healthy, 60% men	At hospitalization	14% anti-β2GPI + in ACS (18% in men, 8% in women: ns difference, higher % in <50 years old and in those who died) vs 2% in controls. Higher titers IgA anti-β2GPI in ACS. No difference in aCL. Nobody was LA+. IgA anti-β2GPI associated with previous stroke, not with hypertension or previous MI, and higher concentration in UA and STEMI, not NSTEMI. IgG and IgM anti-β2GPI correlate with IgG and IgM aCL
Meroni, J Thromb & Hemostasis 2007[257]	aCL and anti-β_2GPI IgG, IgM, ANA. Cut off 10 GPL and MPL for aCL, > 99th percentile for anti-β_2GPI (>0.13 IgG, >0.28 IgM)	172 first MI premenopausal women < 45 years old, mean age 39: 62% significant stenosis, 16% non-significant, 22% no stenosis. 50 unselected MI, 86% men, mean age 53	172 matched for age, sex and geographical origin	3-12 months after (for unselected MI within 3 weeks)	anti-β_2GPI IgG: 9 vs 2% anti-β_2GPI IgM: 11 vs 3% OR adjusted for hypertension and smoking IgG 2.47, IgM 3.68 for the fourth quartile, cut off 13.9%. No positivity in 50 unselected MI, 86% men

Davies, Int J Clin Practice 2007[258]	Cases report (n=5) Various tests repeated +	5 MI < 50 years old with normal coronary arteries, 80% men, mean age 35	-	various	All LA+
Urbanus, Lancet Neurology 2009[259]	LA, aCL IgG, anti-β_2GPI IgG, aPT IgG	203 first MI women < 50 years old (first phase 1990-1995)	628 healthy, frequency matched for age, residence, index year	Second phase (1998-2002)	3% LA+ in MI vs 0.7% in controls, OR of MI 5.3 in LA+ vs 2.3 in LA-, 21.6 if also Oral Contraceptives, 34 LA+ smoke. No interaction tested. aCL and β_2GPI non-significant. High cut offs for traditional CV risk factors
Grosso, Ann Int Med 2018[266,267]	aCL IgG, IgM, IgA, anti-β_2GPI IgG, IgM, IgA. Cut-offs 10 GPL, 30 MPL, 20 APL	805 first MI <75 years old, 81% men, mean age 62, from 17 Swedish hospitals, 2010-2014	805 individual matched for sex, age, geography	6-10 weeks	11% IgG aCL and anti-β_2GPI + in MI vs controls, OR 13, 7.8 adjusted. No difference in type/severity of MI between MI aPL+ vs aPL-. IgG aPL+ more frequent among smokers and females (non-significant)

1.10 Complement, complement regulators and anticoagulants in APS

1.10.1 Interaction between coagulation, fibrinolysis and complement

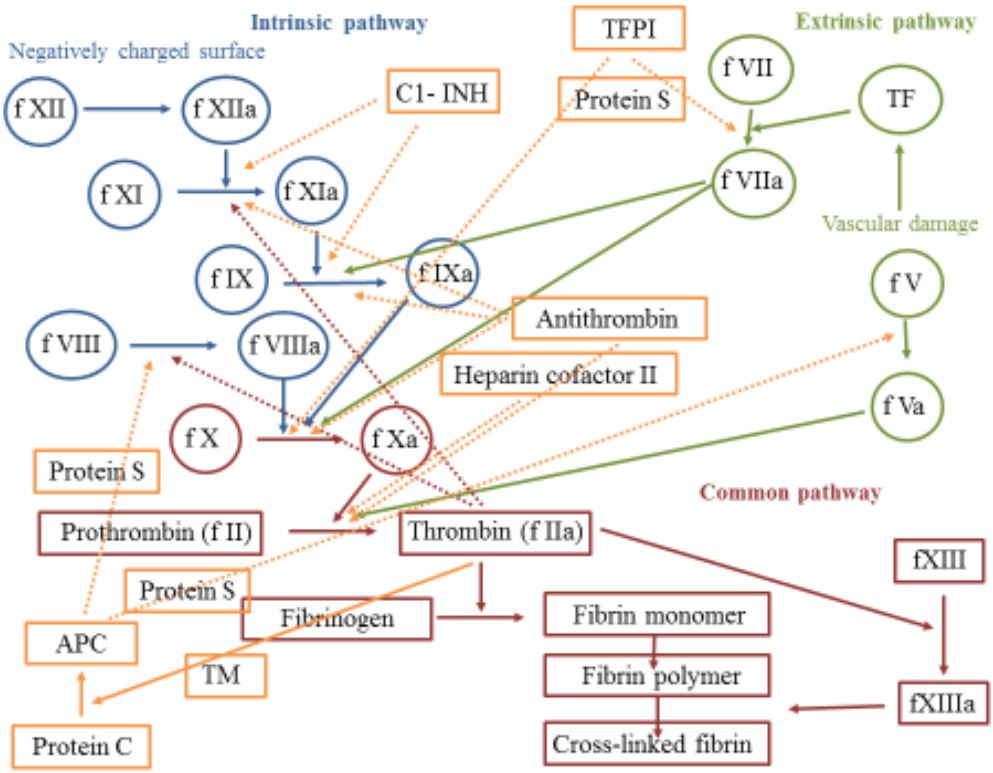
Coagulation, fibrinolysis and complement cascades derive from a single common ancestral pathway, composed of serine proteases and SERPINs. Serine proteases are enzymes that cleave peptide bonds at a specific site in order to activate their substrates. On the contrary, SERPINs are serine protease inhibitors. These systems are tightly regulated, and they cross-interact.

The **coagulation** cascade[183] (figure 4) starts in response to vascular injury, when platelets adhere, aggregate and expose the phospholipids necessary for the binding of clotting enzymes. There are two pathways of activation: the intrinsic (fXII) and extrinsic (fVII-TF) pathways, which join into the common pathway at fXa. The cascade ends with the formation of cross-linked fibrin, stabilized by fXIIIa through the binding with α2-antiplasmin. Positive and negative feedback loops regulate coagulation along the pathway. There are three main inhibitory mechanisms. The first is antithrombin (AT), a SERPIN that forms a complex with thrombin (TAT) and inhibits its actions. AT is potentiated by the help of natural heparin sulfate proteoglycans produced by the endothelium. The second is made of Activated Protein C (APC), helped by the cofactor PS, to inactivate fVIIIa and fVa, making them unavailable for fXa and thrombin formation, respectively. The third is Tissue Factor Pathway Inhibitor

(TFPI) that inhibits the extrinsic pathway at the level of TF-fVII catalytic complex. Its functions are enhanced by heparin and protein S.

Figure 4 – The coagulation system and natural anticoagulants

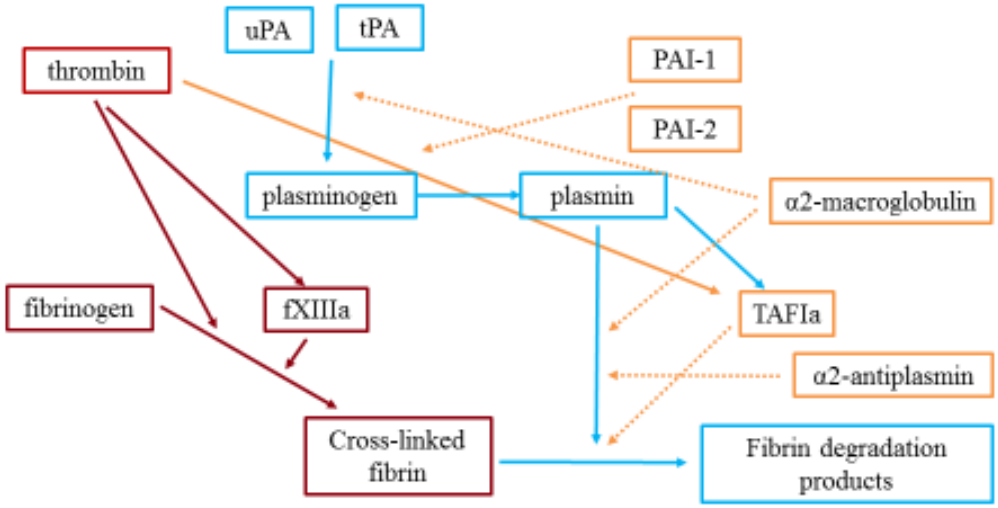
In blue the intrinsic pathway of coagulation activation, in green the extrinsic pathway, in red the common pathway. Natural anticoagulants are illustrated in orange.



Fibrinolysis (figure 5) has the goal of breaking down the clot, in order to preserve blood flow. The central enzyme of this cascade is plasmin, which main function is to disintegrate fibrin. Plasmin derives from the conversion of plasminogen, a circulating pro-enzyme, by the action of tissue (t) and urokinase-type (u) Plasminogen Activator (PA). u-PA binds to a specific cellular receptor (u-PAR, CD87), expressed by monocytes/macrophages, T cells, neutrophils, endothelial cells, epithelial cells (especially renal), smooth muscle cells, and fibroblasts, whereas t-PA is mainly synthesized by endothelium, neurons, microglial cells, and smooth muscle cells[93]. tPA concentrations in plasma are usually low, but they increase during stress and physical exercise. The binding of t-PA to fibrin considerably increases its affinity for plasminogen by 1000 to 1500-fold, further enhanced by annexin II, which also protect plasmin from inhibition[268]. The main inhibitors of the fibrinolytic pathway are

Plasminogen Activator Inhibitor (PAI)-1 and -2, Thrombin Activatable Fibrinolysis Inhibitor (TAFIa), α2-antiplasmin, α2-macroglobulin.

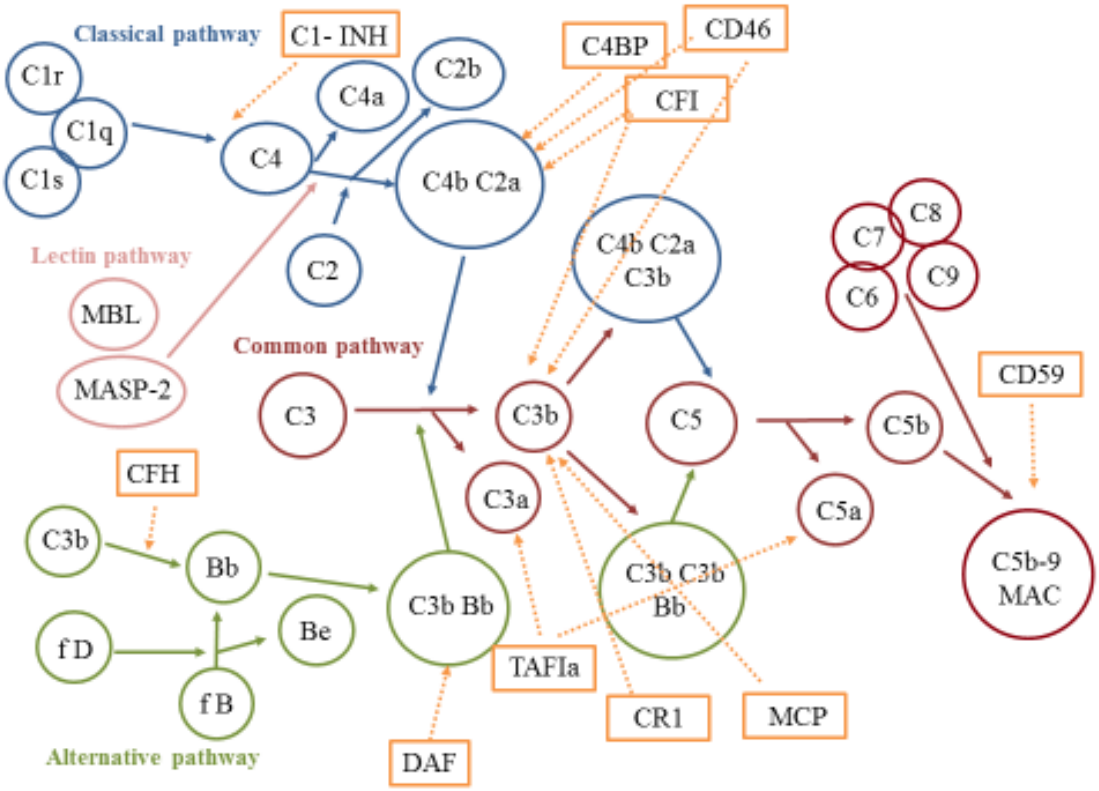
Figure 5 – The fibrinolytic system and its main inhibitors
In light blue the fibrinolytic pathway, in red the final pathway of the coagulation cascade. Natural anti-fibrinolytic factors are illustrated in orange.



The **complement** system (figure 6) was discovered more than 100 years ago[269]. It is part of the innate immune system and its functions are to protect the organism from infectious agents and to recognize and remove modified self-cells. It also represents one of the effector arms of antibody-mediated immunity. The complement cascade can be divided into four main steps: initiation, C3 convertase and C5 convertase activation and amplification, and terminal complex formation. The initiation phase starts with the activation of any or all of three different pathways, depending on the substrate that is recognized: lectin (by carbohydrates on microbial surfaces and on apoptotic/necrotic cells), classical (mostly, but not only, by antigen-antibody complexes) and alternative (by spontaneous activation of C3). The more than 35 small components of the complement system are zymogens that circulate inactive, until they are enzymatically modified to, in turn, activate other substrates by proteolytic cleavage, in a cascade manner. In this way, the complement system can be activated only when and where needed. These reactions can be amplified in order to generate a potent inflammatory response when required, in particular with the formation of the anaphylatoxins C3a and C5a.

Figure 6 – The complement system and its main inhibitors

In blue the classical pathway of complement activation, in pink the lectin pathway, in green the alternative pathway, in red the common pathway. Natural complement regulators are illustrated in orange.



There are several examples of molecular interactions between the coagulation, fibrinolysis and complement cascades[106,270-273] (figure 7).

Complement can activate the coagulation cascade, for example the Terminal Complement Complex (TCC) is able to catalyze the cleavage of prothrombin to thrombin even without fVa. Mannose-associated serine protease 2 (MASP-2), a component of the lectin pathway, can activate thrombin. C5a upregulates Plasminogen activator inhibitor-1 (PAI-1) and induces TF on endothelial cells and neutrophils[274,275], thereby contributing to the activation of the extrinsic coagulation pathway.

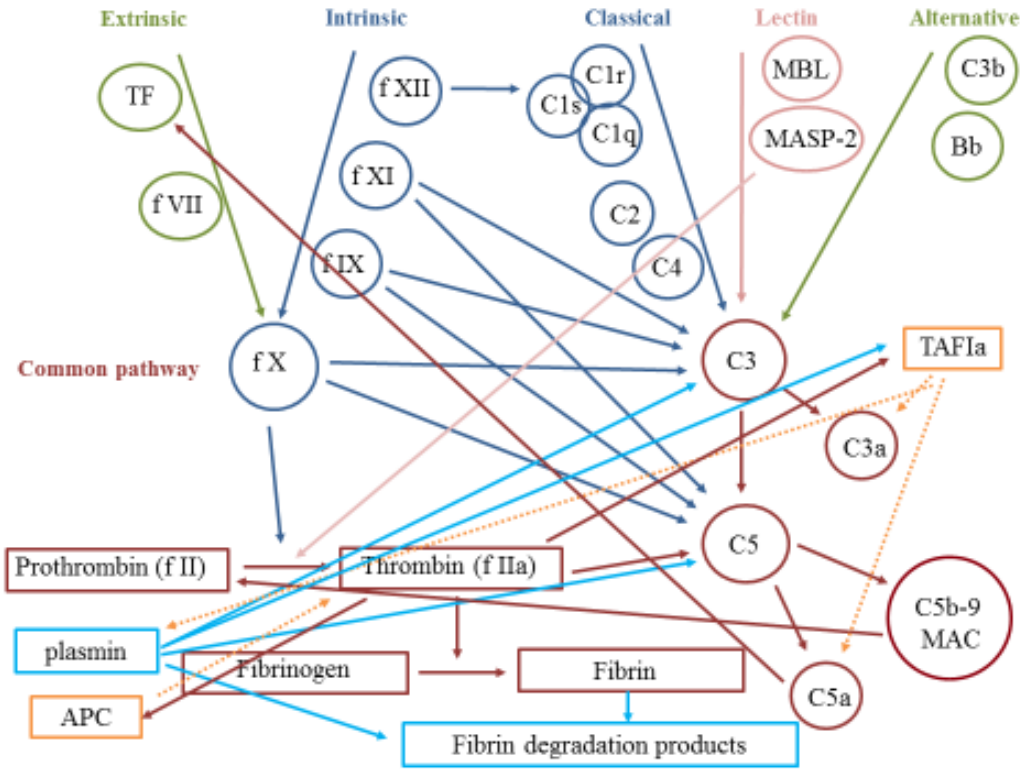
On the other hand, coagulation/fibrinolysis proteases have been shown to cleave C3 and C5 into the anaphylatoxins C3a, C5a in vitro and ex vivo. FXa is considered the most potent factor to mediate this proteolytic activation, but also thrombin, which can generate C5a even in the absence of C3[276], plasmin, fIXa and fXIa[272]. In vitro, inhibition of fXa by

fondaparinux or enoxaparin reduces also fXa-mediated C3 cleavage, in a concentration-dependent manner[272]. TAFI, generated by thrombin-TM complex, inhibits fibrinolysis and inactivates C3a and C5a. FXIIa can activate C1r.

Other mechanisms apart from complement cleavage can explain the pro-inflammatory properties of some coagulation factors. For example, TF has been shown to play a role in pregnancy morbidity, but more through enhanced inflammation than coagulation. In an experimental study, Girardi et al[275] could demonstrate that TF causes trophoblast injury, placental dysfunction and damage to the developing placenta and embryo by enhancing neutrophil activity. In fact, fibrin depositions and thrombi were not present after IgG aPL injection in pregnant mice, despite abundant TF staining throughout the decidua and on embryonic debris.

Figure 7 – some interactions between the pathways

The extrinsic pathway of coagulation and the alternative complement pathway are illustrated in green, in blue the intrinsic coagulation pathway and the classical complement pathway, in pink the lectin complement activation pathway, in red the common pathways of both complement and coagulation. Fibrinolysis is depicted in light blue and inhibitors in orange.



It is intuitive that, if these cascades are so tightly linked and can influence each other, using treatments that target one pathway can have effect on another (more on this in 1.10.6). A further evidence of these tight interactions is given by the fact that anticoagulated plasma has lower levels of complement activation products than serum[277], because coagulation factors activate complement. That's the reason why EDTA plasma is used in complement measurement, because it is more efficient than citrate or heparin in blocking complement activation in vitro. Also the correct handling and preservation of blood samples is extremely important, since even room temperature can activate complement, despite EDTA plasma.

1.10.2 Complement in APS

Research so far talks for a role of complement activation in the pathogenesis of APS[37,103,105,122,274,278-301].

In 1992 Davis et al demonstrated higher levels of sC5b-9 in aPL+ patients affected by stroke compared to other stroke patients aPL-[299].

The following years were characterized by research on APS mice models, where thrombosis or fetal injury could be induced by passive transfer of IgG aPL isolated from human sera of aPL+ patients. It was important to demonstrate a direct pathogenic role of aPL, but also the role of complement was studied in this context. Mice were treated with C3, C5a or fBb inhibitors, like C3 convertase inhibitor complement receptor 1 related gene/protein y (Crry)-Ig[103,282], or knocked out for C3, C4 or C5[279,280,283]. Through these experiments, it was possible to show that, when complement is impaired/blocked, mice are protected from fetal loss and growth retardation. Moreover, they have a decreased thrombus size and less endothelial activation[283,284], as expressed by less leucocyte adhesion on endothelial cells.

Even the anti-complement properties of heparin supported the role of complement in the pathogenesis of fetal injury in APS[110,280,282,283,293] (see section 1.10.6).

After mice models, placentas of aPL+ pregnant women were examined for complement deposits and signs of damage, with controversial results[122,278]. Through these studies, it was possible to demonstrate the presence of aPL deposits in human placentas.

Finally, plasma levels of complement factors and inhibitors were measured in primary and secondary non-pregnant APS[287,289,293,294,302], showing signs of complement activation. In particular, Oku et al[293] in 2009 demonstrated higher mean levels of complement activation products C4a, C3a and Bb, and lower C3, C4 and CH_{50} (complement hemolytic serum activity defined by the serum concentration that results in complement-mediated lysis of

50% of sensitized sheep erythrocytes) in pAPS compared to healthy as well as other non-SLE autoimmune disease-affected controls. No defects in complement factor I (CFI) or complement factor H (CFH) were found, but these complement regulators were not measured in the whole cohort and they were not compared with controls. The presence of anticoagulant treatment was not taken into account in the analysis. However, no patients receiving heparin or its derivatives were included in the study. Devreese et al in 2010[289] and Breen et al in 2012[287] found higher levels of C3a both in LA+ and aPL+ patients. The former study did not consider treatment in the analysis and the findings were independent of previous thrombotic events, whereas the latter showed no difference according to both treatment and clinical manifestations. Stachowicz et al[190] in 2018 showed higher C3a, C5a, sC5b-9 levels in plasma and more C5b-9 deposits on clot from APS patients affected by VTE, especially TP and LA+, compared to VTE non-APS and healthy controls.

Other studies focused on complement split products deposits on platelets and erythrocytes, correlating them with aCL and LA. Navratil et al in 2006[303] noticed an increase in C4d deposits on platelets (P-C4d) in SLE that was associated with LA and aCL, SLE disease activity (SLEDAI) and C4d deposits on erythrocytes; there was no adjustment for anticoagulant treatment. Svenungsson et al[304] confirmed these findings on a larger sample by showing more P-C4d in SLE compared to matched controls. P-C4d positively correlate with plasma C3dg and negatively with C3 and C4. P-C4d associate with thrombosis independently of traditional CV risk factors, renal function and steroid treatment. The Odds Ratio for vascular events is higher in concomitant LA+, and an interaction was shown between P-C4d and LA+. Even here, anticoagulant treatment was not considered in the analysis.

Table 4 – Studies on complement in APS

Author, Journal, Year	What	Study subjects	Controls	Findings
Davis, Clin Exp Rheumatol 1992[299]	sC5b-9	13 stroke aPL+	13 stroke aPL-	Higher in aPL+ stroke vs aPL- (p=.0018)
Stewart, Br J Haematol. 1997[286]	C5b-9, platelets activation (ATP release) and destruction	aPL+ sera	aPL- sera	aPL+ sera induced C5b-9 production, binding to CL-beads, and platelets activation and destruction. Higher titers, higher C5b-9 production and binding. Monoclonal antibodies anti-C5b-9 inhibited platelets activation and destruction
Salmon, Annals of the Rheumatic Diseases 2002[103]	C3 decidual deposits	Pregnant mice injected with aPL IgG	Pregnant mice injected with control IgG or aPL IgG +, Crry-Ig or C3-/-	Fetal resorption and growth retardation aPL IgG-induced, prevented by Crry-Ig. C3-/- protected. Absence of C3 deposits after Crry-Ig
Holers, The Journal of Experimental Medicine 2002[282]	Surgically-induced thrombosis	Mice injected with aPL IgG	Mice injected with healthy control IgG	5-fold increase in thrombus size in aPL IgG, reversed by Crry-Ig
Girardi, Journal of Clinical Investigation 2003[280]	Fetal injury	Pregnant mice injected with human aPL IgG	Pregnant mice injected with F(ab)2 fragments of aPL IgG	C4-/-, C5-/-, anti-C5 mAb, C5aR-/- and neutrophil depletion protected from fetal loss and growth restriction aPL IgG-induced (classical pathway activation by Fc portion)
Girardi, Nature medicine 2004[281]	Fetal resorption	Pregnant mice injected with human aPL IgG	Pregnant mice injected with control healthy human IgG	Increased frequency in aPL IgG (p<.001), same to controls when treated with heparin (complement inhibitory properties), but not with hirudin or fondaparinux. Higher plasma C3a and C3 placental deposits in mice injected with aPL IgG, normalized after UFH/LMWH.
Pierangeli, Arthritis Rheum. 2005[283]	Surgically induced thrombosis and endothelial activation	C3+/+ and C5+/+ mice injected with aPL IgG	C3+/+ and C5+/+ injected with control IgG. C3-/- and C5-/- mice or anti-C5 mAb	Larger thrombi and more leukocyte adhesion in aPL+ vs controls. Smaller thrombus size in C3-/- and C5-/- or in mice treated with anti-C5 mAb

Fischetti, Blood 2005[279]	Platelets-leukocytes aggregates (PLAs) and thrombotic occlusions (TOs) in intraperitoneal injection of LPS 3 hours before	Mice injected with aPL IgG	Mice injected with anti-β_2GPI-depleted aPL IgG. C6-/- mice. anti-C5 monoclonal antibodies (mAb)	Higher PLAs and TOs in aPL IgG, more C3 and C9 deposits. Less PLAs and TOs in C6-/- and in anti C5-mAb treated mice
Navratil, Arthritis & Rheumatism 2006[303]	C4d on platelets (P-C4d)	105 SLE, mean age 49.5 years, all women, 50% aPL+ (just 50% of the patients were tested)	115 AI, 65% women (including HCV n=23, hematologic malignancies n=8, sickle cell anemia n=8, primary Raynaud n=5, Sjögren n=5), 100 healthy, 85% women, mean age 40 years	P-C4d specific for SLE, 18% of SLE, associated with LA+ (p<.0001) and IgG aCL+ (p=0.035), low serum C4 (p=0.046) and C4d on erythrocytes (p<.0001). 31% of aPL+ had P-C4d vs 11% aPL-. No correction for /info on treatment
Cavazzana, J of Autoimmunity 2007[278]	C3c, C4d placental deposits	2 APS abortions >20weeks (treated with ASA and LMWH)	2 therapeutic abortions >20weeks aPL-	Infarcts and fibrin deposition in APS. No difference in complement deposits (APS treated with LMWH)
Shamonki, Am J Obstet Gynecol. 2007[122]	C4d, C3b, C5b-9 placental deposits	47 aPL+ placentas	23 normal placentas	Higher C4d (p<.001) and C3b (p=.005) in aPL+ trophoblasts vs controls (that had some anyway). Higher C5b-9 (p=.005) in controls. 62% of aPL+ had pathological placentas (infarcts, inflammation, thrombosis, vasculopathy) vs 0 controls (p<.001). Those abnormalities correlated with C4d deposits on trophoblasts (p<.001)
Oku, Ann Rheum Dis. 2009[293]	C3, C4, CH_{50}, C3a, C4a, C5a, CFI, CFH (EDTA)	36 pAPS, 75% women, mean age 46	42 AI non-SLE (88% women, mean age 52), 36 healthy	C3, C4, CH_{50} lower (p<.001) and C3a, C4a (measured in 17 pAPS, 9 AI non-SLE and 15 healthy) higher in pAPS vs other groups (p<.001). No diff in C5a. No low CFI, CFH (just measured in 13-16 pAPS).

				CFH ↑ for ↑ C3a, NS (r=0.43, p=0.097), CFI ↑ for ↑ C3a, NS (r=0.38, p=0.22). Serum immune-complex levels higher in pAPS vs AI (70% vs 36%, p=0.012). High LA+ (OR 6.42, 95%CI 1.37-30.1) and IgG aPS/PT (OR 8.0, 95%CI 1.69-38.0) inversely correlated with CH_{50}. No difference according to APS manifestations. No adjusted analysis for treatment (but no heparin-treated)
Peerschke, Lupus 2009[302]	C1q, C4d depositions on heterologous platelets (in-situ complement fixation), aPL, no LA determined in SLE patients	91 SLE-aPL-, 27 SLE-aPL+, 51 sAPS, 57 aPL carriers, 96 pAPS (Sapporo). Mean age ca 40, women ca 80%	50 healthy	IgG aPL and IgG anti-β_2GPI ($p<.05$), platelets activation ($p<.005$) and arterial thrombosis in SLE-aPL+ (p=.04) positively associated with complement fixation. No treatment adjustment (heparin-treated patients excluded, more warfarin treated in apL+), no adjustment for serum complement levels (C4)
Devreese, Thrombosis and Haemostasis 2010[289]	C3a (citrate plasma)	37 LA+ with thrombosis (32 venous, 5 arterial)	19 LA+ w/o thrombosis, 10 healthy, 12 AI aPL-	Higher in LA+ vs AI and controls (p=.0001), higher in AI vs controls (p=.0127). No difference between LA+ thrombosis vs LA+ non-thrombosis (p=0.47)
Cohen, J Pathol. 2011[288]	C4d, C1q, properdin	Mice intraperitoneally injected with human IgG aPL. 26 human SLE/APS placentae from 21 women (15/21 SLE, 6/15 sAPS, 6/21 pAPS)	Mice treated with normal human IgG; mice deficient in C1q and factor D. 40 human healthy placenta controls. 22 Intra-Uterin fetal Death (IUFD) non APS/SLE	Increased C4d deposits in mice aPL+ vs normal IgG or complement deficient, associated with adverse fetal outcome. C4d diffuse staining in SLE/APS ($p<.001$) vs focal staining in IUFD vs none in controls, related to fetal death (p=.03)

Breen, Thrombosis and haemostasis 2012[287]	Bb, C3a (EDTA)	186 aPL+: 39/186 aPL carriers, 147/186 pAPS (Sydney). No SLE.	30 healthy	Higher vs controls. No difference between clinical phenotypes of aPL+ or according to treatment
Stachowicz, Sci Rep 2018[190]	C3a, C4a, C5a, C5b-9 in plasma, C5-9 within clots prepared ex vivo	23 APS, 65% TP	19 VTE (no difference in treatment vs APS), 20 healthy, age and sex matched	Higher C3a, C5a, C5b-9 in plasma APS vs VTE and healthy controls (p<.0001). Higher C5-9 content and decreased antithrombin in clots APS, exp LA+ and TP, vs VTE and healthy. Plasma C5b-9 positively correlated with C5-9 deposits in clots in APS (r=0.46, p=.031)
Svenungsson, Rheumatology 2020[305]	P-C4d (flow cytometry), aPL, LA	308 SLE	308 age, sex and region matched controls	Increased in SLE (50% vs 5%, p<.0001). Positively correlated with C3dg (r_s=0.46, p<.0001) and negatively with C3 (r_s=-0.28, p<.0001) and C4 (r_s=-0.35, p<.0001). Adjusted OR for thrombosis 2.4 (95%CI 1.3-4.6, p=0.006), interaction with LA+, OR 12.0 (5.4-28.3), AP 0.8 (0.4-1.1), RERI 9.2 (-0.7-19.2). No correction for/info on anticoagulants
Chaturvedi, Thrombosis and Hemostasis 2020[36]	Cell surface C5b-9 deposits. Complement-mediated cell killing (mHam assay, positive when >20% cell killing)	59 pAPS (37% TP), 10 CAPS	74 SLE	85.7% in CAPS, 35.6% mHam+ in pAPS, vs 6.8% in SLE (p<.001, for trend). 60% in TPs vs 33% in others (p=.002). 66.7% in recurrent thrombosis vs 33.3% in single event (p=.001). anti-β_2GPI from pAPS induced C5b-9 deposition, which correlated with mHam assay, inhibited by anti-C5 monoclonal antibodies. No effect on mHam or inhibition of C5b-9 deposition by factor D-inhibitor

1.10.3 Complement inhibitors in APS

The main function of complement inhibitors is the regulation of complement activation. Additionally, some of them interact with the enzymes involved in the coagulation process, cell adhesion and extracellular matrix[306-308]. C4BP, Complement factor I (CFI), C1 inhibitor (C1INH) act preferentially on the classical and lectin pathways, whereas Complement factor H (CFH) and Factor H-like protein 1 (FHL1), among others, act on the alternative pathway. TAFIa directly inactivates the split products C3a and C5a of the common final pathway of the complement cascade. All these aforementioned molecules are fluid phase complement inhibitors. In addition, Decay Accelerating Factor (DAF, or CD55), Complement receptor type 1 (CR1, also known as CD35), Complement regulator of the Immunoglobulin superfamily (CRIg), Membrane cofactor protein (MCP, or CD46) and protectin (CD59) are some examples of membrane-bound regulators (figure 6).

Impaired function of complement inhibitors may represent one of the additional mechanisms leading to excess complement activation and development of an inflammatory process in APS. It is therefore of relevance to investigate if aPL have a direct inhibitory effect or if they are associated with a decrease in complement inhibitors and if this could contribute to the pathogenesis of thrombosis and/or obstetric morbidity in APS.

In an old study by Hammond et al[309], decreased numbers of CR1 molecules on erythrocytes in SLE patients with positive aCL and in pAPS was shown. CR1 is a cofactor for factor I in the catabolism of C3b, iC3b and C4b. Its impairment may contribute to increased complement activation. This study reported a negative correlation between aCL titers and CR1, and a positive association between aCL and C3d deposits on erythrocytes.

The studies that followed were conducted on pregnant mice. In rodents, C3 convertase inhibitor complement receptor 1 related gene/protein y (Crry) is a membrane-bound complement classical pathway inhibitor, which blocks C3 and C4 activation on self-membranes. It is essential for early embryonic development and survival[310]. If absent, embryonic damage occurs leading progressively to death, since these embryos are not able to suppress the spontaneous complement activation and tissue damage promoted by C3. This was shown by both the presence of increased C3 split products deposits on placentas in case of absence of Crry, and also by the normal pregnancy outcomes in mice knocked out for both Crry and C3. An experimental study by Holers et al[282] was able to demonstrate that the simultaneous administration of IgG aPL and Crry-Ig in mice during pregnancy totally prevented fetal resorptions and growth retardation as compared with IgG aPL treatment only, which instead caused a nearly four-fold increase in fetal loss. Moreover, the decidual tissue,

which after the injection of IgG aPL was damaged with increased cell density, focal necrosis, apoptosis, and scattered clusters of neutrophils throughout, was normal in mice who received Crry-Ig together with IgG aPL.

Subsequent studies on obstetric APS reported a significant reduction in the endometrial expression of DAF/CD55 in aPL+ women with recurrent pregnancy loss[37], and mutations in genes encoding for complement regulatory proteins in pre-eclamptic aPL+/SLE women[38]. Finally, a recent study by Chaturvedi et al[36] reported a 60% frequency of mutations in genes encoding for some complement regulators in CAPS (see table 5 for details).

Table 5 – Studies on complement inhibitors in APS

Author, Journal, Year	What	Study subjects	Controls	Findings
Hammond, Arthritis rheum 1989[309]	CR1 and C3d on erythrocytes	8 pAPS, 19 SLE aCL+ (tot 27 aCL+) and 34 SLE aCL-	33 healthy	Reduced number of CR1 molecules in aCL+ vs aCL- and in SLE aCL-vs ctrls (trend). CR1 negatively correlated with aCL titers. Higher C3d deposits on erythrocytes in aCL+ vs aCL- and in SLE aCL-vs ctrls (trend). C3d correlated with aCL titers.
Xu, Science 2000[310]	Crry	Crry-/- mice	C3-/- and Crry-/- mice	Total embryonic lethality, more C3 deposits, reversed when also C3-/-
Francis, Molecular Human Reproduction 2006[37]	mRNA of DAF/CD55	aPL+ women with recurrent miscarriages in endometrium before conception	aPL- women	Lower expression
Salmon, PLoS Medicine 2011[38]	MCP/CD46, CFI, CFH	PROMISSE: 250 pregnant SLE and/or aPL+	34 matched	7/40 (18%) pre-eclamptic had heterozygous mutations vs 5/59 (8%) non-autoimmune pre-eclamptic. No mutations in controls or non-pre-ecamptic
Chaturvedi, Thrombosis and Hemostasis 2020[36]	15 genes of complement regulatory proteins, e.g. CFH, CFI, CD46, CR1	10 CAPS, 55 APS	21 SLE aPL-, 33 acquired Haemolitic Uremic Syndrome (aHUS), 43 healthy	60% of CAPS had mutations in genes involved in complement regulation, 51.5% in aHUS, 21.8% in APS (p=.01), 28.6% in SLE aPL- (p=.09), 23.3% in healthy (p=.02)

1.10.4 C4b-binding protein in APS and similarities with β2 glycoprotein I

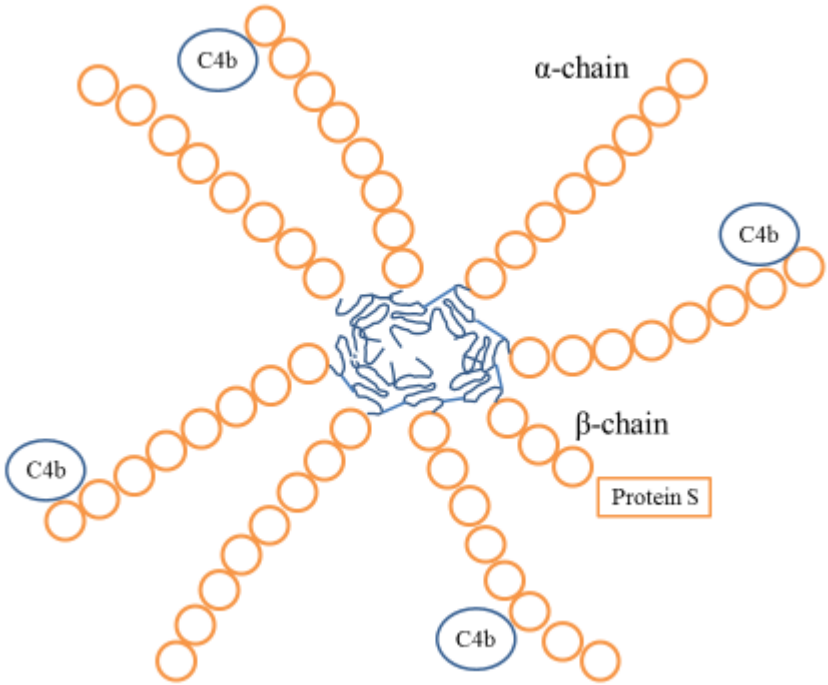
C4b-binding protein (C4BP)[311-325] is a soluble classic/lectin pathway complement inhibitor with anticoagulant properties. Like CFH, the main soluble inhibitor of the alternative pathway, it acts on the C3 and C5 convertases in two ways:

1. with a "decay accelerating activity", speeding up the dissociation of C4b from C2a
2. with a "factor I-cofactor activity", helping CFI in the cleavage and consequent deactivation of C4b into fragments (C4c, C4d)[326]

C4BP is a glycoprotein synthesized in the liver. It has an octopus shape with a central, ring-like core, and seven long tentacles (α-chains), plus one shorter (β-chain) in its major isoform (α7/β1), which constitutes the 80% of the total (t) C4BP present in circulation (figure 8). Other isoforms lack the β-chain or have just six α-chains: α6/β1 (that we call C4BPβ+ together with α7/β1, to indicate the presence of the β-chain), α6/β0 and α7/β0. The α-chains are composed of eight and the β-chain of three Complement Control Protein (CCP) modules[312,314]. The β-chain forms a complex with anticoagulant protein S[327], whereas each α-chain binds to C4b and has high affinity for negatively charged phospholipids[311,313,316]. This feature allows C4BP to localize its complement inhibitory activity near membranes[319]. During inflammation, the synthesis of C4BP isoforms lacking the β chain increases. That's why C4BP α6/β0 and α7/β0 are considered acute-phase reactants. C4BP binds to late apoptotic cells to avoid excessive inflammation, by limiting local C3 and C9 deposition[321,324,325]. Deficiency in C4BP expression can lead to miscarriages[328] and the genetic locus C4BPB/C4BPA (separate for the β- and α-chains, respectively) has been linked to venous thrombosis, independently of protein S[322].

Figure 8 - C4b-Binding Protein (C4BP)

C4BPβ+ is a spider-like molecule composed of multiple identical α-chains made of eight CCPs each and one β-chain made of three CCPs. The chains are disulfide linked in the center. The illustrated isoform is the most abundant in the circulation (α7/β1). Each α-chain contains a binding site for C4b involving the first three CCP domains, whereas the first two CCPs of the β-chain contain the binding site for protein S.



In a pilot proteomic study[329] we recently noted that C4BP is lower in aPL+ SLE patients as compared to SSA/SSB+ SLE patients and controls. Barnum et al[330] showed higher plasma levels of C4BPt in inflammation and in active SLE compared to healthy controls. This can be explained by the fact that the C4BP isoforms lacking the β-chain behave as acute-phase reactants. Forastiero et al[331] in 1994, measured C4BP in different aPL+ subgroups, showing that lower levels are associated with aCL positivity but not with LA+, independently of thrombosis or SLE.

By molecular structure and functions, C4BP is very similar to β_2GPI. They both belong to the CCP superfamily, together with CFH, CR1/CD35, MCP/CD46, DAF/CD55, protectin (CD59), CRIg. The proteins of this particular family are characterized by a structure made of CCPs, also called short consensus repeats (SCRs) or sushi-domains, and by flexible conformations that have an impact on their functional activity. Conformational changes are

influenced by the microenvironment composition, altered by bacterial or viral products, inflammation and oxidative stress. As a member of this superfamily, β_2GPI regulates complement by facilitating C3 degradation[332]. Moreover, it is a scavenger of lipopolysaccharide, playing a role in innate immunity[333]. Since its plasma levels have been shown to be inversely correlated with markers of inflammation as well as temperature rise[333], it is considered a negative acute-phase reactant. It binds liposomes and microparticles (MPs)[96] and it is involved in regulation of apoptosis and angiogenesis[61]. It is involved in hemostasis because it interferes with platelets adhesion[85,86], ADP-induced aggregation[334], fXa, fXII and the intrinsic coagulation pathway[335]. It seems to interact with anticoagulant protein C in vitro[220] and with tPA, plasminogen and plasmin, enhancing fibrinolysis[107]. Nevertheless, a nicked form of β_2GPI, cleaved by plasmin in domain V, has been found to bind to plasminogen and to prevent its conversion to plasmin by tPA[336], in a sort of negative feedback loop to inhibit further plasmin generation. It is downregulated in the acute phase of thrombosis in vivo, especially in DIC together with protein C and antithrombin (AT)[337]. Despite all these indications of interaction, it is yet not clear what role exactly β_2GPI plays in the coagulation network in vivo. Moreover, β_2GPI is involved in the regulation of protein S activity, because it interferes with the binding of protein S to C4BP[338,339], increasing the portion of circulating free protein S available for anticoagulation. Human monoclonal aCL/anti-β_2GPI were shown in vitro to reverse this effect by binding to β_2GPI, thereby increasing the affinity of PS to C4BP[338].

Due to the similarities between β_2GPI and C4BP, the presence of antibodies against C4BP in APS is a justified hypothesis. Such antibodies have been detected by Guerin et al[340] and Arvieux[173] in the late -90s, together with antibodies directed towards other CCPs, such as anti-CR1 and anti-CFH. Nevertheless, these antibodies haven't been included in the classification criteria, because of their not proven association with thrombosis. Antibodies directed to C4BP could possibly contribute to the lower levels of C4BP in aPL+, together with the concomitant effect of warfarin and the presence of anti-protein S antibodies.

Table 6 – C4BP in APS

Author, Journal, Year	What	Patients	Controls	Findings
Barnum, Complement inflamm 1990[330]	C4BP	21 SLE, 100% women	19 patients 24-72h after joint replacement surgery (acute phase) and 33 healthy	Higher in acute-phase vs SLE and controls (p<.0001), higher in SLE vs controls (p<.0001). No correlation between C-reactive protein and C4BP
Parke, the American Journal of Medicine 1992[341]	C4BP, total PS, free PS	11 obstetric aPL+, all women, childbearing age. 6/11 also had other thrombotic events. No SLE.	Healthy women (number not known), childbearing age, no contraceptive	Lower free PS in aPL+ vs controls (p<.05), further decrease in pregnancy. Also the ratio free/total PS was lower in aPL+, positively correlated with C4BP. No difference in total PS and C4BP. No antibody to PS was found.
Forastiero, Blood coagulation and fibrinolysis 1994[331]	C4BP, total and free PS	73 aPL+ (65/73 LA+, 53/73 aCL+, 45/73 both, 20/73 only LA+, 8/73 only aCL+), 56% women, mean age 47 years, 16/73 SLE	44 healthy, 55% women, mean age 39 years, 10 SLE-aPL-, 17 recurrent thrombosis aPL-	Lower levels of C4BP, free and total PS in aCL+ vs aCL- and controls. C4BP lower in aPL+ independently of thrombosis (p<.05) or SLE (p<.001). Free PS lower in aPL+ independently of thrombosis (p<.01), in SLE aPL+ vs SLE aPL- (p<.001), and in SLE aPL- vs controls (p<.05). Only free PS was different in LA+ vs LA-. No subject was on anticoagulation
Guerin, Lupus 1998[340]	Anti-C4BP, anti-CFH, anti-CR1 antibodies	19 APS anti-β_2GPI	-	74% (14/19) anti-CFH antibodies 74% (14/19) anti-CR1 42% (8/19) anti-C4BP 32% (6/19) all four
Arvieux, Blood 1999[173]	aCL specificities	6 aCL+ women anti-β_2GPI negative	-	Antibodies anti-C4BP, LBP (LPS-binding protein), Thrombin Anti-Thrombin (TAT) complex

1.10.5 C4BP, protein S and coagulation

The β chain on C4BP contains the single binding site for protein S (PS). Circa 60% of PS is bound to C4BP in plasma, whereas 40% circulates free. Calcium potentiates the interaction between C4BP and PS, increasing the strength of their association. PS has high affinity for negatively charged phospholipids, therefore localizing the anti-complement actions of C4BP near membranes.

PS is produced by the liver, endothelial cells and megakaryocytes. The main function of PS is anticoagulation through acting as a co-factor for activated protein C (APC) in the proteolytic cleavage and inactivation of fVa and fVIIIa[342]. FVa accelerates the conversion of prothrombin to thrombin catalyzed by fXa in the prothrombinase complex. Levels of anticoagulant PS are reduced in aPL+ patients[221,331,343-346] and during warfarin treatment[342,347-351] (table 7), independently of thrombosis or SLE features. Antibodies against PS have been found among the heterogeneous pool of aPL[352-354]. The presence of these autoantibodies creates an acquired deficiency that may partially explain the pro-coagulant state characteristic of APS[341,346].

The anticoagulant properties of PS change according to its distinct forms. Free PS is believed to be more efficient than the bound state, PS-C4BPβ+. This is explained by the fact that C4BPβ+ modifies the activity of PS as cofactor for APC on fVa inactivation.

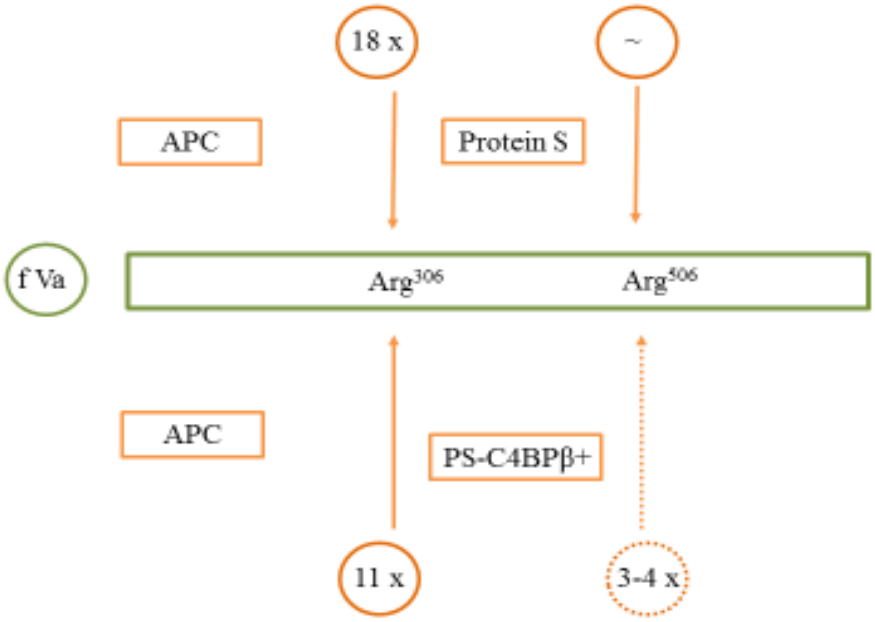
APC cleaves fVa at three sites, Arg^{506}, Arg^{679} and Arg^{306}, and proceeds via a biphasic reaction: a rapid proteolysis at Arg^{506} that results in the formation of an intermediate with partial cofactor activity for fXa, followed by a slow cleavage at Arg^{306}, which leads to a total inactivation of fVa.

FXa, in a sort of positive feedback loop, protects APC-mediated cleavage of fVa at Arg^{506}. One of the functions of PS is to abolish this inhibition by fXa on APC. Free PS also accelerates the APC-mediated cleavage at Arg^{306}. PS also increases the affinity of APC for anionic phospholipids, which are necessary for fVa cleavage.

In the ‘90s[313,318,320] it was believed that PS cofactor activity was totally impaired by the binding to C4BP. In 2008 Maurissen et al[323], by means of different experimental conditions, were able to show that the complex PS-C4BPβ+ actually increases Arg^{306} proteolysis more than 10-fold and inhibits Arg^{506} cleavage by APC by 3-4-fold. Free PS instead stimulates Arg^{306} proteolysis more than 20-fold and has no effect on Arg^{506}, although a 4- to 5-fold stimulation of the proteolysis on Arg^{506} by APC can be achieved, depending on the

experimental design. The total net effect of PS-C4BPβ+ complex is therefore just a 6-8-fold reduction in fVa inactivation as compared to free PS (figure 9). The different amino acid targets between free PS and PS-C4BPβ+ also influence the speed of fVa inactivation.
To summarize, both bound and free PS inactivate fVa, but the site of proteolysis, and the amount and speed of inactivation differ between free PS and the complex PS-C4BPβ+, consequently affecting the degree of anticoagulation.

Figure 9 – APC-mediated inactivation of fVa by free and bound protein S at Arg^{306} and Arg^{506}
Both PS and PS-C4BPβ+ are cofactors for APC in the proteolysis of fVa. PS enhances the cleavage at Arg^{306} by 18-fold, bound PS by 11-fold. While PS has no effect on APC-mediated inactivation at Arg^{506} in fVa, the complex PS-C4BPβ+ specifically inhibits APC-mediated cleavage at Arg^{506} by 3- to 4-fold. In green fVa, in orange the anticoagulant factors.



1.10.6 Anticoagulants[355] and complement

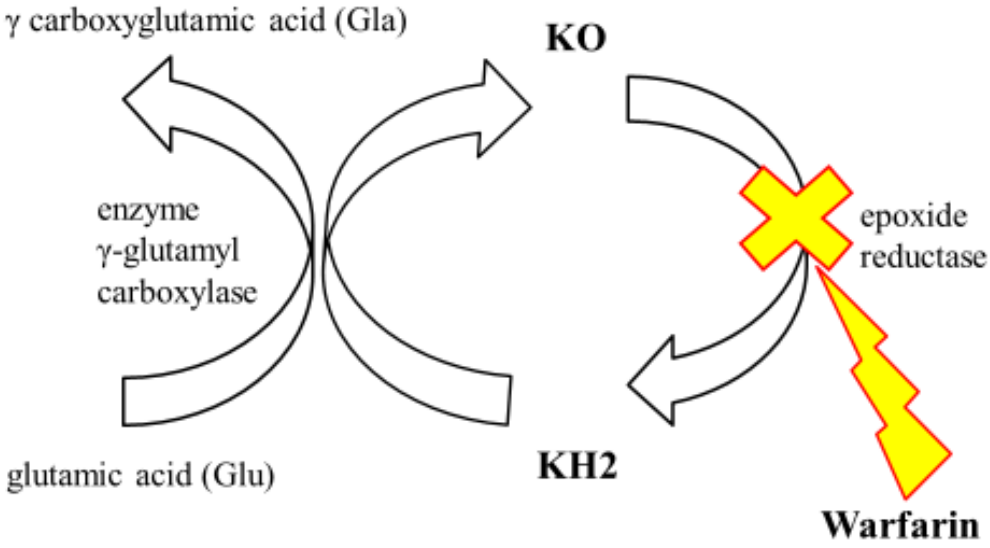
Heparin: Heparin is a natural, highly sulfated polysaccharide that needs binding to antithrombin (AT), an endogenous anticoagulant glycoprotein synthetized by the liver, to exert its anticoagulant effects, therefore it is considered an indirect anticoagulant. The complex heparin-AT inhibits fXa and, to a minor extent in LMWH compared to UFH, thrombin (fIIa). It is known since 1929 that heparin has both anticoagulant and complement inhibitory properties, thanks to its Oxygen (O) and Nitrogen (N) sulfation[356-358]. A relevant finding in the field of APS was brought by Girardi et al [281] with their experimental study conducted on pregnant mice in vivo and confirmed in vitro. They reported that even sub-

anticoagulant doses of heparin are able to block the complement activation induced by IgG aPL, consequently preventing miscarriages in mice. In contrast, other anticoagulants like hirudin or fondaparinux that lack complement inhibitory properties, are not able to prevent fetal injury, despite efficacious anticoagulation. They also demonstrated a negative association between heparin/LMWH and the complement activation product C3a in mice. The complete lack of benefit of anticoagulation on fetal resorption has been a striking and unexpected finding, because the treatment for thrombosis in APS has been based on anticoagulation since the discovery of the syndrome. Moreover, experimental studies show that heparin is able to bind to β_2GPI via its fifth domain and enhance plasmin-mediated cleavage of the Lys^{317}-Thr^{318} site in β_2GPI. In this way, heparin competes with β_2GPI's phospholipid-binding site, making it less able to recognize phospholipids, thus decreasing its immunogenicity[62].

Warfarin: Studies on the direct effect of warfarin on complement are lacking. Nevertheless, by understanding its mechanism of action, it is possible to hypothesize how warfarin might influence the complement system. Warfarin inhibits all vitamin K-dependent factors, both pro- (fII, fVII, fIX, fX) and anti-coagulants (protein S and C) through the block of the vitamin K epoxide reductase complex 1 (VKORC1). This enzyme has the function of bringing back vitamin K from an oxidative state (KO) to a reduced state (KH2). The reverse process of vitamin K epoxidation is performed by another enzyme, the gamma-glutamyl carboxylase, which, at the same time, is involved in the post-translational modification[359] of vitamin K-dependent proteins. The epoxidation and carboxylation reactions are called "coupled reactions", because carboxylation can only proceed if the gamma-glutamyl carboxylase is able to oxidize vitamin K at the same time. The gamma-carboxylation of the glutamic acid (Glu) residues in the NH2 terminal molecular region of a protein is necessary for its proper release and activation. Since warfarin blocks VKORC1, KH2 is no more available to start another epoxidation/carboxylation reaction. This results in the synthesis of uncarboxylated or partially carboxylated forms that are biologically inactive. Thus, warfarin cannot inhibit proteins that are already in the circulation, rather its anticoagulant effect will start after some time. This time variates depending on the half-life of each vitamin K-dependent factor. Since protein C and S have a shorter half-life compared to vitamin K-dependent coagulation factors, a paradoxical hypercoagulable state characterizes the first few days of warfarin therapy.

Figure 10 – Warfarin mechanism of action

KO is the oxidative state of vitamin K, KH2 the reduced form. The epoxide reductase is the enzyme inhibited by warfarin, which brings back KO to its reduced state. Gamma-glutamyl carboxylase has a coupled function: the oxidation of KH2 together with the γ-carboxylation of glutamic acid residues on the vitamin K-dependent proteins, necessary for their release and activation.



C4BP is linked to protein S in a complex, so it is reasonable to hypothesize that the whole complex may be reduced by warfarin treatment. Some old studies confirm this hypothesis (table 7). Takahashi[348,349] and Bertina[342] in the '80s reported between 6% and 14% decrease in C4BP plasma levels in patients treated with warfarin, as well as in liver disease. Zöller et al[360] in 1995 showed that plasma levels of both C4BPt and C4BPβ+ are 25% reduced by warfarin, a somewhat higher percentage compared to the previous findings. This difference can be due to different analytical methods, intensity of anticoagulation and patient selection.

Direct Oral Anti Coagulants (DOACs): DOACs are small molecules that directly inhibit a specific coagulation factor: Xa for Rivaroxaban, Apixaban, Edoxaban, thrombin (IIa) for Dabigatran. They have a much shorter half-life compared to warfarin, they are eliminated by the kidneys to some extent (80% dabigatran, 25-50% the others) and they do not require coagulation monitoring in clinical practice. Moreover, they interact much less than warfarin with food or other drugs. The only study that draws conclusions about an effect of rivaroxaban on complement is by Arachchillage et al[361], where they hypothesize that rivaroxaban may have anti-complement properties through the inhibition of fXa. They show that complement factors C3a, C5a, sC5b-9 are increased in APS patients affected by

previous VTE as compared to healthy controls, irrespective of the anticoagulant used. Moreover, they report a reduction of complement activation markers (apart from factor Bb) after the switch from warfarin to rivaroxaban. Nevertheless, no correlations were found between complement activation factors and rivaroxaban. They concluded in favor of their initial hypothesis. On the contrary, an alternative interpretation, based on the mechanism of action of these two different classes of anticoagulants, might be that it is warfarin that activates complement, whereas the switch to rivaroxaban promotes a normalization of complement activation product levels (C3a, C5a, sC5b-9). It's also interesting to notice that mainly the classical pathway is activated, since levels of factor Bb are unchanged. This may confirm that the mechanism by which warfarin increases complement activation markers involves the downregulation of C4BP.

Table 7 – warfarin, protein S and C4BP

Author, Journal, Year	Patients	Controls	Findings
Bertina, Thromb Haemost 1985[342]	93 long-term warfarin	9 liver disease, 13 DIC, 45 healthy	Reduced C4BP (6-14%) on warfarin, DIC and in liver disease. PS ca 40% reduced on warfarin, only slightly reduced in liver disease. The intensity of anticoagulation (INR) did not affect the amount of reduction of any of the tested proteins
Takahashi, Thrombosis Research 1988[349]	60 long-term warfarin	-	Reduced PS (ca 40%) and C4BP (6%) on warfarin. C4BP positively correlated with total (r=0.7) and free PS (r=0.4). PS correlated with all the others vitamin K-dependent coagulation factors, also decreased on warfarin. PS reduction on warfarin seemed to be dependent on the intensity of anticoagulation (but high variability)
Takahashi, Clin chim acta 1989[348]	51 warfarin	39 DIC, 34 liver disease, 17 autoimmune disease (11 SLE), 17 diabetes, 43 healthy	Reduced C4BP (6-14%) and reduced PS total and free (ca 40%) on warfarin. The reduction of both free and total PS on warfarin seemed to be dependent on the intensity of anticoagulation. Reduced C4BP and PS in liver disease and higher in diabetes. Elevated C4BP in DIC. C4BP positively correlated with total and free PS. PS slightly higher in men vs women
Zöller, Blood 1995[360]	117 non anticoagulated protein S-deficient relatives, 34 anticoagulated protein S-deficient	190 healthy relatives, 40 anticoagulated controls, 60 non-anticoagulated controls	C4BPt and C4BPβ+ 25% lower in anticoagulated controls vs non-anticoagulated controls, even lower in anticoagulated protein S-deficient (ca 30%). PS was 34% lower in anticoagulated controls vs non-anticoagulated controls

1.11 Treatment

aPL profile (e.g. titers, single, double or triple positivity, isotypes, repeated positivity, presence of LA), history of past thrombotic/obstetric events, presence of SLE or other rheumatic diseases and traditional risk factors for arterial or venous thrombosis or obstetric morbidity, are the major determinants for the choice of treatment in APS/aPL carriers. The most recent treatment guidelines for APS/aPL have been recently published by the European League Against Rheumatism (EULAR)[362].

Primary thromboprophylaxis: For all aPL+ individuals, the presence of other thrombotic risk factors or other autoimmune diseases must be considered and treated accordingly. Smoking cessation, a proper diet and regular physical activity, counselling on oral contraceptive use/postmenopausal hormone therapy, and management of diabetes, hypertension and dyslipidaemia are the mainstay. Besides the known effects on blood pressure control and kidney function protection, ACE-inhibitors/Angiotensin Receptor Blockers also seem to downregulate monocytes TF synthesis and expression in vitro[363]. Statins are potent inhibitors of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, involved in cholesterol synthesis in the mevalonate pathway. Besides their lipid-lowering properties, they can also act as anti-inflammatory and anti-coagulant molecules. Experimental studies[364-366] have shown that they can reduce the aPL-mediated induction of TF, IL-6 and adhesion molecules expression on cultured ECs.

Hydroxychloroquine (HCQ) should be given to all patients with SLE[138]. In addition to its modulation of the immune system, it is also believed to have anti-thrombotic properties.

Low dose aspirin (LDA) is recommended in “high risk” aPL profiles (defined as any of the following: multiple aPL positivity, LA+ or persistently high aPL titres), and it should be considered in non-pregnant women with a history of obstetric APS only and in patients with concomitant SLE and low risk aPL profile.

Low Molecular Weight Heparin (LMWH) at prophylactic dosage is recommended in high-risk situations such as surgery, hospitalization, prolonged immobilization, pregnancy and puerperium.

Secondary thromboprophylaxis: Warfarin represents the standard and first choice treatment of APS. In case of arterial thrombotic events or unprovoked VTE, the treatment should be continued indefinitely, requiring regular INR (prothrombin time) monitoring to achieve INR values between 2 and 3. It is debated if patients with a history of arterial vs venous events

should receive the same treatment or if the target INR should be kept higher in arterial thrombosis. In case of provoked VTE and/or low risk aPL profile, shorter treatment may be considered in selected cases.

In case of recurrent event despite reaching the target INR, the addition of LDA, or increase INR goal to 3-4 or switch to LMWH is recommended. The choice depends on the individual risk profile between bleeding and thrombosis. It should also be taken into account that Prothrombin (PT) time is affected by the patients' diet, other drugs and even by PL/LA, although this depends on the reagent used that can be more or less sensitive to LA[367]. Anyway, the influence of LA on the PT test is considered less than on the activated Partial Thromboplastin Time (aPTT) assay, because of the much higher PL concentration in the PT reagent.

In this sense, **Direct Oral Anticoagulants (DOACs)** are easier to manage, since they are prescribed in a fixed dose and they do not require monitoring. Nowadays they are indicated for VTE and atrial fibrillation in the general population, because they are considered safe and effective. Nevertheless, recent randomized controlled trials[368,369], case series[370-372], and a meta-analysis[373] have suggested that DOACs should not be used at least in "high-risk" APS patients, due to the high occurrence rates of especially arterial thrombosis and even reported cases of CAPS[374] after the switch from warfarin to rivaroxaban. The "high risk" APS patients are defined in the presence of a triple positive aPL profile, a history of arterial thrombosis, microthrombosis or organ involvement or in the presence of heart valve disease according to Sydney criteria[176]. DOACs are also contraindicated in APS patients that are not adherent to warfarin or with recurrent thrombosis while on therapeutic intensity warfarin. In case of "low risk" APS patients, single or double positives, affected by a single episode of VTE, further studies are needed to establish the non-inferiority and safety of DOACs as compared to warfarin[373].

These are the last recommendations from the Lupus Anticoagulant/Antiphospholipid Antibodies Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) published last year[176], after other recommendations issued from different societies (EULAR, EMA, ESC, BSH).

The first trial, Rivaroxaban in the Antiphospholipid Syndrome (RAPS)[375-379], was a non-inferiority randomized controlled trial (RCT) on APS patients, with and without SLE, affected by previous VTE and on permanent warfarin treatment. The triple positive patients were 28%. Participants were randomized 1:1 to remain on standard intensity warfarin with

target INR 2-3 or to switch to rivaroxaban for 6 months. The primary aim was to demonstrate that the intensity of anticoagulation achieved with rivaroxaban is not inferior to that of warfarin. The intensity of anticoagulation was assessed by measuring the percentage of change from randomization to day 42 of the Endogenous Thrombin Potential (ETP), i.e. the Area Under the Curve of thrombin generation, triggered by TF in vitro. Secondary aims were to compare rates of recurrent thrombosis, bleeding and the quality of life in patients on rivaroxaban with those on warfarin.

Since the mechanisms of actions of the two drugs are different, the authors wanted to choose a laboratory surrogate outcome measure that could be comparable, but the choice of ETP for monitoring the intensity of anticoagulation in DOACs has been a subject of debate. Some studies support its use as appropriate, even though ETP is intrinsically large in rivaroxaban, not really reflecting the degree of anticoagulation in vivo. In fact, rivaroxaban has a targeted, specific inhibitory action on fXa, affecting mostly the initiation and propagation of thrombin generation, because of a delay in the formation of the prothrombinase complex. This is shown by other parameters, such as lag time (time to generate thrombin from TF), time to peak thrombin generation (TG) and peak TG (max TG), intrinsically prolonged and lower during rivaroxaban treatment. At the endpoint of the trial, the ETP was significantly larger in the rivaroxaban group as compared to warfarin, despite the mean percentage change in ETP didn't reach the non-inferiority threshold. Since the peak TG was significantly lower, and lag time and time to peak TG were prolonged in the rivaroxaban group, the authors concluded for the non-inferiority of rivaroxaban regarding the intensity of anticoagulation.

Additionally, there were no new thrombotic events, no major bleeding episodes and better quality of life was registered in the rivaroxaban group than in the warfarin group. The authors concluded that the treatment with rivaroxaban is safe and effective in APS patient with previous VTE, irrespective of the aPL profile.

These conclusions led Pengo et al to start the Trial of Rivaroxaban in the Antiphospholipid Syndrome (TRAPS)[380,381], a non-inferiority, prospective, randomized trial in high-risk, triple positive patients with APS, affected by previous venous, arterial thromboembolism, micro-thrombosis or obstetric APS. The primary outcome was to assess the non-inferiority of rivaroxaban in terms of prevention of thromboembolic events, major bleeding, and vascular death. The trial was prematurely stopped because of a much higher rate of thromboembolism, all in the arterial circulation (12% vs 0%) and major bleeding (7% vs 3%) in the rivaroxaban as compared to the warfarin group. Moreover, a 2-year follow-up from the

interruption of the trial showed that the difference in outcomes was even higher, especially in terms of thromboembolisms[381]. The possible causes of rivaroxaban failure may depend on a poor compliance or on an insufficient drug concentration, due to a higher inter-individual variability in drug metabolism. Moreover, rivaroxaban has a shorter half-life as compared to warfarin, which may reduce the anticoagulant effect in patients with poor compliance. In the RAPS trial[377], only 51% of the patients reached the minimum therapeutic concentration of rivaroxaban at their peak, whereas 11% were far below the range. This may imply that DOACs are less effective and safe than warfarin when compliance is not good and adherence to treatment is even more important than with warfarin, because of their shorter half-life and less constant plasma levels. Moreover, the different mechanism of action and a different impact on the complement system may contribute to explain the different outcomes of the two drugs.

Apixaban for the Secondary Prevention of Thromboembolism among patients with the AntiphosPholipid Syndrome (ASTRO-APS) is an ongoing clinical trial where definite results are not available yet, but they seem to be in line with data reported so far, according to the last Forum on APS on the 26th of March 2021 in Belgrade.

In case of **obstetric APS**[382] with recurrent pregnancy complications despite combination treatment with LDA and LMWH at prophylactic dosage, LMWH dose can be increased to therapeutic dose or HCQ may be added or low-dose prednisolone in the first trimester. Intravenous immunoglobulin might be considered in highly selected cases. In women with a history of thrombotic APS, combination treatment of LDA and LMWH at therapeutic dosage during pregnancy is recommended.

Long-term treatment with LMWH has apparent disadvantages since it has to be given subcutaneously and heparin-induced thrombocytopenia is a rare but life-threatening side effect. Nevertheless, in a case series, Vargas-Hitos et al observed no thrombotic recurrences in 23 patients on LMWH who failed or did not tolerate warfarin[383].

CAPS merits a special and more aggressive treatment, consisting of a triple therapy with anticoagulation, glucocorticoids, plasma exchange and/or intravenous immunoglobulins. This therapy is recommended over single agents or other combinations of drugs, according to the data collected in the CAPS registry[32,384]. Additionally, any triggering factor (eg, infections, gangrene or malignancy) should be treated accordingly.

Future perspectives: Since new pathogenic mechanisms of disease have been elucidated, new targets for treatment, apart from coagulation, have been hypothesized and tested in clinical practice, mainly in cases of refractory or rapidly progressing CAPS. The two principal tested mechanisms are B cell depletion through Rituximab and complement inhibition through Eculizumab. Rituximab is a chimeric monoclonal antibody against a surface protein expressed on the membrane of B cells (CD20), currently approved for the treatment of chronic lymphocytic leukemia, diffuse large B-cell, advanced follicular lymphoma, refractory rheumatoid arthritis and severe vasculitis. Eculizumab is a humanized monoclonal antibody against C5 convertase, so far successfully employed in case reports with thrombotic microangiopathy (TMA) secondary to APS and CAPS[300,385-391].

2 AIMS

2.1 General aims

The aim of this project was to investigate important mechanisms behind thrombotic complications in aPL positive individuals and to address differences between patients affected by arterial and venous thrombosis. For this purpose, we aimed to study the interactions between coagulation, fibrinolysis and complement system in APS patients.

More specifically, we focused our investigations on the role of the two complement inhibitors involved in regulation of coagulation and fibrinolysis, i.e. C4b-binding protein (C4BP) and Thrombin activatable fibrinolysis inhibitor (TAFI), respectively.

Our results led us also to study the effects of different anticoagulants on complement activation, bearing in mind that hemorrhage is a quite common side effect, representing ca 11% of causes of death in APS.

We were also interested in understanding if APS is really a rare syndrome, or if it's an "under-recognized condition", since aPL are not routinely tested in clinical practice in several specialties that deal with occlusive vascular diseases. According to the epidemiological studies by Cervera et al[23-25], despite MI was present in 5.5% of patients at enrollment, it was reported to be the second most frequent cause of death. Thus, we analyzed aPL in a large cohort of patients from the general population affected by myocardial infarction (MI) and in matched controls, to study the prevalence of aPL in this condition. Moreover, we aimed to see if the presence of aPL makes a difference in terms of severity of events or if it can be considered a marker of increased cardiovascular risk.

2.2 Specific aims

2.2.1 Paper I

- Study TAFI and TAFIa in mainly pAPS classified according to Miyakis criteria
- Correlate TAFI and TAFIa levels to markers of fibrinolysis (CLT and Ks) and complement activation (C5a) in different clinical APS manifestations (arterial vs venous thrombosis/obstetric morbidity)

2.2.2 Paper II

- Assess the prevalence of anti-β_2GPI and aCL IgG/A/M in a large multicenter case-control study comprising 805 first-time MI patients and 805 population controls, matched for age, sex and geographical region
- Compare aPL positive versus aPL negative MI patients in terms of MI characteristics, traditional CV risk factors and previous history/comorbidities

2.2.3 Paper III

- Study complement activation, and in particular the soluble complement inhibitor C4BPt, in primary and secondary APS patients, and in different subgroups of patients affected by SLE
- Study complement activation, with focus on C4BPt, in relation to anticoagulant treatment

2.2.4 Paper IV

- Explore the effects of anticoagulants on complement activation, by comparing complement protein levels and C4BP in patients from the general population affected by previous venous thrombosis, during and after treatment with warfarin and DOACs

3 MATERIALS AND METHODS

3.1 Study design and population

3.1.1 Paper I

This is a cross-sectional study on 52 patients (43 women and 9 men, mean age 44 years) recruited from the Rheumatology, Haematology and Women's and Children's Health clinics, Karolinska University Hospital, between 2009-2012. At inclusion, patients filled out a detailed questionnaire about their daily habits, clinical history, signs, symptoms and treatment, helped by a specialist physician and a nurse; all records were then verified through medical file review. Blood samples were collected at the same time.

Definition of APS clinical manifestations

Arterial thrombosis: myocardial infarction (MI) was defined as at least two of the following: chest pain of typical intensity and duration, ST segment elevation or depression of ≥1 mm in any limb lead of the electrocardiogram, of ≥2 mm in any precordial lead, or both, or at least a doubling in cardiac enzymes CK, CK-MB, Troponin T. MI was also considered after PTCA/CABG intervention. Abrupt onset of a neurological deficit that resolved completely in < 24 h was diagnosed as transient ischemic attack. Patients with new neurological deficits persisting for >24 h underwent cerebral computerized tomography (CT) scan or magnetic resonance imaging to discriminate hemorrhagic and ischemic strokes. Acute peripheral arterial thrombosis was diagnosed in the presence of a typical clinical picture (pain, absence of peripheral pulse) and confirmed by Doppler ultrasound, angiogram and/or surgical intervention.

Venous thromboembolism: deep vein thrombosis (DVT) confirmed by Doppler ultrasound, pulmonary embolism (PE) detected by scintigraphy or angio-CT scan

Obstetric complications: defined according to criteria[2], detected by the obstetric Unit.

Arterial hypertension was considered present if patients were receiving antihypertensive treatment or if arterial blood pressure was > 140/90mmHg at inclusion; hyperlipidemia if the available values of total or fractioned cholesterol were not in the reference range at the time of enrollment. This choice was based by the fact that not all patients treated with statins are dyslipidemic: many are on lipid-lowering treatment as secondary prevention after an arterial thrombosis.

Definition of laboratory criteria

aPL positivity was considered when at least one test among lupus anticoagulant (LA), anti-cardiolipin IgG/IgM isotype (aCL IgG/IgM) at medium/high titer or anti-β_2 glycoprotein I IgG/IgM isotype (anti-β_2GPI IgG/IgM) at titers >99th percentile of healthy blood donors (free from thrombosis/obstetric morbidity and matched for age, sex and place of residence) was positive twice. Confirmation tests were performed after a minimum of 12 weeks. We also required at least 12 weeks and no more than 5 years between positive testing and the clinical manifestation.

General characteristic of the investigated subjects

All 52 patients were free from SLE, which was an exclusion criterium. Eight patients also fulfilled criteria for other rheumatic diseases: 6 rheumatoid arthritis, 1 ankylosing spondylitis with ulcerative colitis, and 1 Sjögren's syndrome. The 15 healthy controls were recruited from our laboratory and none of them took non-steroidal anti-inflammatory drugs (NSAID) or acetylsalicylic acid (ASA) for at least two weeks before blood sampling.

Clinical APS manifestations: 33 (63.5%) patients were affected by a venous thromboembolic event, of which 22 (42.3%) deep venous thrombosis (DVT), 18 (34.6%) pulmonary embolism (PE). Fifteen patients (28.8%) suffered from ischemic stroke, one of them also had a myocardial infarction (MI). Twenty patients (38.5%) had experienced obstetric morbidity.

Antibody profile: single positives were present in 7/52 (13.5%) for LA, one for anti-β2GPI IgG, no single positives for aCL. Half of the patients were triple positives (26/52).

Further demographic, clinical and laboratory characteristics of the investigated patients are presented in table 1 and 2, paper I.

3.1.2 Paper II

This study has a cross-sectional, case-control design, and uses the samples of patients and controls enrolled in the PAROKRANK study[392], a multicenter case-control study on 805 patients less than 75 years old, recruited between May 2010 and February 2014 from the coronary care units of 17 Swedish hospitals. The study was coordinated from the Cardiology Unit, Department of Medicine at Karolinska Institutet, Stockholm, Sweden.

Exclusion criteria were prior MI, prior heart valve replacement and any other condition that could limit the ability to cope with the study protocol.

Controls were individually matched for age, gender and region of living, using the Swedish population registry, coded for date of birth and sex. Controls were contacted by a phone call by trained research personnel at the PAROKRANK coordinating center, providing study information. Controls had to be free from prior MI and heart valve replacement and be

willing to participate. Contact information was subsequently sent to the local study center where oral and written informed consent to participate was obtained.
Patients were recruited while hospitalized for MI and scheduled for outpatient visits 6-10 weeks later at the local department of cardiology. The matched control was recruited from the same region as soon as a patient had undergone the follow-up visit at the cardiology department in order to perform the investigations during the same season.
All participants fasted and abstained from smoking for 12 hours before visiting the cardiology department where a physical examination including heart rate, blood pressure following five minutes of rest in sitting position, height, body weight and waist circumference was performed. Questionnaires comprising extensive information on family and medical history, risk and health preserving factors were completed both at the time of hospital admission for the patients/recruitment for the controls and at follow up.
Smoking habits were defined as current, previous (stopped >1 month ago) or never.
Treatment after MI was according to guidelines.

Clinical and serological characteristics of the investigated subjects are shown in the table in paper II.

3.1.3 Paper III

This is a cross-sectional study on different subgroups:

- 67 patients affected by pAPS according to Miyakis criteria, and 15 individuals with repeated positivity (++) for aPL/LA but without SLE or any thrombotic/obstetric manifestations (aPL carriers) were recruited at the Rheumatology, Haematology and Women's and Children's Health clinics, Karolinska University Hospital, between 2009-2014.
- 487 patients diagnosed with SLE according to the 1982 revised American College of Rheumatology classification criteria were recruited from the Rheumatology Department, Karolinska University and Danderyd's Hospitals between 2004-2014. Of those, 118 were repeatedly positive for aPL/LA (SLE-aPL++), of which 56 affected by sAPS, 291 tested negative for aPL/LA (SLE-aPL-), and 77 were only aPL+ once. This last subgroup was excluded from the study to avoid misclassifications, since we couldn't know if these patients would have been repeatedly positives, if tested.
- 322 controls were identified through the National population registry and a subgroup of 67 was individually matched for age and gender to pAPS patients. No pregnant women were included.

All participants filled out a detailed questionnaire, including data on smoking history (previous smokers if ≥ 1 year without smoking) and treatment, and underwent a physical examination, including measurement of systolic and diastolic blood pressure. Hypertension was defined as >140/90mmHg or current antihypertensive treatment. All records were verified through medical file review. Data on APS clinical manifestations were classified as arterial thrombosis, venous thromboembolism (including Deep Venous Thrombosis (DVT) and Pulmonary Embolism (PE)) and obstetric manifestations according to criteria[2] (see also point 3.1.1). We collected data on lupus nephritis (LN) in SLE patients at the time of sampling/enrollment, and we recorded it according to both presence and activity, using the British Isles Lupus Assessment Group (BILAG) renal score (A most active, E no history of LN)[393].

Clinical and serological characteristics of the investigated subjects are presented in table 2 and 3, paper III.

3.1.4 Paper IV

This is a study with a crossover design: each patient was sampled twice, during treatment and after withdrawal, so that each patient served as his or her own control (paired samples). Comparisons were also made between independent groups of patients with/after different treatments. All the enrolled patients belonged to the general population, they had been diagnosed with venous thrombosis (VTE) (indication for treatment) and were free from autoimmune diseases and cancer. No woman was pregnant at the time of sampling.
Samples from 22 patients treated with warfarin were collected between 2009-2015. Duration between VTE diagnosis and first sampling was in median 120 days and between warfarin withdrawal and second sampling 19 days.
A second cohort of 33 patients on DOACs (11 on apixaban and 22 on rivaroxaban) were sampled between 2014-2019, with first sampling in median 74 days after VTE diagnosis and second sampling 21 days after DOAC withdrawal.

Clinical and serological characteristics of the investigated subjects are presented in table 1, paper IV.

3.2 Laboratory investigations

3.2.1 Paper I

Blood samples from the APS patients and healthy controls were drawn into 0.129 mol/L trisodium citrate and centrifuged at room temperature (for 20min at 2570xg and 2000xg respectively) to obtain platelet-poor plasma that was then deep frozen in aliquots of 0.5 mL at -70°C to -80°C. Samples were thawed in water bath at 37°C before testing.

Antibodies against cardiolipin (aCL IgM/IgG) and β_2 glycoprotein-I (anti-β_2GPI IgG) were analyzed by ELISA (Orgentec, Mainz, Germany). Anti-β_2GPI IgM were tested with a multiplex immunoassay (BioPlex 2200, Bio-Rad Laboratories, Hercules, California, USA)

Lupus anticoagulant by dilute Russell Viper Venom (dRVVT) method with reagents from Biopool, Umeå, Sweden and Gradipore, North Ryde, Australia.

TAFI (proenzyme) was determined by a chromogenic substrate method (STA® - Stachrom® TAFI kit, Diagnostica Stago, France).

TAFIa (enzyme) was quantified by measuring, through ELISA (Asserachrom TAFIa/TAFIai antigen kit, Diagnostica Stago, France), the complex TAFIa/TAFIai, made of both the active and inactive forms of the enzyme, which corresponds to the concentration of the active form that cannot be measured because of instability.

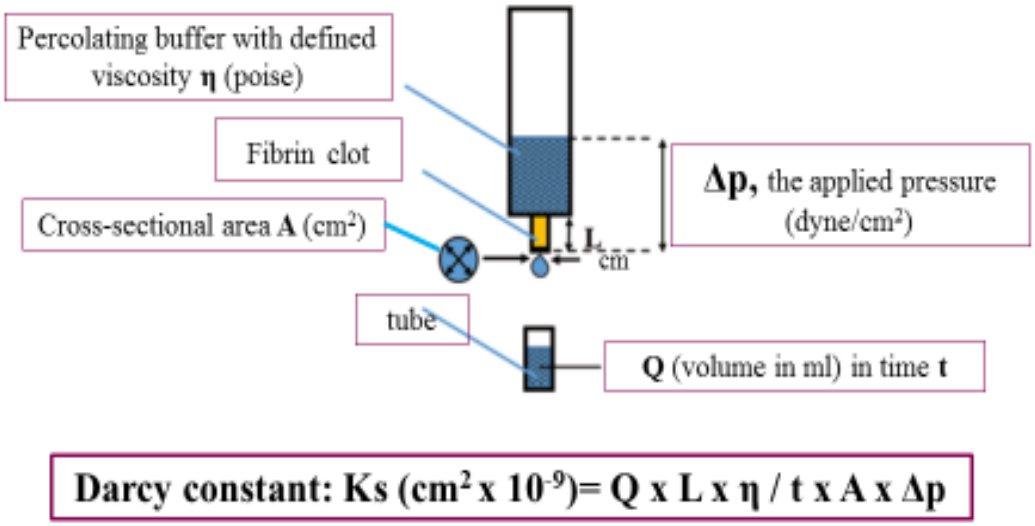
C5a was analyzed with a direct-capture immunoassay (MicroVue C5a Enzyme Immunoassay, Quidel Corporation, San Diego, USA).

Thrombomodulin was determined by quantitative sandwich ELISA (Human Thrombomodulin/BDCA-3 Quantikine ELISA Kit, R&D Systems Inc. USA).

Fibrin clot structure was studied in citrated plasma samples by measurement of the permeability coefficient (Ks, Darcy constant) through a liquid permeation technique[394], described below (figure 11). 200 µL of plasma were supplemented with $CaCl_2$ and thrombin at final concentrations of 20 mmol/L and 0.2 U/mL respectively, and left in a moisture atmosphere overnight in order to allow clot formation in all samples. Thereafter, a percolating buffer (pH 7.4, 0.02 mol/L Tris, 0.02 mol/L imidazole, 0.1 mol/L NaCl) was passed through the clots at 5 different hydrostatic pressures and the volume of collected eluate was measured after an indicated time. Analyses were performed in duplicate.

The Darcy constant reflects the permeability of the clot by percolating a volume of buffer (Q) with a certain viscosity (η) through a fibrin gel (with length L and cross-sectional area A) at a given hydrostatic pressure (p) in a given time (t). The formula to calculate Ks is: $Q \times L \times \eta / t \times A \times \Delta p$. Its normal values range between 6-10 $cm^2 \times 10^{-9}$.

Figure 11 – Permeability coefficient or Darcy constant Ks



Turbidimetric clotting and lysis assays were performed to assess fibrin formation and degradation respectively, according to the methods described by Carter et al[181]. Seventy-five µL of assay buffer (pH 7.4, 0.05 mol/L Tris-HCl, 0.15 mol/L NaCl) was added to 25 µl of citrated plasma (in duplicate) in a microtiter plate. Fifty µl of a mixture of thrombin (final concentration 0.03 IU/mL) and $CaCl_2$ (final concentration 7.5 mMol/L) was added to the plasma samples and the absorbance at 340 nm was read every 18 sec (240 cycles for each sample). In the turbidimetric lysis assay, recombinant tPA (Technoclone, GmbH, Vienna, Austria) was added to the Tris-HCl buffer at a final concentration of 83 ng/ml. CLT was defined as the time from the midpoint of the clear–to–maximum-turbidity transition (which is defined as clotting time), to the midpoint of the maximum turbid–to–clear transition. It is used to estimate fibrin porosity[181]: the higher time it takes to lyse the clot, the less the permeability[180,181,394-396]. The inter-assay coefficient of variation was 11.4%, intra-assay 2.3%.

Fibrinogen was analyzed by nephelometry (BN Prospec nephelometer, Dade Behring, Deerfield, IL, USA for 29 patients and Sysmex® CS2100i, Sysmex, Kobe, Japan with reagent Dade Thrombin from the Siemens Healthcare Diagnostics Inc, Tarrytown, NY, USA for 23 patients and healthy controls), a technique based on the quantification of the intensity of the scattering of a light/radiation, proportional to the concentration of the analyzed molecule.

3.2.2 Paper II

The local laboratories performed the following analyses: complete blood count, P-lipids (total and HDL-cholesterol and triglycerides), P-creatinine, P-fibrinogen, P-glucose and glycated hemoglobin A1c (HbA1c). Study participants without known diabetes underwent an oral glucose tolerance test (75 g glucose in 200 ml water) with venous P-glucose measured in the fasting state and two hours after glucose intake. The point-of-care HemoCue® 201 System (HemoCue AB, Ängelholm, Sweden) was used for the P-glucose analysis. High sensitivity C Reactive Protein (hsCRP) was analysed at a central laboratory (Redhot diagnostics, Södertälje, Sweden) by an ELISA method (MP Biomedicals, New York, USA) intended for quantitative determination CRP, with the functional sensitivity of 0.1 mg/L.

Antibodies to specific nuclear antigens (dsDNA, SSA Ro-52, SSA Ro-60, SSB, Sm) and phospholipids/phospholipid-binding proteins (cardiolipin and β_2-glycoprotein I IgG, IgM, IgA) were analyzed by multiplexed bead technology (Luminex) using BioPlex 2200 system (Bio-Rad, Hercules, CA, USA) according to the specifications of the manufacturer. The cut off for aCL and anti-β_2GPI fulfills the 99th percentile as described[397].

3.2.3 Paper III

At the time of inclusion, blood samples were collected by direct venipuncture, centrifuged at 2000xg at room temperature for 20 minutes before aliquotation and stored at -80°. Plasma levels of inflammatory markers (CRP, ESR, fibrinogen, albumin), renal function (creatinine), lymphocytes and platelets were measured according to hospital routines[398].

Antiphospholipid antibodies were analyzed by multiplexed bead technology (Luminex) using BioPlex 2200 system (Bio-Rad, Hercules, CA, USA) according to the specifications of the manufacturer.

Lupus anticoagulant was determined using a modified Dilute Russel Viper Venom method (Biopool, Umea, Sweden) using Bioclot lupus anticoagulant.

The total amount of C4BP (C4BPt) was measured using magnetic carboxylated microspheres coupled with a monoclonal antibody against the α-chain of human-C4BP (BIO-RAD, Hercules, CA, USA). To detect C4BPt the same antibody was used, biotinylated. The binding of biotinylated antibodies was detected by streptavidin-phycoerythrin (PE, BIO-RAD) and data were collected using a MAGPIX Multiplex Reader (BIO-RAD). The results are expressed in mg/L.

Factor I was detected by a magnetic bead assay using a mouse monoclonal anti-Factor I antibody (Abcam, Cambridge, UK) for capture and a biotinylated polyclonal anti-Hu-factor I

antibody (Abcam) followed by streptavidin-PE for detection using a MAGPIX Multiplex Reader.

C1q was detected by magnetic bead-based sandwich immunoassay (MBSI)[399].

C2 serum concentration was measured by electroimmunoassay[400] and the results are given in arbitrary units (% of normal human serum pool).

C3, C3dg, C4 were analyzed according to clinical routine using nephelometry at the Department of Clinical Chemistry at the Karolinska University Hospital. The results are reported as g/L (normal ranges: C3 0.77–1.62 g/L, C3dg <8 g/L, C4 0.13-0.32 g/L).

C3a was determined by sandwich ELISA, using mAb 4SD17.3 for capture and biotinylated polyclonal anti-C3a, followed by HRP-conjugated streptavidin (GE Healthcare, Uppsala, Sweden) for detection. Data are given as µg/L.

sC5b-9 was quantified with an in-house magnetic bead-based assay using mAb anti-human-neo-C9 aEII (Bioporto Diagnostics A/S, Hellerup, Denmark) (1.5 µg/1.25 *10^6 beads) for capture and polyclonal sheep anti-Hu-C5 antibody (BP373, OriGene, Herford, Germany; 4 µg/mL, biotinylated) followed by streptavidin-PE (1:100) for detection. The assay was calibrated against a commercially available kit (Quidel, San Diego, CA, USA) and data are given as µg/L.

Free protein S antigen (PS Ag) in citrated plasma samples was performed using Innovance® Free PS Ag turbidimetric assay on automated coagulation analyzer BCS®XP, Siemens Healthcare Diagnostics (Germany).

Total protein S was analyzed using Asserachrom® Total Protein S one-step enzyme immunoassay by ELISA method, Diagnostica Stago (France).

3.2.4 Paper IV

Samples were provided by the Biological Resources Center, Assistance Publique - Hôpitaux de Marseille in France (CRB AP-HM, certified NF S96-900 & ISO 9001 v2015). Blood was collected in both EDTA and citrated tubes (0.105M sodium citrate): EDTA blood was used for routine analysis, complement assays and DNA preparation; citrate plasma was used for coagulation assays. All samples were centrifuged within one hour by double centrifugation at 2000xg for 15 min at room temperature, then stored at -80˚C in aliquots until analyzed. Prior to analysis, the samples were thawed in a 37°C water bath, then transferred to an ice bath where all dilutions took place. EDTA (10 mM final concentration) was added the first time the samples were thawed in order to minimize the risk of pre-analytical complement activation, since it has been demonstrated that in citrate plasma one single 5 min cycle of thawing at 37°C can increase the levels of C3a and sC5b-9 by ≈ 5-20 fold compared to EDTA plasma[30].

Platelet counts and measurement of coagulation parameters, concentration of DOACs, aPL (negative for all patients) were performed along with DNA analysis for Factor V Leiden (R506Q) and the 20210G-A prothrombin mutation.

FVIII:C was analyzed using human FVIII-deficient plasma in a one-stage assay using the STA®-ImmunoDef VIII kit (Diagnostica Stago, France). Fibrinogen levels were assayed using the STA Fibrinogen kit (Clauss method). D-Dimer was measured by the particle-enhanced immunoturbimetric assay STA®-Liatest®, D-Di PLUS (Diagnostica Stago). Free protein S was measured by enzyme-linked immunosorbent assay (ELISA) using the Asserachrom FPS assay (Diagnostica Stago, France). INR, derived from Prothrombin Time was obtained by using the STA®-Neoplastine CI reagent (Diagnostica Stago) and was used to monitor anticoagulant effect of warfarin. Thrombospondin-1 (TSP-1) was measured with a commercial ELISA-kit (Duo-Set DY-3074, R&D 9 Barton Lane, Abingdon Science Park, Abingdon, UK). Plasmin-α2-antiplasmin (PAP) was measured with a commercial ELISA-kit (Duo-Set DY-1407-05, from R&D Systems).

Plasma concentration of rivaroxaban and apixaban was based on anti-Xa specific activities, and measured with an anti-Xa assay calibrated to the specific drug (Biophen Heparin™, Hyphen Biomed, Neuville-sur-Oise France). All parameters were determined in one sample on an automated coagulometer (STA-R; Diagnostica Stago).

Thrombin generation at 5pM TF and 4μM of phospholipids was measured using the Calibrated Automated thrombography (CAT) method[401], according to manufacturer's instructions. Four parameters were collected from the Thrombinoscope software: lag time (minutes), peak (nM), time to peak (minutes) and endogenous thrombin potential (ETP in nM/minute) corresponding to the area under the curve which reflects the total amount of thrombin generation.

The CP functional test was assessed by lysis of sensitized sheep erythrocytes[402].

C3a was determined by sandwich ELISA, using mAb 4SD17.3 for capture and biotinylated polyclonal anti-C3a, followed by HRP-conjugated streptavidin (GE Healthcare, Uppsala, Sweden) for detection. C4a was measured only in warfarin patients with a commercial ELISA kit (A036, Quidel, San Diego, CA, USA). sC5b-9 (surrogate marker for C5a generation) was quantified with an in-house magnetic bead-based assay using mAb anti-human neo-C9 aEII (Bioporto Diagnostics A/S, Hellerup, Denmark) for capture and polyclonal sheep biotinylated anti-Hu-C5 antibody (BP373, OriGene, Herford, Germany) followed by streptavidin-phycoerythrin (PE; BIO-RAD, Hercules, CA, USA) for detection. The assay was calibrated against a commercially available kit (Quidel). C1q was detected by

magnetic bead assay as previously described[399]. C3 and C4 (C4 only in warfarin patients) were determined with an Immage nephelometer (Beckman Coulter, Bromma, Sweden). Since C4BP can exist in different isoforms, in house assays were constructed to quantitate the total amount of C4BP. C4BPt (total, including all isoforms), the C4BP isoform that contains the β-chain (C4BPβ+), and the complexes between C4BPβ and protein S (PS-C4BPβ+) were all measured by magnetic bead-based assays using a BioPlex MAGPIX Multiplex Reader (BIO-RAD). For all assays, magnetic carboxylated microspheres (Bio-Plex, Pro Magnetic COOH Beads, BIO-RAD) were coupled with mAb against the α-chain of human-C4BP (MCA2609, BIO-RAD) and used to capture the antigen. C4BPt was quantified using the same biotinylated mAb. Detection of C4BPβ+ was performed using a rabbit biotinylated polyclonal antibody against the β-chain (MBS712775, MyBioSource, San Diego, CA, USA), and PS-C4BPβ+ was detected with a biotinylated polyclonal sheep antibody against human PS (7861-1009, BIO-RAD). The binding of biotinylated antibodies was detected by streptavidin-PE (BIO-RAD). The raw data from the BioPlex MAGPIX Multiplex Reader, expressed as mean fluorescence intensity (MFI) and the standard curves were calculated with a five-parameter regression model using Bio-Plex Manager MP Software and Bio-Plex Manager 6.1 (BIO-RAD).

3.3 Statistics

Data are presented as mean values ± standard deviation for normally distributed continuous variables, and as median (Interquartile Range, IQR) for non-normally distributed continuous variables. Data with skewed distributions were logarithmically transformed to obtain normality. Percentages and proportions are used to present categorical variables.
For continuous, normally distributed variables, comparisons were made using paired (when matched data) or independent Student's t-test. Fishers' exact test and χ^2 test, when appropriate, were used to compare independent categorical variables, McNemars' test for categorical, matched variables. To assess dependence and strength of associations among continuous variables, we used standard least squares linear regression and correlation analysis, respectively, to calculate regression coefficients (β) and Pearson's correlation coefficients (r) for continuous parametric variables; Spearman´s rank correlation coefficient (ρ) was used for continuous non-parametric variables. Bonferroni correction was applied to multiple comparisons. Multivariable linear/logistic regression analyses were performed to analyse associations between a dependent continuous/categorical variable and multiple predictor variables.

Odds Ratios, crude and adjusted for confounders, and corresponding 95% confidence intervals were calculated by use of logistic regression, conditional in case of matched pairs. Mediation analysis was performed as in Vanderweele and Vansteelandt[403] in the SLE patient group. The repeated presence of aPL was considered as exposure and C4BPt levels as the outcome. Warfarin is a mediator, since its long-term treatment is mostly given to exposed (aPL++) patients and its reducing effect on C4BPt has been described in previous studies in the general population in the absence of aPL. Warfarin occurs in the causal pathway between the exposure and the outcome and it is not a confounder because it is not believed to affect the exposure (aPL++). Among the SLE patients, there are both exposed (aPL++) and unexposed (aPL-), with and without mediator (warfarin).

The significance level α was set at 0.05 for hypothesis testing (two-sided), a 95% Confidence Interval was reported for the estimation.

JMP software (SAS Institute, Carey, North Caroline, USA) was used for statistical analysis. Mediation analysis was performed with R. GraphPad Prism 8 was used to make graphs.

3.4 Ethical considerations

Paper I, II and III were approved by the Regional Ethical Committee in Stockholm, and all the studies were performed respecting the Declaration of Helsinki.

All the subjects in all the studies gave voluntary informed consent for participating and for their blood to be stored anonymously (with an assigned code) in the biobank at Medicine Institution, Karolinska University Hospital.

Patients' data were handled anonymously by using codes and numbers.

The access to each patients' journal was approved by the patients and just authorized doctors reviewed medical files. Access to the database was limited to researchers involved in the studies and it was protected by a password.

All the studies included in this thesis are observational and descriptive, none experimental.

For paper II different registers have been used to collect patients' information: the National quality registry SWEDEHEART (www.swedeheart.se), which includes all consenting patients when diagnosed with MI, the Swedish Register for myocardial infarctions (RIKS-HIA) at time of hospitalization, the Swedish register for secondary prevention (SEPHIA) at the secondary prevention follow-up visit 6-10 weeks post-MI, a Register for coronary-angiography and angioplastics (SCAAR), a Coronary-Bypass Register (CABG), and a Register for causes of Death. The Swedish population Registry was used for controls.

Samples from paper IV were collected in France at the Biological Resources Center, Assistance Publique - Hôpitaux de Marseille as part of other research projects in coagulation. They were sent to Sweden after authorization of the French Minister and further analysed in Kalmar and Uppsala, without having access to patients' information here in Sweden.

4 RESULTS

4.1 Paper I

The levels of TAFI and TAFIa, expressed as mean±SD, are higher in APS patients compared to healthy controls (TAFI 111.1±18.2% n=50 APS vs 88.0±16.1% n=15 controls, p<0.0001; TAFIa 18.8±11.6 ng/ml n=52 APS vs 12.3±2.7 ng/ml n=15 controls, p=0.02). CLT is prolonged and Ks is lower in APS, indicating reduced clot permeability (CLT 1698.1±453.0 sec n=50 APS vs 1452.2±459.4 sec n=15 controls, p=0.04; Ks 7.2±3.3 cm^2x10^{-9} n=44 APS vs 9.8±2.4 cm^2x10^{-9} n=15 controls, p=0.004). Fibrinogen and C5a are increased (fibrinogen 4.0±0.9 g/L n=52 vs 2.7±0.5 g/L n=15, p<.0001; C5a 34.3±7.0 ng/ml n=52 vs 27.2±5.3 ng/ml n=15, p=0.001).

The levels of TAFIa (25.4±13.9 ng/ml arterial n=16 vs 15.8±9.1 ng/ml others n=36, p=0.001) and fibrinogen (4.3±0.8 ng/ml arterial n=16 vs 3.8±0.9 ng/ml others n=36, p=0.03) are higher in APS patients affected by arterial thrombosis compared to the levels in patients with other clinical manifestations. Traditional cardiovascular risk factors are present in higher extent as well (age p<.0001, hypertension p=0.0005, actual smoking p=0.03, hyperlipidemia p=0.03). More patients affected by arterial thrombosis are treated with statins and ACE-inhibitors/Angiotensin Receptor Blockers, as part of secondary prevention.

TAFI positively correlates to C5a (p=0.0026, r=0.41) and age (p=0.003, r=0.41), TAFIa to TM (p<.0001, r=0.62) and age (p<.0001, r=0.62). TAFI and TAFIa positively correlate with each other (p=0.0011, r=0.45). Age and TM are the major determinants of TAFIa (p<.0001, R^2adj=0.72; p<.0001 for age, p=0.005 for TM, p=0.004 for Ks, p=0.04 for fibrinogen).

Patients affected by hypertension have higher levels of TAFIa compared with normotensive patients (25.4±13.6 ng/ml n=20 hypertensive vs 14.7±7.8 ng/ml n=32 normotensive, p=0.0001), but probably these data are confounded by age (hypertensive patients are older than normotensive: 55 vs 38 years, p<.0001). Hypertensive patients have also higher levels of TM (6369.5±1906.8 ng/ml n=19 vs 5230.6±2903.9 ng/ml n=31, p=0.02), but in this case TM does not correlate with age. TAFIa positively correlates with Ks (p=0.01, r=0.39) but not with CLT. Ks does not correlate with age, nor with TM, fibrinogen, cholesterol. TAFI does not correlate with neither CLT nor Ks. Fibrinogen positively correlate with both TAFI (p<.0001, r=0.61) and TAFIa (p=0.0001, r=0.50). After adjustment for age, C5a and fibrinogen remain associated with TAFI (p<.0001, R^2adj=0.48; p=0.01 for age, p=0.003 for C5a, p=0.0009 for fibrinogen). There are no differences in markers of fibrinolysis according to clinical APS manifestations, but patients treated with heparin have higher Ks compared to others (10.6 ±3.4 cm^2x10^{-9} n=5 vs 6.8±3.1 cm^2x10^{-9} n=39, p=0.018). A negative correlation is present between TAFIa and levels if IgG aPL (p=0.028, r=0.32), but not with other isotypes. IgG aPL

titers are lower in patients affected by arterial thrombosis (10.7±2.0 μg/ml n=16 vs 13.1±4.3 μg/ml n=34, p=0.04), but lose their significance when put into a multivariable model with TAFIa as dependent variable.

4.2 Paper II

IgG aCL and IgG anti-β_2GPI (positivity in any of these is referred to IgG aPL) are more prevalent in first MI patients at 6-10 weeks post-MI compared to matched controls (11.1% vs 1.3%, p<.0001), with no difference in the other aPL isotypes (IgM and IgA). The majority of IgG aPL positive first-time MI patients presented with high titers.

Each isotype of the two different target antigens (aCL and anti-β_2GPI) present a strong positive correlation: IgG (ρ=0.88, r=0.98, p<.0001), IgA (ρ=0.85, r=0.98, p<.0001), IgM (ρ=0.91, r=0.97, p<.0001). None of the investigated specificities of antinuclear antibodies (ANA) differ between patients and controls.

Present smoking, family history of cardiovascular disease (CVD), but also rheumatic and pulmonary diseases are more common in MI patients, whereas hypertension, Body Mass Index (BMI) and diabetes do not differ from matched controls.

After multivariable conditional logistic regression analysis, IgG aPL positivity [OR 7.8 (95% CI: 4.0-15.3), p<0.001] and present smoking [OR 2.6 (95% CI: 1.9-3.5), p<0.001] remain associated with MI, not so for diabetes, hypertension, BMI or family history of CVD.

When comparing IgG aPL+ (n=88) vs IgG aPL- (n=704) MI patients, IgG aPL positivity is non-significantly more prevalent among females (26.1 vs. 17.9%, p=0.06) and smokers (33.7% vs. 25.1%, p=0.08), and significantly more prevalent in patients affected by pulmonary disease, in particular chronic obstructive pulmonary disease (COPD). Age (63.1 years in IgG aPL+ vs. 61.8 years in IgG aPL-, p=0.146), presence of diabetes or rheumatic diseases, BMI, previous venous or arterial thrombosis, family history of CVD, or even MI characteristics (STEMI vs NSTEMI) do not differ between IgG aPL+ vs IgG aPL- MI. Nevertheless, readmission < 10 weeks after MI was more common among the IgG aPL+ MI patients.

Table 1 – paper II

Clinical and serological characteristics in patients with myocardial infarction IgG aPL+ vs IgG aPL-

Characteristics	IgG aPL + MI	IgG aPL - MI	p-value
Traditional cardiovascular risk factors			
Mean age (SD), years	63 (6)	62 (8)	0.11
Gender, female (%)	23/88 (26.1)	126/704 (17.9)	0.06
Present smokers (%)	29/86 (33.7)	173/690 (25.1)	0.08
Hypertension (%)	25/87 (28.7)	258/702 (36.7)	0.14
Mean BMI (SD), kg/m^2	27.4 (4.7)	27.0 (3.7)	0.35
Diabetes (%)	10/87 (11.5)	70/702 (10.0)	0.70
Family history of known CVD (%)	29/78 (37.2)	266/607 (43.8)	0.26
Medical history (self-reported), (%)			
Venous thrombosis	2/88 (2.3)	25/704 (3.6)	0.76
Stroke	1/88 (1.1)	21/701 (3.0)	0.50
Peripheral artery disease	2/88 (2.3)	16/704 (2.3)	1
Cancer	7/88 (8.0)	56/704 (8.0)	1
Osteoarthritis	9/88 (10)	64/704 (9)	0.73
Any Rheumatic diseases	24/88 (27.3)	152/704 (21.6)	0.23
Systemic Lupus Erythematosus	0 (0)	0 (0)	na
Rheumatoid arthritis	2/88 (2.3)	13/704 (1.8)	0.68
Psoriatic arthritis	5/88 (5.7)	37/704 (5.3)	0.80
Pulmonary disease (any)	23/88 (26.1)	100/704 (14.2)	**0.0036**
COPD	8/88 (9.1)	26/704 (3.7)	**0.0185**
Asthma	7/88 (7.9)	31/704 (4.4)	0.14
Laboratory results, mean (SD) ‡			
Total cholesterol, mmol/L	3.9 (1.0)	3.9 (0.8)	0.49
HDL, mmol/L	1.2 (0.4)	1.2 (0.3)	0.61
Total/HDL cholesterol ratio	3.3 (0.9)	3.3 (0.9)	0.93
Triglycerides, mmol/L	1.3 (0.7)	1.3 (0.9)	0.54
hsCRP, nmol/L	2.3 (2.4)	2.2 (2.7)	0.71
Fibrinogen, g/L	3.4 (1.0)	3.4 (0.8)	0.84
Creatinine, μmol/L	80.3 (16.8)	83.6 (21.3)	0.08
Hemoglobin, g/L	142.4 (10.9)	141.7 (11.4)	0.60
Platelets, x10^9 cells/L	234.8 (49.5)	240.1 (64.8)	0.46
Leukocytes, x10^9 cells/L	7.8 (11.0)	6.5 (3.4)	0.56

Autoantibodies targeting, (%)			
β_2GPI IgG+	83/88 (94.3)	0 (0)	**<0.001**
β_2GPI IgM+	5/87 (5.7)	4/704 (0.6)	**0.0013**
β_2GPI IgA+	8/88 (9.1)	4/704 (0.6)	**<.0001**
CL IgG+	86/88 (97.7)	0 (0)	**<.0001**
CL IgM+	4/87 (4.6)	7/704 (1.0)	**0.0246**
CL IgA+	8/88 (9.1)	4/704 (0.6)	**<.0001**
dsDNA+	2/87 (2.3)	13/683 (1.9)	0.68
Sm+	0 (0)	1/702 (0.1)	na
Ribosomal P+	0 (0)	0 (0)	na
RNP 68+	0 (0)	1/702 (0.1)	1
RNP A+	3/88 (3.4)	27/702 (3.8)	1
SSA Ro52+	3/88 (3.4)	8/702 (1.1)	0.11
SSA Ro60+	3/88 (3.4)	6/702 (0.85)	0.07
SSB+	2/88 (2.3)	8/702 (1.1)	0.31

‡ samples were analyzed 6-10 weeks after MI: results could be affected by the post-MI treatment

β_2GPI= β_2 glycoprotein I, aPL= antiphospholipid antibodies, BMI= Body Mass Index, CL= cardiolipin, COPD= Chronic Obstructive Pulmonary Disease, CVD= Cardiovascular Disease, HDL= high-density lipoproteins, dsDNA= double stranded DNA, Sm= Smith antigen, RNP= ribonucleoprotein, SSA= Sjögren antigen A, SSB= Sjögren antigen B, na= not applicable

4.3 Paper III

C4BPt is 20-23% lower in patients with repeated positivity for aPL, independently of previous thrombotic events or nephritis, as well as in patients treated with warfarin.
No difference is present according to double or triple positivity for aPL (mean 168.5 mg/L, n=97 TP vs 173.9 mg/L, n=103 non-TP, p=0.23), neither depending on aPL titers or isotype. There are no statistically significant differences in the levels of C4BPt between arterial, venous and obstetric complications in either pAPS or sAPS. Similar results are found also when considering just patients not treated with warfarin, where the difference in C4BPt levels is further reduced (table 1). In general, obstetric pAPS patients tend to have higher levels of C4BPt compared to other groups, and they are less often treated with warfarin (approximately 10% of obstetric APS patients are treated with warfarin vs 50-60% of arterial or venous APS). aPL carriers present only a slight reduction in C4BPt compared to pAPS (mean 202.1 mg/L vs 175.9 mg/L), although not significantly different (p=0.8). When we consider the subgroups of pAPS and SLE-aPL++ not on warfarin, they present higher levels of C4BPt compared to the whole group (that includes also warfarin treated patients), but still

lower than controls (table 2). These observations further support that warfarin contributes to the reduced levels of C4BPt.

Complement proteins in disease subtypes and controls

C1q, C4, C3 are decreased in all SLE patients, with lower levels in SLE-aPL++ versus SLE-aPL- and controls. C1q is reduced in pAPS vs matched controls (mean 237.2 mg/L vs 267.5 mg/L, p=0.003).

sC5b-9 is increased in pAPS, SLE-aPL++ and aPL- patients compared to controls.

C3dg is increased in SLE-aPL++ versus SLE-aPL-, as well as in warfarin treated patients (9.0 μg/L on warfarin n=36 vs 6.2 μg/L not on warfarin n=245, p=0.0003).

C4BPt and complement proteins

C4BPt positively correlates with C1q and complement inhibitor Factor I in all subjects, and with C3, C4 (and C2 in SLE – not tested in controls), in SLE patients and controls. FI levels are not associated with aPL++ or with warfarin treatment.

C4BPt negatively correlates with complement activation product C3dg in both SLE groups.

C4BPt and treatment

C4BPt is lower in pAPS and SLE-aPL++ patients treated with warfarin, compared to patients not on warfarin.

On the other hand, C4BPt is higher in pAPS patients treated with LMWH as compared to non-treated patients, after excluding those treated with warfarin.

No difference in C4BPt according to Low dose Aspirin (LDA) treatment was recorded.

In SLE, C4BPt is not associated with treatment with prednisolone and its dosage, mycophenolate mophetil, hydroxycloroquine, metotrexate, rituximab and cyclophosphamide.

SLE patients treated with azathioprine (AZA) have lower levels of C4BPt (161.5 (219.0-127.5) mg/L on AZA n=32 vs 186.3 (251.1-147.6) mg/L not on AZA n=154, p=0.0221).

Protein S in pAPS

Both free and bound Protein S (PS) in pAPS correlate positively with C4BPt and both are reduced by warfarin. C4BPt, free and total PS are lower in pAPS patients affected by DVT compared to other clinical manifestations as previously defined. Total PS is also increased in patients treated with LMWH compared to non-treated pAPS, with no significant difference in free PS.

Relative contribution of aPL++ and warfarin to depressed levels of C4BPt

C4BPt is 20% reduced in SLE-aPL++ compared to SLE-aPL- and in pAPS compared to controls, 23% in SLE-aPL++ compared to controls. Applying mediation analysis to the SLE group, we assess that 45% of this reduction is mediated through warfarin. More specifically, aPL++ has a direct reducing effect on C4BPt of 11%, while warfarin contributes to 9% of the

observed reduction. An interaction effect between aPL++ and warfarin seems to be present, but it does not reach statistical significance.

Table 1 – paper III

Independent t-tests for C4BPt levels (mg/L) between different clinical manifestations

All sAPS (n=56)	**Arterial (n=33)**	**p-value arterial vs venous**	**Venous (n=23)**	**p-value venous vs obstetric**	**Obstetric (n=14‡, pure* n=6)**	**p-value obstetric vs arterial**
C4BPt	186.8 ± 101.2	0.97	180.0 ± 95.8	0.21	211.5 ± 54.4*	0.30
NOT on warfarin sAPS (n=23)	**Arterial (n=13)**	**p-value arterial vs venous**	**Venous (n=8)**	**p-value venous vs obstetric**	**Obstetric (n=9‡, pure* n=4)**	**p-value obstetric vs arterial**
C4BPt	236.2 ± 115.5	0.97	241.9 ± 134.4	0.96	221.5 ± 67.1*	0.97
All pAPS (n=67)	**Arterial (n=24)**	**p-value arterial vs venous**	**Venous (n=44)**	**p-value venous vs obstetric**	**Obstetric (n=23‡, pure* n=11)**	**p-value obstetric vs arterial**
C4BPt	181.7 ± 69.6	0.6	169.4 ± 67.2	**0.04**	214.3 ± 65.6*	0.1
NOT on warfarin pAPS (n=34)	**Arterial (n=15)**	**p-value arterial vs venous**	**Venous (n=17)**	**p-value venous vs obstetric**	**Obstetric (n=14‡, pure* n=10)**	**p-value obstetric vs arterial**
C4BPt	212.7 ± 67.8	0.7	208.6 ± 65.6	0.8	221.6 ± 64.2*	0.6

* Calculations are made on the pure obstetric APS patients vs other clinical APS manifestations (i.e. when a patient has an overlap obstetric + arterial or venous, we consider it arterial or venous).
‡ Overlap obstetric + arterial or venous
In case of overlap arterial + venous, patients are considered alternatively arterial or venous in a total equal amount. The number of patients reported in each group is the total number of patients affected by that specific clinical APS manifestation. Conversely, mean and SD are those that have been used to run the t-test, and not those of all the patients with the named event because, in case of overlap, the patients were considered in either one or the other group/clinical APS manifestation

Table 2 – paper III

Direct comparisons of C4BPt levels, expressed in mg/L, between: a) pAPS and SLE-aPL++ not on warfarin vs controls, b) pAPS, SLE-aPL++ and aPL carriers not on warfarin vs controls, c) pAPS not on warfarin vs controls, d) pAPS not on warfarin vs matched controls, e) SLE-aPL++ not on warfarin vs controls. The independent t-test on lnC4BPt was used in a), b), c), e), paired t-test in d)

a) **NOT on warfarin**	**pAPS and SLE-aPL++ (n=114)**	**Controls (n=321)**	**p-value**
C4BPt	206.9 ± 78.8	226.8 ± 63.4	**0.0008**
b) NOT on warfarin	**pAPS, SLE-aPL++ and aPL carriers (n=127)**	**Controls (n=321)**	**p-value**
C4BPt	212.1 ± 85.0	226.8 ± 63.4	**0.0038**
c) NOT on warfarin	**pAPS (n=34)**	**Controls (n=321)**	**p-value**
C4BPt	217.1 ± 64.3	226.8 ± 63.4	0.4
d) NOT on warfarin	**pAPS (n=34)**	**Matched controls (n=34)**	**p-value**
C4BPt	217.1 ± 64.3	220.5 ± 50.9	0.7
e) NOT on warfarin	**SLE-aPL++ (n=80)**	**Controls (n=321)**	**p-value**
C4BPt	202.6 ± 84.2	226.8 ± 63.4	**0.0003**

4.4 Paper IV

Thrombin generation and coagulation

Both investigated classes of anticoagulants (warfarin and DOACs) suppress the endogenous thrombin generation potential (ETP), although warfarin is more effective ($p<.0001$). Warfarin also reduces the levels of D-dimer, prothrombin, protein C, protein S and thrombospondin. fVIII is reduced in both treatment groups. Platelets are higher in warfarin compared to DOACs.

Complement activation markers

During warfarin treatment, C1q is reduced (consumed), C3a (also presented as C3a/C3) and sC5b-9 are greatly increased, C4BP, both total and in complex with protein S (C4BPβ+ and PS-C4BPβ+), is reduced, compared to after treatment.

When comparing on versus off DOACs, no difference is seen in complement activation markers.

When comparing the two treatments, C1q is reduced, C3a (also presented as C3a/C3) and sC5b-9 are increased in the warfarin treated group, whereas no difference is seen in C4BP, both total and as PS-C4BPβ+. However, a significant difference is seen after treatment, when levels of C4BPt and PS-C4BPβ+ are higher in patients previously treated with warfarin compared to those who received DOACs. C3a and sC5b-9 remain higher in patients post-warfarin treatment compared to post-DOACs. The Classical Pathway (CP) functional test is not affected by treatment.

Univariate and multivariate regression analysis

There is a weak positive correlation between platelets and C3a in all treated patients combined ($p=0.0013$, $r = 0.43$), and in patients on warfarin treatment ($p=0.0213$, $r = 0.45$), but not in the DOAC treated group.

In all treated patients taken together, free PS has a negative correlation with sC5b-9 ($p<.0001$, $r = -0.75$)

We observe weak inverse correlations between C1q and C3a ($p=0.0033$, $r= -0.39$), and between C1q and sC5b-9 ($p<.0001$, $r = -0.50$).

Platelets, free PS and C1q are no longer significantly associated to C3a or sC5b-9 in a multivariable model that includes treatment group.

C3a levels are associated with treatment (higher in warfarin treated group), sex (higher in women) and sC5b-9 ($p<.0001$, R^2 adj 0.74; $p=0.0002$ for treatment, $p=0.0263$ for sex and $p=0.0281$ for sC5b-9). The higher levels in women are explained by treatment (more women on warfarin compared to DOACs, 64% vs 36%). In fact, there is no difference between men and women in C3a levels (and also C1q and sC5b-9) when taking each treatment group separately (treatment stratification). C3a positively correlates with sC5b-9 ($p<.0001$, $r = 0.82$), in all treated patients.

C4BPβ+ and PS-C4BPβ+

C4BPβ+ positively correlates with PS-C4BPβ+ in all patients off treatment ($p<.0001$, $r = 0.63$), on treatment ($p<.0001$, $r = 0.69$), just on warfarin ($p = 0.0202$, $r = 0.46$), just on DOACs ($p<.0001$, $r = 0.93$), and off DOACs ($p<.0001$, $r = 0.81$).

C4BPβ+ positively correlates with sC5b-9 ($p<.0001$, $r = 0.50$), and with C3a ($p=0.0004$, $r = 0.45$) after, but not during treatment, and just when taking all patients together.

C4BPβ+ positively correlates with number of days between drug withdrawal and sample 2 ($p= 0.0057$, $r = 0.61$): the more time passes, the higher the levels. C3a, C5b-9, C4BPt are not associated with days from withdrawal.

5 DISCUSSION

5.1 Paper I

In order to understand the effect of TAFI and TAFIa in APS, markers of fibrinolysis and complement activation should be investigated as well, since TAFIa influences both. No study so far has included these variables. With this study we are able to confirm that APS patients have a lower clot permeability and prolonged CLT as compared to healthy controls, in line with Vikerfors et al[189]. We tried to identify a possible biomarker that could be associated with this impaired fibrinolysis. Since TAFIa is a natural fibrinolysis inhibitor that also plays a role in complement regulation, we hypothesized that it could be associated with markers of fibrinolysis, but no association was found. Nevertheless, we were able to demonstrate an increased complement activation and subclinical inflammation present in pAPS by showing higher levels of C5a, fibrinogen, TAFI and TAFIa compared to controls.

When we divided our sample according to clinical APS manifestations, we became soon aware that patients affected by arterial thrombotic events have higher levels of TAFIa, fibrinogen and TM compared to patients with other clinical APS manifestations. They also have more traditional CV risk factors, as expected.

APS thromboembolism can happen at any site in the vascular tree and there is no strict concordance between arterial or venous thrombosis at diagnosis and that of the recurrent thrombotic event. So far, no biomarker has been found to be able to precisely predict which vessel would be hit by thromboembolism in the course of APS, or which APS manifestation would characterize each and every patient. We identify TAFIa levels as higher in arterial thrombosis, and positively associated with TM, a marker of endothelial damage/activation[114,404]. We partly confirm previous findings by Ieko et al[219] of an inverse correlation between IgG aPL titers and TAFIa, but since we didn't compare our cohort with a group of patients APS-negative and affected by another autoimmune disease, we couldn't draw any other conclusion. We observe slightly lower titers of IgG aPL in the arterial subgroup but, after running a multivariable analysis in the whole cohort with TAFIa as the dependent variable, we see that they lose significance. Unfortunately, it was not possible to run a multivariable analysis in the arterial subgroup only, due to the small sample size.

5.2 Paper II

Thanks to the large PAROKRANK cohort, the SWEDEHEART quality registry and the Swedish population registry, we were able to collect data from a large number of patients from the general population affected by a first time MI, well matched with controls free from

previous MI and heart valve replacements. A strong association is shown between IgG aPL and first-time MI, independently of traditional CV risk factors. We confirm previous reports that the IgG aPL isotype is more strongly associated with occlusive vascular events than IgM or IgA aPL[253,405,406]. We cannot confirm the findings by Bili et al[255], that patients belonging to the lowest quartile of IgM anti-CL are at increased risk of recurrent cardiac events. Moreover, previous studies[243,257-259] suggest that aPL are overrepresented in young patients with MI. Considering the present data, obtained from a population with a mean age of 62 years, this assumption may need some revision towards accepting aPL positivity as a potential risk factor also in older age groups. The fact that IgG aPL+ MI patients tend to be smokers more often than controls, although not significantly, is in line with previous reports on positive associations between aPL and smoking[47,257,259,406]. This observation may also explain the positive association between Chronic Obstructive Pulmonary Disease (COPD), a proxy for smoking habits, and IgG aPL.

This study is important because it demonstrates that aPL are common in patients with MI in the general population. Our results create the premises for a follow-up study where, by repeating aPL measurement and comparing outcomes, we will be able to establish if aPL are transient or permanent, if isotypes differ or switch during long-term observations and if they have prognostic significance. These findings may have an impact on treatment, because, in case of persistent aPL positivity, patients should receive indefinite anticoagulants, according to the present APS guidelines[362], thus changing the current screening and treatment strategies for patients with MI at coronary care units.

5.3 Paper III

Our findings demonstrate that levels of complement inhibitor C4BPt are depressed in persons with persistent presence of aPL as compared to aPL negative subjects, irrespective of underlying SLE, lupus nephritis, thrombotic manifestations or aPL profile. Further reductions of C4BPt are seen in patients who are treated with warfarin, whereas higher levels are present in patients treated with LMWH. There are no statistically significant differences in the levels of C4BPt between arterial, venous and obstetric complications in either pAPS or sAPS, although obstetric pAPS patients tend to have higher levels compared to other groups. This may be explained by the fact that they are less often treated with warfarin (approximately 10% of obstetric pAPS patients are on warfarin vs 50-60% of patients with previous arterial or venous thrombotic events). In the same way, aPL carriers present only a slight and non-significant reduction in C4BPt as compared to controls, because they are less often treated with warfarin. Among aPL carriers 2/15 (13%) are on warfarin as compared to the other groups of aPL++ with pAPS or SLE (50% and 34% respectively on warfarin).

Therefore, infrequent warfarin use may explain why we observe less C4BPt reduction in aPL carriers and in obstetric pAPS.

Moreover, low C4BPt levels are associated with markers of enhanced complement activation (C2, C3, C4, and negatively with C3dg), indicating that the reduction of C4BPt may have a functional impact. We also measured free and total protein S in pAPS, showing lower levels of both fractions in warfarin treated patients, in the subgroup affected by DVT (more often treated with warfarin) and in correlation to C4BPt.

This work can have a considerable impact in the research field of APS and SLE, not only for the observation that C4BPt is depressed in patients with aPL++/APS, but especially for the observed effects of anticoagulation on the complement system, an aspect that has not been taken into account in the majority of the studies so far. If C4BP is reduced in warfarin-treated patients and in aPL+, does this lead to complement activation? Or maybe other complement inhibitors are upregulated to compensate for the C4BP reduction? Is the complement activation that was reported in previous APS studies, due also to anticoagulant treatment with warfarin? What about the C4d deposits on platelets or erythrocytes? Are they an effect of C4BP reduction or a secondary effect of warfarin? If this is the case, the results of many studies on APS and complement done so far should be reconsidered.

5.4 Paper IV

This study is important in general for patients in need of anticoagulant treatment, and in particular for APS patients. We demonstrate for the first time that warfarin, in contrast to DOACs, is strongly associated with markers of complement activation (increased levels of C3a and sC5b-9, consumption of C1q), not only during the actual treatment but also 2-3 weeks after withdrawal.

What was already known by previous studies in the general population[342,348,349,360], confirmed in aPL++ patients in paper III[407], was that warfarin reduces C4BP. This study adds that levels of C4BPt and PS-C4BPβ+ become higher after warfarin withdrawal, as a rebound effect, and remain persistently high for at least 19 days on average. Also C3a and sC5b-9 remain higher post-warfarin compared to post-DOACs, positively correlating to C4BPβ+.

We are able to clarify that DOACs have no effect on complement, but, in contrast, we demonstrate that warfarin is associated with complement activation, partly but probably not only through inhibition of C4BP.

These results give new perspectives, possibly of importance for the choice of anticoagulants, especially in inflammatory systemic diseases.

5.5 Limitations and strengths

5.5.1 Limitations

The three first studies are cross-sectional, a study design that does not allow to infer causality because it presents just a moment in time, not showing the temporal relationship between the measured variables. This is of particular relevance for paper II, where we cannot provide information about the presence of aPL before the MI event, or know if aPL developed as a response to myocardial damage, making it impossible to infer causality. Nevertheless, we know that the frequency of previous venous and arterial thromboses is not higher in the aPL+ MI compared to the aPL- subset. The samples were taken just once at 6-10 weeks post-MI, so aPL cannot be characterized as transient or permanent. For the same reason, the observed differences between MI patients and controls regarding lipids and blood pressure levels are not reliable for comparison, because they are all affected by post-MI treatment.

In all papers, the samples were collected in a quiescent phase of APS. Thus, we don't know how TAFIa, TM, C5a and markers of fibrinolysis (paper I) behave at the time of an acute thrombosis, e.g. MI (paper II). We even don't know how C4BPt (paper III and IV) changes or relates to aPL profile/complement activation markers during acute phases of thrombotic/obstetric events.

The small number of patients in paper I and IV is a limitation: if we had more patients, we would have had more power to run a multivariable analysis just in the arterial subset of patients (paper I). The lack of a significant difference in C4BP levels during treatment with warfarin versus DOACs in paper IV may be due to lack of power.

Finally, not having enough samples: in paper II, due to lack of citrated plasma, the LA test could not be performed, and in paper III we couldn't measure PS in controls, so that we lacked a reference group, as well as in SLE patients, which prevented us from running a mediation analysis to quantify the direct and controlled effect of aPL++ by warfarin on PS reduction. The lack of samples from controls free from thrombosis and its treatment in paper IV precluded us the possibility to compare C4BP and complement factors in patients on/off warfarin/DOACs with reference values taken from the same source population.

5.5.2 Strengths

Patients from studies I and III are well characterized and they fulfill Miyakis criteria for primary or secondary APS[2]. aPL are determined according to standardized cut-offs and detection methods.

The main strength of paper I is the fact that we studied well-characterized, mostly pAPS patients, free from SLE. We investigated TAFI and TAFIa also in relation to CLT, Ks and markers of complement activation, and analyzed these molecules separately in different clinical APS manifestations.

The number of subjects studied in paper II is large and representative of both sexes, with a broad age-span, including well-matched controls, with an extensive collection of patients' characteristics. All events were first-time MIs. aPL are also differentiated according to isotype and target antigen, and that allowed us to demonstrate the unique association of IgG aPL with first-time MI, whereas we didn't see any association with the other isotypes, as well as the strong correlation between the same isotype of different target antigens (aCL and anti-β_2GPI). In previous studies, IgG was either analyzed in isolation[408,409] or pooled with IgM[410,411], making it difficult to dissect the role of each isotype.

Also in paper III, considering the rarity of APS/aPL++, we were able to study a relatively large number of subjects, who were well characterized regarding their clinical manifestations and aPL profiles, representing both primary and secondary APS and aPL carriers. We also investigated a large number of complement proteins representing different steps in the classical pathway of the complement cascade.

Thanks to the study design in paper IV, we could limit bias by studying the same patients during and after treatment.

Paper II, despite having, at present, a cross-sectional design, it has the potential to become a large longitudinal study, thanks to an already planned follow-up of the same subjects.

All the four studies presented in this thesis try to answer crucial questions in APS by analyzing known proteins (TAFI, C4BP, complement factors):

- in a relatively new group of patients: well characterized APS, precisely sub-classified according to more recent criteria
- in relation to old and new anticoagulant treatments
- by studying the prevalence of aPL positivity of different isotypes in a large cohort representing both sexes and a broad spectrum of ages with modern and standardized detection methods

6 CONCLUSIONS AND FUTURE PERSPECTIVES

6.1 Conclusions

- TAFI and TAFIa are increased in pAPS
- TAFIa is particularly increased in patients with pAPS affected by arterial thrombosis, independently of traditional CV risk factors
- Thrombomodulin, a marker of endothelial damage/activation, is increased in pAPS patients affected by arterial thrombosis, and positively correlates with TAFIa
- There is a subclinical inflammation in patients with pAPS, marked by higher levels of TAFI and complement activation
- pAPS patients have an impaired fibrinolysis, measured by a prolonged CLT and lower permeability coefficient Ks
- There is a detectable complement activation in APS: higher levels of C5a, C3a, C3dg, sC5b-9, and consumed C1q
- The complement inhibitor C4BP is reduced by both warfarin treatment and persistently positive aPL
- Warfarin, DOACs and LMWH have a different impact on the complement system and C4BP
- 6-10 weeks after a first-time MI, the prevalence of IgG aPL is 11%, i.e. ten times higher as compared to matched controls

6.2 Future perspectives

- Longitudinal follow-up study of paper II, to explore the prognostic value of IgG aPL in patients with MI (now being performed)
- Personal follow-up with new measurements of aPL in patients who were aPL positive after their first MI, to investigate if aPL are persistent after 6-10 years
- Measure plasma C4d in SLE/APS in relation to anticoagulant treatment and C4BP levels
- Measure C4d deposits on platelets or in kidney biopsies in primary and secondary APS, also in relation to anticoagulant treatment and C4BP levels
- Measure antibodies against C4BP in APS
- Further studies on the effects of warfarin on the complement system
- Extend and update APS and SLE database by continuously recruiting new patients

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8 REFERENCES

1. Ames PRJ, Alves JD, Gentile F. Coagulation and complement in antiphospholipid syndrome. Thrombosis Research; 2017. p. 149-51.
2. Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS. *Journal of Thrombosis and Haemostasis* 2006; **4**(2): 295-306.
3. Hojnik M, George J, Ziporen L, Shoenfeld Y. Heart Valve Involvement (Libman-Sacks Endocarditis) in the Antiphospholipid Syndrome. *Circulation* 1996; **93**(8): 1579-87.
4. Karen S, Savino S, Philip GDG, et al. Antiphospholipid syndrome. *Nature Reviews Disease Primers* 2018; **4**.
5. Deane KD, West SG. Antiphospholipid Antibodies as a Cause of Pulmonary Capillaritis and Diffuse Alveolar Hemorrhage: A Case Series and Literature Review. *Semin Arthritis Rheum* 2005; **35**(3): 154-65.
6. Espinosa G, Cervera R, Font J, Asherson RA. The lung in the antiphospholipid syndrome. *Annals of the Rheumatic Diseases* 2002; **61**(3): 195.
7. Harris EN, Gharavi AE, Boey ML, et al. Anticardiolipin antibodies: detection by radioimmunoassay and association with thrombosis in systemic lupus erythematosus. *Lancet (London, England)* 1983; **2**(8361): 1211.
8. Hughes GRV. Thrombosis, Abortion, Cerebral Disease, And The Lupus Anticoagulant. *Br Med J (Clin Res Ed)* 1983; **287**(6399): 1088-9.
9. Hughes GR, Harris NN, Gharavi AE. The anticardiolipin syndrome. *J Rheumatol* 1986; **13**(3): 486.
10. Galli M, Barbui T, Comfurius P, et al. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. *The Lancet (British edition)* 1990; **335**(8705): 1544-7.
11. Kandiah DA, Krilis SA. Beta2-glycoprotein I. 1994. p. 207-12.
12. McNeil H, Simpson R, Chesterman C, Krilis SA. Antiphospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation - beta-2-glycoprotein-I (apolipoprotein-H). *Proceedings Of The National Academy Of Sciences Of The United States Of Ame* 1990; **87**(11): 4120-4.
13. Wilson WA, Gharavi AE, Koike T, et al. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: Report of an International workshop. *Arthritis & Rheumatism* 1999; **42**(7): 1309-11.
14. Harris EN. Special report. The Second International Anti-cardiolipin Standardization Workshop/the Kingston Anti-Phospholipid Antibody Study (KAPS) group. *Am J Clin Pathol* 1990; **94**(4): 476.
15. Harris EN, Gharavi AE, Patel SP, Hughes GR. Evaluation of the anti-cardiolipin antibody test: report of an international workshop held 4 April 1986. *Clin Exp Immunol* 1987; **68**(1): 215.
16. Harris EN, Pierangeli SS. Revisiting the anticardiolipin test and its standardization. Thousand Oaks, CA; 2002. p. 269-75.
17. Pierangeli SS, Groot PGd, Dlott J, et al. 'Criteria' aPL tests: Report of a Task Force and preconference workshop at the 13th International Congress on Antiphospholipid Antibodies, Galveston, Texas, April 2010. *Lupus* 2011; **20**(2): 182-90.
18. Willis R, Lakos G, Harris EN. Standardization of antiphospholipid antibody testing--historical perspectives and ongoing initiatives. *Seminars in thrombosis and hemostasis* 2014; **40**(2): 172.
19. Duarte-García A, Pham MM, Crowson CS, et al. The Epidemiology of Antiphospholipid Syndrome: A Population-Based Study. *Arthritis & rheumatology (Hoboken, NJ)* 2019; **71**(9): 1545-52.

20. Pierangeli SS, Chen PP, Raschi EO, et al. Antiphospholipid Antibodies and the Antiphospholipid Syndrome: Pathogenic Mechanisms. *Seminars In Thrombosis And Hemostasis* 2008; **34**(03): 236-50.
21. Petri M. Update on anti-phospholipid antibodies in SLE: the Hopkins' Lupus Cohort. *Lupus* 2010; **19**(4): 419.
22. Cervera R, Boffa MC, Khamashta MA, Hughes G. The Euro-Phospholipid project: epidemiology of the antiphospholipid syndrome in Europe. *Lupus* 2009; **18**(10): 889-93.
23. Cervera R, Khamashta MA, Shoenfeld Y, et al. Morbidity and mortality in the antiphospholipid syndrome during a 5-year period: a multicentre prospective study of 1000 patients. *Ann Rheum Dis* 2009; **68**(9): 1428-32.
24. Cervera R, Piette JC, Font J, et al. Antiphospholipid syndrome: clinical and immunologic manifestations and patterns of disease expression in a cohort of 1,000 patients. *Arthritis Rheum* 2002; **46**(4): 1019-27.
25. Cervera R, Serrano R, Pons-Estel GJ, et al. Morbidity and mortality in the antiphospholipid syndrome during a 10-year period: a multicentre prospective study of 1000 patients. *Ann Rheum Dis* 2015; **74**(6): 1011-8.
26. Espinosa G, Cervera R. Morbidity and mortality in the antiphospholipid syndrome. *Curr Opin Pulm Med* 2009; **15**(5): 413-7.
27. Asherson RA. The catastrophic antiphospholipid syndrome. *J Rheumatol* 1992; **19**(4): 508.
28. Vora SK, Asherson RA, Erkan D. Catastrophic antiphospholipid syndrome. *Journal of intensive care medicine* 2006; **21**(3): 144-59.
29. Ortega-Hernandez OD, Agmon-Levin N, Blank M, Asherson RA, Shoenfeld Y. The physiopathology of the catastrophic antiphospholipid (Asherson's) syndrome: compelling evidence. *J Autoimmun* 2009; **32**(1): 1-6.
30. Cervera R, Espinosa G. Update on the Catastrophic Antiphospholipid Syndrome and the “CAPS Registry”. *Semin Thromb Hemost* 2012; **38**(04): 333-8.
31. Asherson RA, Cervera R, de Groot PG, et al. Catastrophic antiphospholipid syndrome: international consensus statement on classification criteria and treatment guidelines. *Lupus* 2003; **12**(7): 530-4.
32. Rodríguez-Pintó I, Moitinho M, Santacreu I, et al. Catastrophic antiphospholipid syndrome (CAPS): Descriptive analysis of 500 patients from the International CAPS Registry. *Autoimmunity reviews* 2016; **15**(12): 1120-4.
33. Lundström E, Gustafsson JT, Jönsen A, et al. HLA-DRB104/13 alleles are associated with vascular disease and antiphospholipid antibodies in systemic lupus erythematosus. *Annals of the rheumatic diseases* 2013; **72**(6): 1018-25.
34. Sebastiani GD, Iuliano A, Cantarini L, Galeazzi M. Genetic aspects of the antiphospholipid syndrome: An update. *Autoimmunity reviews* 2016; **15**(5): 433-9.
35. Sebastiani GD, Minisola G, Galeazzi M. HLA class II alleles and genetic predisposition to the antiphospholipid syndrome. *Autoimmunity Reviews* 2003; **2**(6): 387-94.
36. Chaturvedi S, Braunstein EM, Yuan X, et al. Complement activity and complement regulatory gene mutations are associated with thrombosis in APS and CAPS. *Blood* 2020; **135**(4): 239-51.
37. Francis J, Rai R, Sebire NJ, et al. Impaired expression of endometrial differentiation markers and complement regulatory proteins in patients with recurrent pregnancy loss associated with antiphospholipid syndrome. *Molecular Human Reproduction* 2006; **12**(7): 435-42.
38. Salmon JE, Heuser C, Triebwasser M, et al. Mutations in Complement Regulatory Proteins Predispose to Preeclampsia: A Genetic Analysis of the PROMISSE Cohort (Complement Mutations in Preeclampsia). *PLoS Med* 2011; **8**(3): e1001013.
39. Svenungsson E, Gustafsson J, Leonard D, et al. A STAT4 risk allele is associated with ischaemic cerebrovascular events and anti-phospholipid antibodies in systemic lupus erythematosus. *Annals of the rheumatic diseases* 2010; **69**(5): 834-40.
40. Yasuda S, Atsumi T, Matsuura E, et al. Significance of valine/leucine247 polymorphism of β2-glycoprotein I in antiphospholipid syndrome: Increased reactivity of anti–β2-

glycoprotein I autoantibodies to the valine247 β2-glycoprotein I variant. *Arthritis & Rheumatism* 2005; **52**(1): 212-8.
41. Atsumi T, Tsutsumi A, Amengual O, et al. Correlation between beta2-glycoprotein I valine/leucine247 polymorphism and anti-beta2-glycoprotein I antibodies in patients with primary antiphospholipid syndrome. *Rheumatology (Oxford)* 1999; **38**(8): 721-3.
42. Hirose N, Williams R, Alberts AR, et al. A role for the polymorphism at position 247 of the beta2-glycoprotein I gene in the generation of anti-beta2-glycoprotein I antibodies in the antiphospholipid syndrome. *Arthritis and rheumatism* 1999; **42**(8): 1655-61.
43. Prieto GA, Cabral AR, Zapata-Zuñiga M, et al. Valine/valine genotype at position 247 of the β2-glycoprotein I gene in Mexican patients with primary antiphospholipid syndrome: Association with anti–β2-glycoprotein I antibodies. *Arthritis and rheumatism* 2003; **48**(2): 471-4.
44. Camilleri RS, Mackie IJ, Humphries SE, Machin SJ, Cohen H. Lack of association of beta2-glycoprotein I polymorphisms Val247Leu and Trp316Ser with antiphospholipid antibodies in patients with thrombosis and pregnancy complications. *British Journal of Haematology* 2003; **120**(6): 1066-72.
45. de Groot PG, Urbanus RT. The significance of autoantibodies against beta2-glycoprotein I. *Blood* 2012; **120**(2): 266-74.
46. Levy Y, Almog O, Gorshtein A, Shoenfeld Y. The environment and antiphospholipid syndrome. *Lupus* 2006; **15**(11): 784-90.
47. Gustafsson JT, Gunnarsson I, Kallberg H, et al. Cigarette smoking, antiphospholipid antibodies and vascular events in Systemic Lupus Erythematosus. *Ann Rheum Dis* 2015; **74**(8): 1537-43.
48. McIntyre JA, Wagenknecht DR, Faulk WP. Redox-reactive autoantibodies: Detection and physiological relevance. *Autoimmunity reviews* 2006; **5**(1): 76-83.
49. Asherson RA, Cervera R. Antiphospholipid antibodies and infections. *Annals of the Rheumatic Diseases* 2003; **62**(5): 388-93.
50. Epstein FH, Albert LJ, Inman RD. Molecular Mimicry and Autoimmunity. *The New England Journal of Medicine* 1999; **341**(27): 2068-74.
51. Abdel-Wahab N, Lopez-Olivo MA, Pinto-Patarroyo GP, Suarez-Almazor ME. Systematic review of case reports of antiphospholipid syndrome following infection. *Lupus* 2016; **25**(14): 1520-31.
52. Male C, Foulon D, Hoogendoorn H, et al. Predictive value of persistent versus transient antiphospholipid antibody subtypes for the risk of thrombotic events in pediatric patients with systemic lupus erythematosus. *Blood* 2005; **106**(13): 4152-8.
53. de Groot PG, Urbanus RT. Antiphospholipid Syndrome--Not a Noninflammatory Disease. *Semin Thromb Hemost* 2015; **41**(6): 607-14.
54. Levine JS, Koh JS, Subang R, Rauch J. Apoptotic Cells as Immunogen and Antigen in the Antiphospholipid Syndrome. *Experimental and Molecular Pathology* 1999; **66**(1): 82-98.
55. Andreoli L, Fredi M, Nalli C, Franceschini F, Meroni PL, Tincani A. Antiphospholipid antibodies mediate autoimmunity against dying cells. *Autoimmunity* 2013; **46**(5): 302-6.
56. Bondanza A, Rovere-Querini P, Zimmermann VS, et al. Requirement for dendritic cells in the establishment of anti-phospholipid antibodies. *Autoimmunity* 2007; **40**(4): 302-6.
57. Michlewska S, McColl A, Rossi AG, Megson IL, Dransfield I. Clearance of dying cells and autoimmunity. *Autoimmunity* 2007; **40**(4): 267-73.
58. Palomo IG, Segovia FM, Alarcon ML, et al. An insight into the pathophysiology of thrombosis in antiphospholipid syndrome. *Frontiers in bioscience : a journal and virtual library* 2007; **12**: 3093.
59. Giannakopoulos B, Krilis SA. The pathogenesis of the antiphospholipid syndrome. *N Engl J Med* 2013; **368**(11): 1033-44.
60. Rand JH, Wu XX, Quinn AS, Taatjes DJ. The annexin A5-mediated pathogenic mechanism in the antiphospholipid syndrome: role in pregnancy losses and thrombosis. *Lupus* 2010; **19**(4): 460.
61. de Groot PG, Meijers JC. beta(2) -Glycoprotein I: evolution, structure and function. *J Thromb Haemost* 2011; **9**(7): 1275-84.

62. Guerin J, Sheng Y, Reddel S, Iverson GM, Chapman MG, Krilis SA. Heparin inhibits the binding of beta 2-glycoprotein I to phospholipids and promotes the plasmin-mediated inactivation of this blood protein. Elucidation of the consequences of the two biological events in patients with the anti-phospholipid syndrome. *The Journal of biological chemistry* 2002; **277**(4): 2644.
63. Agar C, van Os GM, Morgelin M, et al. Beta2-glycoprotein I can exist in 2 conformations: implications for our understanding of the antiphospholipid syndrome. *Blood* 2010; **116**(8): 1336-43.
64. Giannakopoulos B, Mirarabshahi P, Krilis SA. New insights into the biology and pathobiology of beta2-glycoprotein I. *Curr Rheumatol Rep* 2011; **13**(1): 90-5.
65. Kuwana M, Matsuura E, Kobayashi K, et al. Binding of beta 2-glycoprotein I to anionic phospholipids facilitates processing and presentation of a cryptic epitope that activates pathogenic autoreactive T cells. *Blood* 2005; **105**(4): 1552-7.
66. Ruiz-Irastorza G, Crowther M, Branch W, Khamashta MA. Antiphospholipid syndrome. *The Lancet* 2010; **376**(9751): 1498-509.
67. Espinosa G, Cervera R. Antiphospholipid syndrome. *Arthritis Res Ther* 2008; **10**(6): 230.
68. de Groot PG, de Laat B. Mechanisms of thrombosis in systemic lupus erythematosus and antiphospholipid syndrome. *Best Practice & Research Clinical Rheumatology* 2017; **31**(3): 334-41.
69. de Groot PG, Urbanus RT, Derksen R. Pathophysiology of thrombotic APS: where do we stand? *Lupus* 2012; **21**(7): 704-7.
70. Brandt KJ, Kruithof EK, de Moerloose P. Receptors involved in cell activation by antiphospholipid antibodies. *Thromb Res* 2013; **132**(4): 408-13.
71. de Groot PG, Urbanus RT. Cellular signaling by antiphospholipid antibodies. *J Thromb Haemost* 2014; **12**(5): 773-5.
72. Raschi E, Testoni C, Bosisio D, et al. Role of the MyD88 transduction signaling pathway in endothelial activation by antiphospholipid antibodies. *Blood* 2003; **101**(9): 3495.
73. Pierangeli SS, Vega-Ostertag M, Harris EN. Intracellular signaling triggered by antiphospholipid antibodies in platelets and endothelial cells: a pathway to targeted therapies. *Thromb Res* 2004; **114**(5-6): 467-76.
74. Oku K, Amengual O, Atsumi T. Pathophysiology of thrombosis and pregnancy morbidity in the antiphospholipid syndrome. *Eur J Clin Invest* 2012; **42**(10): 1126-35.
75. Ling Q, Jacovina AT, Deora A, et al. Annexin II regulates fibrin homeostasis and neoangiogenesis in vivo. *The Journal of clinical investigation* 2004; **113**(1): 38.
76. Ma K, Simantov R, Zhang JC, Silverstein R, Hajjar KA, McCrae KR. High affinity binding of beta 2-glycoprotein I to human endothelial cells is mediated by annexin II. *The Journal of biological chemistry* 2000; **275**(20): 15541-8.
77. Romay-Penabad Z, Montiel-Manzano MG, Shilagard T, et al. Annexin A2 is involved in antiphospholipid antibody-mediated pathogenic effects in vitro and in vivo. *Blood* 2009; **114**(14): 3074-83.
78. Satta N, Kruithof EKO, Fickentscher C, et al. Toll-like receptor 2 mediates the activation of human monocytes and endothelial cells by antiphospholipid antibodies. *Blood* 2011; **117**(20): 5523-31.
79. Pierangeli S, Vega-Ostertag, E. M, et al. Toll-like receptor and antiphospholipid mediated thrombosis: in vivo studies. *Annals of the Rheumatic Diseases* 2007; **66**(10): 1327.
80. Simantov R, LaSala JM, Lo SK, et al. Activation of cultured vascular endothelial cells by antiphospholipid antibodies. *J Clin Invest* 1995; **96**(5): 2211-9.
81. Zhou. Annexin A2 mediates anti-β2GPI/β2GPI-induced tissue factor expression on monocytes. *International Journal of Molecular Medicine* 2009; **24**(04).
82. Cuadrado MJ, López-pedrera C, Khamashta MA, et al. Thrombosis in primary antiphospholipid syndrome. A pivotal role for monocyte tissue factor expression. *Arthritis & Rheumatism* 1997; **40**(5): 834-41.

83. 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. *Thrombosis and haemostasis* 1998; **79**(2): 276.
84. Urbanus RT, Derksen RHWM, de Groot PG. Platelets and the antiphospholipid syndrome. *Lupus* 2008; **17**(10): 888-94.
85. van Lummel M, Pennings MT, Derksen RH, et al. The binding site in {beta}2-glycoprotein I for ApoER2' on platelets is located in domain V. *J Biol Chem* 2005; **280**(44): 36729-36.
86. Hulstein JJJ, Lenting PJ, de Laat B, Derksen RHWM, Fijnheer R, de Groot PG. beta2-Glycoprotein I inhibits von Willebrand factor dependent platelet adhesion and aggregation. *Blood* 2007; **110**(5): 1483.
87. 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**(1): 51-6.
88. Bravo-Barrera J, Kourilovitch M, Galarza-Maldonado C. Neutrophil Extracellular Traps, Antiphospholipid Antibodies and Treatment. *Antibodies (Basel, Switzerland)* 2017; **6**(1): 4.
89. Volker B, Ulrike R, Christian G, et al. Neutrophil Extracellular Traps Kill Bacteria. *Science (American Association for the Advancement of Science)* 2004; **303**(5663): 1532-5.
90. Redecha P, Franzke CW, Ruf W, Mackman N, Girardi G. Neutrophil activation by the tissue factor/Factor VIIa/PAR2 axis mediates fetal death in a mouse model of antiphospholipid syndrome. *J Clin Invest* 2008; **118**(10): 3453-61.
91. Bucciarelli P, Martinelli I, Artoni A, et al. Circulating microparticles and risk of venous thromboembolism. *Thromb Res* 2012; **129**(5): 591-7.
92. Morel O, Toti F, Hugel B, et al. Procoagulant microparticles: disrupting the vascular homeostasis equation? *Arterioscler Thromb Vasc Biol* 2006; **26**(12): 2594-604.
93. Lacroix R, Dubois C, Leroyer AS, Sabatier F, Dignat-George F. Revisited role of microparticles in arterial and venous thrombosis. *J Thromb Haemost* 2013; **11 Suppl 1**: 24-35.
94. Dignat-George F, Camoin-Jau L, Sabatier F, et al. Endothelial microparticles: a potential contribution to the thrombotic complications of the antiphospholipid syndrome. *Thromb Haemost* 2004; **91**(4): 667-73.
95. Greer IA. Endothelial cell microparticles and antiphospholipid syndrome: pathologic explanations and diagnostic opportunities. *Thrombosis and haemostasis* 2004; **91**(4): 636.
96. Nomura S, Komiyama Y, Matsuura E, Kokawa T, Takahashi H, Koike T. Binding of beta 2-glycoprotein I to platelet-derived microparticles. *British journal of haematology* 1993; **85**(3): 639-40.
97. Mobarrez F, Gunnarsson I, Svenungsson E. Altered beta2 -glycoprotein I expression on microparticles in the presence of antiphospholipid antibodies. *J Thromb Haemost* 2017; **15**(9): 1799-806.
98. Vikerfors A, Mobarrez F, Bremme K, et al. Studies of microparticles in patients with the antiphospholipid syndrome (APS). *Lupus* 2012; **21**(7): 802-5.
99. Mobarrez F, Vikerfors A, Gustafsson JT, et al. Microparticles in the blood of patients with systemic lupus erythematosus (SLE): phenotypic characterization and clinical associations. *Sci Rep* 2016; **6**: 36025.
100. Dahlback B, Wiedmer T, Sims PJ. Binding of anticoagulant vitamin K-dependent protein S to platelet-derived microparticles. *Biochemistry* 1992; **31**(51): 12769.
101. Salmon J, de Groot PG. Pathogenic role of antiphospholipid antibodies. *Lupus* 2008; **17**(5): 405-11.
102. Salmon JE, Girardi G. Antiphospholipid antibodies and pregnancy loss: a disorder of inflammation. *J Reprod Immunol* 2008; **77**(1): 51-6.
103. Salmon JE, Girardi G, Holers VM. Complement activation as a mediator of antiphospholipid antibody induced pregnancy loss and thrombosis. *Annals of the Rheumatic Diseases* 2002; **61**(suppl 2): ii46.

104. Salmon JE, Girardi G, Lockshin MD. The antiphospholipid syndrome as a disorder initiated by inflammation: implications for the therapy of pregnant patients. *Nat Clin Pract Rheumatol* 2007; **3**(3): 140-7; quiz 1 p following 87.
105. O'Neil K. Role of complement in antiphospholipid antibody-mediated thrombosis. *Current Rheumatology Reports* 2007; **9**(3): 205-11.
106. Oikonomopoulou K, Ricklin D, Ward P, Lambris J. Interactions between coagulation and complement—their role in inflammation. *Semin Immunopathol* 2012; **34**(1): 151-65.
107. Lopez-Lira F, Rosales-Leon L, Martinez VM, Ruiz Ordaz BH. The role of beta2-glycoprotein I (beta2GPI) in the activation of plasminogen. *Biochim Biophys Acta* 2006; **1764**(4): 815-23.
108. Forastiero R, Martinuzzo M. Prothrombotic mechanisms based on the impairment of fibrinolysis in the antiphospholipid syndrome. *Lupus* 2008; **17**(10): 872-7.
109. Krone KA, Allen KL, McCrae KR. Impaired fibrinolysis in the antiphospholipid syndrome. *Curr Rheumatol Rep* 2010; **12**(1): 53-7.
110. Redecha P, Tilley R, Tencati M, et al. Tissue factor: a link between C5a and neutrophil activation in antiphospholipid antibody induced fetal injury. *Blood* 2007; **110**(7): 2423.
111. Forastiero R, Martinuzzo M. The emerging role of multiple antiphospholipid antibodies positivity in patients with antiphospholipid syndrome. *Expert Rev Clin Immunol* 2015; **11**(11): 1255-63.
112. Rosenberg RD, Aird WC. Vascular-bed--specific hemostasis and hypercoagulable states. *The New England journal of medicine* 1999; **340**(20): 1555.
113. Aird WC. Spatial and temporal dynamics of the endothelium. *Journal of thrombosis and haemostasis* 2005; **3**(7): 1392-406.
114. Martin FA, Murphy RP, Cummins PM. Thrombomodulin and the vascular endothelium: insights into functional, regulatory, and therapeutic aspects. *Am J Physiol Heart Circ Physiol* 2013; **304**(12): H1585-97.
115. Meroni PL, Borghi MO, Grossi C, Chighizola CB, Durigutto P, Tedesco F. Obstetric and vascular antiphospholipid syndrome: same antibodies but different diseases? *Nature reviews Rheumatology* 2018; **14**(7): 433-40.
116. Di Simone N, Luigi MP, Marco D, et al. Pregnancies Complicated with Antiphospholipid Syndrome: The Pathogenic Mechanism of Antiphospholipid Antibodies: A Review of the Literature. *Annals of the New York Academy of Sciences* 2007; **1108**(1): 505-14.
117. Pregnolato F, Gerosa M, Raimondo MG, et al. EUREKA algorithm predicts obstetric risk and response to treatment in women with different subsets of anti-phospholipid antibodies. *Rheumatology (Oxford)* 2021; **60**(3): 1114-24.
118. Miyakis S, Robertson SA, Krilis SA. Beta-2 glycoprotein I and its role in antiphospholipid syndrome-lessons from knockout mice. *Clin Immunol* 2004; **112**(2): 136-43.
119. Agostinis C, Biffi S, Tedesco F, et al. In vivo distribution of β2 glycoprotein I under various pathophysiologic conditions. *Blood* 2011; **118**(15): 4231-8.
120. Gerardi MC, Fernandes MA, Tincani A, Andreoli L. Obstetric Anti-phospholipid Syndrome: State of the Art. *Current rheumatology reports* 2018; **20**(10): 1-12.
121. Meroni PL, Borghi MO, Raschi E, Tedesco F. Pathogenesis of antiphospholipid syndrome: understanding the antibodies. *Nat Rev Rheumatol* 2011; **7**(6): 330-9.
122. Shamonki JM, Salmon JE, Hyjek E, Baergen RN. Excessive complement activation is associated with placental injury in patients with antiphospholipid antibodies. *Am J Obstet Gynecol* 2007; **196**(2): 167 e1-5.
123. Martinez-Berriotxoa A, Ruiz-Irastorza G, Egurbide M-V, et al. Transiently positive anticardiolipin antibodies and risk of thrombosis in patients with systemic lupus erythematosus. *Lupus* 2007; **16**(10): 810-6.
124. Galli M, Luciani D, Bertolini G, Barbui T. Lupus anticoagulants are stronger risk factors for thrombosis than anticardiolipin antibodies in the antiphospholipid syndrome: a systematic review of the literature. *Blood* 2003; **101**(5): 1827.

125. Galli M, Luciani D, Bertolini G, Barbui T. Anti-beta 2-glycoprotein I, antiprothrombin antibodies, and the risk of thrombosis in the antiphospholipid syndrome. *Blood* 2003; **102**(8): 2717-23.
126. Levine SR, Salowich-Palm L, Sawaya KL, et al. IgG Anticardiolipin Antibody Titer >40 GPL and the Risk of Subsequent Thrombo-occlusive Events and Death: A Prospective Cohort Study. *Stroke (1970)* 1997; **28**(9): 1660-5.
127. Rance A, Emmerich J, Fiessinger J-N. Anticardiolipin Antibodies and Recurrent Thromboembolism. *Thrombosis and Haemostasis* 1997; **77**(1): 221-2.
128. de Bandt M, Benali K, Guillevin L, et al. Longitudinal determination of antiphospholipid antibodies in lupus patients without previous manifestations of antiphospholipid syndrome. A prospective study. *J Rheumatol* 1999; **26**(1): 91.
129. Schulman S, Svenungsson E, Granqvist S. Anticardiolipin Antibodies Predict Early Recurrence of Thromboembolism and Death Among Patients with Venous Thromboembolism Following Anticoagulant Therapy. *The American journal of medicine* 1998; **104**(4): 332-8.
130. Finazzi G, Brancaccio V, Moia M, et al. Natural history and risk factors for thrombosis in 360 patients with antiphospholipid antibodies: A four-year prospective study from the italian registry. *The American journal of medicine* 1996; **100**(5): 530-6.
131. Pengo V, Biasiolo A, Pegoraro C, Cucchini U, Noventa F, Iliceto S. Antibody profiles for the diagnosis of antiphospholipid syndrome. *Thrombosis and Haemostasis* 2005; **93**(6): 1147-52.
132. Pengo V, Ruffatti A, Legnani C, et al. Clinical course of high-risk patients diagnosed with antiphospholipid syndrome. *Journal of Thrombosis and Haemostasis* 2010; **8**(2): 237-42.
133. Pengo V, Ruffatti A, Legnani C, et al. Incidence of a first thromboembolic event in asymptomatic carriers of high-risk antiphospholipid antibody profile: a multicenter prospective study. *Blood* 2011; **118**(17): 4714.
134. Ruffatti A, Del Ross T, Ciprian M, et al. Risk factors for a first thrombotic event in antiphospholipid antibody carriers: a prospective multicentre follow-up study. *Annals of the rheumatic diseases* 2011; **70**(6): 1083-6.
135. Roger VL, Go AS, Lloyd-Jones DM, et al. Heart disease and stroke statistics--2011 update: a report from the American Heart Association. *Circulation (New York, NY)* 2011; **123**(4): e18-e209.
136. De Carolis S, Tabacco S, Rizzo F, et al. Antiphospholipid syndrome: An update on risk factors for pregnancy outcome. *Autoimmunity reviews* 2018; **17**(10): 956-66.
137. Ahluwalia J, Sreedharanunni S. The Laboratory Diagnosis of the Antiphospholipid Syndrome. *Indian Journal of Hematology and Blood Transfusion* 2017; **33**(1): 8-14.
138. Tektonidou MG, Laskari K, Panagiotakos DB, Moutsopoulos HM. Risk factors for thrombosis and primary thrombosis prevention in patients with systemic lupus erythematosus with or without antiphospholipid antibodies. *Arthritis and rheumatism* 2008; **61**(1): 29-36.
139. Pengo V, Denas G, Banzato A, et al. Interpretation of laboratory data and need for reference laboratories. *Lupus* 2012; **21**(7): 732-3.
140. Galli M, Borrelli G, Jacobsen EM, et al. Clinical significance of different antiphospholipid antibodies in the WAPS (warfarin in the antiphospholipid syndrome) study. *Blood* 2007; **110**(4): 1178-83.
141. Carolyn N, Joyce R, Jeannine K, et al. Antiphospholipid antibodies predict imminent vascular events independently from other risk factors in a prospective cohort. *Thrombosis and haemostasis* 2009; **101**(1): 100-7.
142. Pengo V. Antiphospholipid syndrome: interpretation of laboratory data. *Journal of Thrombosis and Haemostasis* 2011; **9**(2): 402.
143. de Laat B, Pengo V, Pabinger I, et al. The association between circulating antibodies against domain I of beta2-glycoprotein I and thrombosis: an international multicenter study. *J Thromb Haemost* 2009; **7**(11): 1767-73.
144. de Laat B, Derksen RHWM, Urbanus RT, de Groot PG. IgG antibodies that recognize epitope Gly40-Arg43 in domain I of beta 2-glycoprotein I cause LAC, and their presence correlates strongly with thrombosis. *Blood* 2005; **105**(4): 1540.

145. de Laat HB, Derksen RHWM, Urbanus RT, Roest M, de Groot PG. beta2-glycoprotein I-dependent lupus anticoagulant highly correlates with thrombosis in the antiphospholipid syndrome. *Blood* 2004; **104**(12): 3598.
146. Mahler M, Norman GL, Meroni PL, Khamashta M. Autoantibodies to domain 1 of beta 2 glycoprotein 1: A promising candidate biomarker for risk management in antiphospholipid syndrome. *Autoimmunity reviews* 2012; **12**(2): 313-7.
147. De Craemer AS, Musial J, Devreese KMJ. Role of anti-domain 1-β2glycoprotein I antibodies in the diagnosis and risk stratification of antiphospholipid syndrome. *Journal of Thrombosis and Haemostasis* 2016; **14**(9): 1779-87.
148. Atsumi T, Ieko M, Bertolaccini ML, et al. Association of autoantibodies against the phosphatidylserine–prothrombin complex with manifestations of the antiphospholipid syndrome and with the presence of lupus anticoagulant. *Arthritis & Rheumatism* 2000; **43**(9): 1982-93.
149. Sciascia S, Sanna G, Murru V, Roccatello D, Khamashta MA, Bertolaccini ML. Anti-prothrombin (aPT) and anti-phosphatidylserine/prothrombin (aPS/PT) antibodies and the risk of thrombosis in the antiphospholipid syndrome. A systematic review. *Thrombosis and haemostasis* 2014; **111**(2): 354-64.
150. Radin M, Foddai SG, Cecchi I, et al. Antiphosphatidylserine/Prothrombin Antibodies: An Update on Their Association with Clinical Manifestations of Antiphospholipid Syndrome. *Thrombosis and Haemostasis* 2020; **120**(4): 592-8.
151. Forastiero R, Martinuzzo M, Pombo G, et al. A prospective study of antibodies to beta2-glycoprotein I and prothrombin, and risk of thrombosis. *Journal of thrombosis and haemostasis* 2005; **3**(6): 1231.
152. Bizzaro N, Ghirardello A, Zampieri S, et al. Anti-prothrombin antibodies predict thrombosis in patients with systemic lupus erythematosus: a 15-year longitudinal study. *Journal of Thrombosis and Haemostasis* 2007; **5**(6): 1158-64.
153. Sakai Y, Atsumi T, Ieko M, et al. The effects of phosphatidylserine-dependent antiprothrombin antibody on thrombin generation. *Arthritis and rheumatism* 2009; **60**(8): 2457-67.
154. Greco TP, Amos MD, Conti-Kelly AM, Naranjo JD, Ijdo JW. Testing for the antiphospholipid syndrome: importance of IgA anti-beta 2-glycoprotein I. *Lupus* 2000; **9**(1): 33-41.
155. de Laat B, van Berkel M, Urbanus RT, et al. Immune responses against domain I of beta(2)-glycoprotein I are driven by conformational changes: domain I of beta(2)-glycoprotein I harbors a cryptic immunogenic epitope. *Arthritis Rheum* 2011; **63**(12): 3960-8.
156. Pengo V, Ruffatti A, Tonello M, et al. Antibodies to Domain 4/5 (Dm4/5) of β2-Glycoprotein 1 (β2GP1) in different antiphospholipid (aPL) antibody profiles. *Thrombosis research* 2015; **136**(1): 161-3.
157. Pengo V. Anti-β2-glycoprotein I antibody testing in the laboratory diagnosis of antiphospholipid syndrome. *Journal of Thrombosis and Haemostasis* 2005; **3**(6): 1158-9.
158. Núñez-Álvarez CA, Hernández-Molina G, Bermúdez-Bermejo P, et al. Prevalence and associations of anti-phosphatidylserine/prothrombin antibodies with clinical phenotypes in patients with primary antiphospholipid syndrome: aPS/PT antibodies in primary antiphospholipid syndrome. *Thrombosis research* 2018; **174**: 141-7.
159. Hoxha A, Mattia E, Tonello M, Grava C, Pengo V, Ruffatti A. Antiphosphatidylserine/prothrombin antibodies as biomarkers to identify severe primary antiphospholipid syndrome. *Clin Chem Lab Med* 2017; **55**(6): 890-8.
160. Shi H, Zheng H, Yin Y-F, et al. Antiphosphatidylserine/prothrombin antibodies (aPS/PT) as potential diagnostic markers and risk predictors of venous thrombosis and obstetric complications in antiphospholipid syndrome. *Clin Chem Lab Med* 2018; **56**(4): 614-24.
161. Sciascia S, Khamashta MA, Bertolaccini ML. New Tests to Detect Antiphospholipid Antibodies: Antiprothrombin (aPT) and Anti-Phosphatidylserine/Prothrombin (aPS/PT) Antibodies. *Current Rheumatology Reports* 2014; **16**(5): 415.
162. Cattini MG, Bison E, Pontara E, Cheng C, Denas G, Pengo V. Tetra positive thrombotic antiphospholipid syndrome: Major contribution of anti-phosphatidyl-serine/prothrombin antibodies to lupus anticoagulant activity. *J Thromb Haemost* 2020.

163. Samarkos M, Davies KA, Gordon C, Walport MJ, Loizou S. IgG subclasses distribution of antibodies against beta(2)-GPI and cardiolipin in patients with systemic lupus erythematosus and primary antiphospholipid syndrome, and their clinical associations. *Rheumatology* 2001; **40**: 1026-32.
164. Andreoli L, Chighizola CB, Banzato A, Pons-estel GJ, Jesus GR, Erkan D. Estimated Frequency of Antiphospholipid Antibodies in Patients With Pregnancy Morbidity, Stroke, Myocardial Infarction, and Deep Vein Thrombosis: A Critical Review of the Literature. *Arthritis Care & Research* 2013; **65**(11): 1869-73.
165. Chighizola CB, Andreoli L, de Jesus GR, Banzato A, Pons-Estel GJ, Erkan D. The association between antiphospholipid antibodies and pregnancy morbidity, stroke, myocardial infarction, and deep vein thrombosis: a critical review of the literature. *Lupus* 2015; **24**(9): 980-4.
166. Ortel TL. Laboratory Diagnosis of the Lupus Anticoagulant. *Current Rheumatology Reports* 2012; **14**(1): 64-70.
167. Pengo V, Tripodi A, Reber G, et al. Update of the guidelines for lupus anticoagulant detection. Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis. *J Thromb Haemost* 2009; **7**(10): 1737-40.
168. Devreese KMJ, Groot PG, Laat B, et al. Guidance from the Scientific and Standardization Committee for lupus anticoagulant/antiphospholipid antibodies of the International Society on Thrombosis and Haemostasis. *Journal of thrombosis and haemostasis* 2020; **18**(11): 2828-39.
169. Tripodi A, Cohen H, Devreese KMJ. Lupus anticoagulant detection in anticoagulated patients. Guidance from the Scientific and Standardization Committee for lupus anticoagulant/antiphospholipid antibodies of the International Society on Thrombosis and Haemostasis. *Journal of thrombosis and haemostasis* 2020; **18**(7): 1569-75.
170. Pennings MTT, de Groot PG, Meijers JCM, Huisman A, Derksen RHWM, Urbanus RT. Optimisation of lupus anticoagulant tests: should test samples always be mixed with normal plasma? *Thrombosis and haemostasis* 2014; **112**(4): 736-42.
171. Adams M. Measurement of Lupus Anticoagulants: An Update on Quality in Laboratory Testing. *Seminars in Thrombosis and Hemostasis* 2013; **39**(3): 267-71.
172. Froom P, Barak M. Testing for lupus anticoagulants – fresh or frozen? *Clin Chem Lab Med* 2012; **50**(9): 1607-9.
173. Arvieux J, Pernod G, Regnault V, Darnige L, Garin J. Some anticardiolipin antibodies recognize a combination of phospholipids with thrombin-modified antithrombin, complement C4b-binding protein, and lipopolysaccharide binding protein. *Blood* 1999; **93**(12): 4248.
174. Devreese KMJ. Standardization of antiphospholipid antibody assays. Where do we stand? *Lupus* 2012; **21**(7): 718-21.
175. Devreese KMJ, Pierangeli SS, de Laat B, Tripodi A, Atsumi T, Ortel TL. Testing for Antiphospholipid antibodies with Solid Phase Assays: guidance from the SSC of the ISTH. *Journal of thrombosis and haemostasis* 2014; **12**(5): 792-5.
176. Zuily S, Cohen H, Isenberg D, et al. Use of direct oral anticoagulants in patients with thrombotic antiphospholipid syndrome: Guidance from the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. *Journal of thrombosis and haemostasis* 2020; **18**(9): 2126-37.
177. Out HJ, van Vliet M, de Groot PG, Derksen RH. Prospective study of fluctuations of lupus anticoagulant activity and anticardiolipin antibody titre in patients with systemic lupus erythematosus. *Annals of the rheumatic diseases* 1992; **51**(3): 353-7.
178. Yelnik CM, Porter TF, Branch DW, et al. Brief Report: Changes in Antiphospholipid Antibody Titers During Pregnancy: Effects on Pregnancy Outcomes. *Arthritis & rheumatology (Hoboken, NJ)* 2016; **68**(8): 1964-9.
179. Kawaja M, Magder L, Goldman D, Petri MA. Loss of antiphospholipid antibody positivity post-thrombosis in SLE. *Lupus Science & Medicine* 2020; **7**: 1-7.
180. Undas A. How to Assess Fibrinogen Levels and Fibrin Clot Properties in Clinical Practice? 2016; **42**(04): 381-8.

181. Carter AM, Cymbalista CM, Spector TD, Grant PJ, Euro CI. Heritability of clot formation, morphology, and lysis: the EuroCLOT study. *Arterioscler Thromb Vasc Biol* 2007; **27**(12): 2783-9.
182. Undas A, Zawilska K, Ciesla-Dul M, et al. Altered fibrin clot structure/function in patients with idiopathic venous thromboembolism and in their relatives. *Blood* 2009; **114**(19): 4272.
183. La Corte ALC, Philippou H, Ariëns RAS. Role of Fibrin Structure in Thrombosis and Vascular Disease. *Adv Protein Chem Struct Biol* 2011; **83**: 75-127.
184. Undas ASA, Ariëns ASR. Fibrin Clot Structure and Function: A Role in the Pathophysiology of Arterial and Venous Thromboembolic Diseases. *Arterioscler Thromb Vasc Biol* 2011; **31**(12): e88-e99.
185. Zabczyk M, Undas A. Plasma fibrin clot structure and thromboembolism: clinical implications. *Polish archives of internal medicine* 2017; **127**(12): 873-81.
186. Lisman T, de Groot PG, Meijers JCM, Rosendaal FR. Reduced plasma fibrinolytic potential is a risk factor for venous thrombosis. *Blood* 2005; **105**(3): 1102.
187. Collet JP, Allali Y, Lesty C, et al. Altered fibrin architecture is associated with hypofibrinolysis and premature coronary atherothrombosis. *Arterioscler Thromb Vasc Biol* 2006; **26**(11): 2567-73.
188. Celinska-Lowenhoff M, Iwaniec T, Padjas A, Musial J, Undas A. Altered fibrin clot structure/function in patients with antiphospholipid syndrome: association with thrombotic manifestation. *Thromb Haemost* 2014; **112**(2): 287-96.
189. Vikerfors A, Svenungsson E, Agren A, et al. Studies of fibrin formation and fibrinolytic function in patients with the antiphospholipid syndrome. *Thromb Res* 2014; **133**(5): 936-44.
190. Stachowicz A, Zabczyk M, Natorska J, et al. Differences in plasma fibrin clot composition in patients with thrombotic antiphospholipid syndrome compared with venous thromboembolism. *Sci Rep* 2018; **8**(1): 17301.
191. Willemse JL, Hendriks DF. Measurement of procarboxypeptidase U (TAFI) in human plasma: a laboratory challenge. *Clin Chem* 2006; **52**(1): 30.
192. Heylen E, Willemse J, Hendriks D. An update on the role of carboxypeptidase U (TAFIa) in fibrinolysis. *Frontiers in bioscience (Landmark edition)* 2011; **16**: 2427.
193. Antovic JP. Thrombin activatable fibrinolysis inhibitor (TAFI) in different hemorrhagic and thrombotic conditions. Institutionen för kirurgisk vetenskap / Department of Surgical Science; 2010.
194. Makris T. Thrombomodulin levels in patients with arterial hypertension. *The American journal of medicine* 1997; **103**(4): 331-2.
195. Takano S, Kimura S, Ohdama S, Aoki N. Plasma Thrombomodulin in Health and Diseases. *Blood* 1990; **76**(10): 2024-9.
196. Mosnier LO, Meijers JC, Bouma BN. Regulation of fibrinolysis in plasma by TAFI and protein C is dependent on the concentration of thrombomodulin. *Thrombosis and haemostasis* 2001; **85**(1): 5-11.
197. Mosnier LO, Elisen MGLM, Bouma BN, Meijers JCM. Protein C inhibitor regulates the thrombin-thrombomodulin complex in the up- and down regulation of TAFI activation. *Thrombosis and Haemostasis* 2001; **86**(4): 1057-64.
198. Foley JH, Kim PY, Mutch NJ, Gils A. Insights into thrombin activatable fibrinolysis inhibitor function and regulation. *J Thromb Haemost* 2013; **11 Suppl 1**: 306-15.
199. Mosnier LO, Bouma BN. Regulation of fibrinolysis by thrombin activatable fibrinolysis inhibitor, an unstable carboxypeptidase B that unites the pathways of coagulation and fibrinolysis. *Arterioscler Thromb Vasc Biol* 2006; **26**(11): 2445-53.
200. Mosnier LO, Von Dem Borne PA, Meijers JC, Bouma BN. Plasma TAFI levels influence the clot lysis time in healthy individuals in the presence of an intact intrinsic pathway of coagulation. *Thrombosis and haemostasis* 1998; **80**(5): 829.
201. Colucci M, Semeraro N. Thrombin activatable fibrinolysis inhibitor: at the nexus of fibrinolysis and inflammation. *Thromb Res* 2012; **129**(3): 314-9.

202. Leung LL, Myles T, Nishimura T, Song JJ, Robinson WH. Regulation of tissue inflammation by thrombin-activatable carboxypeptidase B (or TAFI). *Mol Immunol* 2008; **45**(16): 4080-3.
203. Eichinger S, Schonauer V, Weltermann A, et al. Thrombin-activatable fibrinolysis inhibitor and the risk for recurrent venous thromboembolism. *Blood* 2004; **103**(10): 3773-6.
204. Meltzer ME, Lisman T, de Groot PG, et al. Venous thrombosis risk associated with plasma hypofibrinolysis is explained by elevated plasma levels of TAFI and PAI-1. *Blood* 2010; **116**(1): 113-21.
205. van Tilburg NH, Rosendaal FR, Bertina RM. Thrombin activatable fibrinolysis inhibitor and the risk for deep vein thrombosis. *Blood* 2000; **95**(9): 2855.
206. de Bruijne EL, Gils A, Guimaraes AH, et al. The role of thrombin activatable fibrinolysis inhibitor in arterial thrombosis at a young age: the ATTAC study. *J Thromb Haemost* 2009; **7**(6): 919-27.
207. Malyszko J, Tymcio J. Thrombin activatable fibrinolysis inhibitor and other hemostatic parameters in patients with essential arterial hypertension. *Polskie Archiwum Medycyny Wewnetrznej* 2008; **118**(1-2): 36-41.
208. Meltzer ME, Doggen CJM, de Groot PG, Meijers JCM, Rosendaal FR, Lisman T. Low thrombin activatable fibrinolysis inhibitor activity levels are associated with an increased risk of a first myocardial infarction in men. *Haematologica* 2009; **94**(6): 811.
209. Santamaría CA, Oliver CA, Borrell CM, et al. Risk of Ischemic Stroke Associated With Functional Thrombin-Activatable Fibrinolysis Inhibitor Plasma Levels. *Stroke: Journal of the American Heart Association* 2003; **34**(10): 2387-91.
210. Tregouet DA, Schnabel R, Alessi MC, et al. Activated thrombin activatable fibrinolysis inhibitor levels are associated with the risk of cardiovascular death in patients with coronary artery disease: the AtheroGene study. *J Thromb Haemost* 2009; **7**(1): 49-57.
211. Ringwald J, Buettner S, Zimmermann R, et al. Thrombin-activatable fibrinolysis inhibitor and activated factor XII in patients with systemic lupus erythematosus. Thrombosis research; 2007. p. 129-31.
212. Peters MJL, Nurmohamed MT, van Eijk IC, Verkleij CJN, Marx PF. Thrombin-activatable fibrinolysis inhibitor and its relation with inflammation in rheumatoid arthritis. *Annals of the Rheumatic Diseases* 2009; **68**(7): 1232.
213. Donmez A, Aksu K, Aydın H, et al. The plasma levels of activated thrombin activatable fibrinolysis inhibitor and thrombomodulin in Behçet Disease and their association with thrombosis. *Thrombosis Research* 2010; **126**(3): 207-10.
214. Donmez A, Aksu K, Celik HA, et al. Thrombin activatable fibrinolysis inhibitor in Behçet's disease. *Thrombosis research* 2005; **115**(4): 287-92.
215. Ricart JM, Ramon LA, Vaya A, et al. Fibrinolytic inhibitor levels and polymorphisms in Behcet disease and their association with thrombosis. *British journal of haematology* 2008; **141**(5): 716-9.
216. Martínez-Zamora MA, Creus M, Tassies D, et al. Thrombin activatable fibrinolysis inhibitor and clot lysis time in women with recurrent miscarriage associated with the antiphospholipid syndrome. *Fertility and Sterility* 2010; **94**(6): 2437-40.
217. Martinez-zamora MA, Tassies D, Carmona F, et al. ORIGINAL ARTICLE: Thrombin Activatable Fibrinolysis Inhibitor and Clot Lysis Time in Pregnant Patients with Antiphospholipid Syndrome: Relationship with Pregnancy Outcome and Thrombosis. *American Journal of Reproductive Immunology* 2009; **62**(6): 381-9.
218. Martínez-Zamora MA, Tassies D, Carmona F, et al. Clot lysis time and thrombin activatable fibrinolysis inhibitor in severe preeclampsia with or without associated antiphospholipid antibodies. *Journal of Reproductive Immunology* 2010; **86**(2): 133-40.
219. Ieko M, Yoshida M, Naito S, et al. Increase in plasma thrombin-activatable fibrinolysis inhibitor may not contribute to thrombotic tendency in antiphospholipid syndrome because of inhibitory potential of antiphospholipid antibodies toward TAFI activation. *International Journal of Hematology* 2010; **91**(5): 776-83.

220. Keeling DM, Wilson AJ, Mackie IJ, Isenberg DA, Machin SJ. Role of beta 2-glycoprotein I and anti-phospholipid antibodies in activation of protein C in vitro. *Journal of Clinical Pathology* 1993; **46**(10): 908.
221. Malia RG, Kitchen S, Greaves M, Preston FE. Inhibition of activated protein C and its cofactor protein S by antiphospholipid antibodies. *British Journal of Haematology* 1990; **76**(1): 101-7.
222. Bokarewa IM, Blombäck IM, Egberg IN, Rosén IS. A new variant of interaction between phospholipid antibodies and the protein C system. *Blood Coagul Fibrinolysis* 1994; **5**(1): 37-42.
223. Conroy RM, Pyörälä K, Fitzgerald AP, et al. Estimation of ten-year risk of fatal cardiovascular disease in Europe: the SCORE project. *European Heart Journal* 2003; **24**(11): 987-1003.
224. D'agostino BR, Vasan SR, Pencina JM, et al. General Cardiovascular Risk Profile for Use in Primary Care: The Framingham Heart Study. *Circulation* 2008; **117**(6): 743-53.
225. Stamatelopoulos KS, Kitas GD, Papamichael CM, et al. Atherosclerosis in Rheumatoid Arthritis Versus Diabetes: A Comparative Study. *Arterioscler Thromb Vasc Biol* 2009; **29**(10): 1702-8.
226. Agca R, Heslinga SC, Rollefstad S, et al. EULAR recommendations for cardiovascular disease risk management in patients with rheumatoid arthritis and other forms of inflammatory joint disorders: 2015/2016 update. *Annals of the Rheumatic Diseases* 2017; **76**(1): 17.
227. Sciascia S, Sanna G, Murru V, Roccatello D, Khamashta MA, Bertolaccini ML. GAPSS: the Global Anti-Phospholipid Syndrome Score. *Rheumatology (Oxford)* 2013; **52**(8): 1397-403.
228. Sciascia S, Sanna G, Murru V, Roccatello D, Khamashta MA, Bertolaccini ML. The global anti-phospholipid syndrome score in primary APS. *Rheumatology* 2015; **54**(1): 134-8.
229. Zuily S, de Laat B, Mohamed S, et al. Validity of the global anti-phospholipid syndrome score to predict thrombosis: a prospective multicentre cohort study. *Rheumatology* 2015; **54**(11): 2071-5.
230. Amaya-Amaya J, Rojas-Villarraga A, Anaya J-M. Cardiovascular disease in the antiphospholipid syndrome. *Lupus* 2014; **23**(12): 1288-91.
231. Ambrosino P, Lupoli R, Di Minno A, Iervolino S, Peluso R, Di Minno MN. Markers of cardiovascular risk in patients with antiphospholipid syndrome: a meta-analysis of literature studies. *Annals of medicine* 2014; **46**(8): 693-702.
232. Jara LJ, Medina G, Vera-Lastra O, Shoenfeld Y. Atherosclerosis and antiphospholipid syndrome. *Clinical reviews in allergy & immunology* 2003; **25**(1): 79.
233. Matsuura E, Hughes GR, Khamashta MA. Oxidation of LDL and its clinical implication. *Autoimmun Rev* 2008; **7**(7): 558-66.
234. Matsuura E, Kobayashi K, Lopez L. Atherosclerosis in autoimmune diseases. *Current Rheumatology Reports* 2009; **11**(1): 61-9.
235. Shoenfeld MY, Gerli JR, Doria LA, et al. Accelerated Atherosclerosis in Autoimmune Rheumatic Diseases. *Circulation* 2005; **112**(21): 3337-47.
236. Doria A, Sherer Y, Meroni PL, Shoenfeld Y. Inflammation and Accelerated Atherosclerosis: Basic Mechanisms. *Rheum Dis Clin North Am* 2005; **31**(2): 355-62.
237. Kajiwara T, Yasuda T, Matsuura E. Intracellular trafficking of beta2-glycoprotein I complexes with lipid vesicles in macrophages: implications on the development of antiphospholipid syndrome. *J Autoimmun* 2007; **29**(2-3): 164-73.
238. Zhang H, Li J, Chen A, Liu Q. Binding of glycoprotein β_2-GPI with oxidized low density lipoprotein. *Shengwu gongcheng xuebao* 2017; **33**(1): 122-31.
239. Hasunuma Y, Matsuura E, Makita Z, Katahira T, Nishi S, Koike T. Involvement of β 2 -glycoprotein I and anticardiolipin antibodies in oxidatively modified low-density lipoprotein uptake by macrophages. *Clinical & Experimental Immunology* 1997; **107**(3): 569-73.
240. Soltesz P, Szekanecz Z, Kiss E, Shoenfeld Y. Cardiac manifestations in antiphospholipid syndrome. *Autoimmun Rev* 2007; **6**(6): 379-86.

241. Matsuura E, Kobayashi K, Hurley BL, Lopez LR. Atherogenic Oxidized Low-Density Lipoprotein/β2-Glycoprotein I (oxLDL/β2GPI) Complexes in Patients with Systemic Lupus Erythematosus and Antiphospholipid Syndrome. *Lupus* 2006; **15**(7): 478-83.
242. Wu R, Nityanand S, Berglund L, Lithell H, Holm G, Lefvert AK. Antibodies against cardiolipin and oxidatively modified LDL in 50-year-old men predict myocardial infarction. *Arterioscler Thromb Vasc Biol* 1997; **17**(11): 3159.
243. Hamsten A, Björkholm M, Norberg R, De Faire U, Holm G. Antibodies to Cardiolipin in young survivors of Myocardial Infarction: an association with recurrent cardiovascular events. *The Lancet* 1986; **327**(8473): 113-6.
244. Cortellaro M, Boschetti C, Cardillo M, Barbui T. Antiphospholipid antibodies in patients with previous myocardial infarction. *Lancet (London, England)* 1992; **339**(8798): 929.
245. De Caterina R, Amp, Apos, et al. Prevalence of anticardiolipin antibodies in coronary artery disease. *The American Journal of Cardiology* 1990; **65**(13): 922-3.
246. Diaz MN, Becker RC. Anticardiolipin Antibodies in Patients with Unstable Angina. *Cardiology* 1994; **84**(6): 380-4.
247. Eber B, Kronberger-Schaffer E, Brussee H, et al. Anticardiolipin antibodies are no marker for survived myocardial infarction. *Klin Wochenschr* 1990; **68**(12): 594-6.
248. Klemp P CR, Strauss FJ, Jordaan ER, Przybojewski JZ. Anti-cardiolipin antibodies in ischemic heart disease. *Clin Exp Immunol* 1988; **74**: 254-7.
249. Phadke KV, Phillips RA, Clarke DT, Jones M, Naish P, Carson P. Anticardiolipin antibodies in ischaemic heart disease: marker or myth? *Br Heart J* 1993; **69**(5): 391.
250. Raghavan C, Ditchfield J, Taylor RJ, Haeney MR, Barnes PC. Influence of anticardiolipin antibodies on immediate patient outcome after myocardial infarction. *Journal of Clinical Pathology* 1993; **46**(12): 1113-5.
251. Sletnes KE, Larsen EW, Stokland O, Wisløff F. Antiphospholipid antibodies detected as anticephalin and anticardiolipin antibodies in patients with acute myocardial infarction: Immunological response to myocardial necrosis? *Thrombosis Research* 1990; **59**(3): 675-80.
252. 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. *The Lancet* 1992; **339**(8791): 451.
253. Vaarala O, Mänttäri M, Manninen V, et al. Anti-cardiolipin antibodies and risk of myocardial infarction in a prospective cohort of middle-aged men. *Circulation* 1995; **91**(1): 23.
254. Yilmaz E, Adalet K, Yilmaz G, et al. Importance of serum anticardiolipin antibody levels in coronary heart disease. *Clin Cardiol* 1994; **17**(3): 117.
255. Bili A, Moss AJ, Francis CW, Zareba W, Watelet LF, Sanz I. Anticardiolipin antibodies and recurrent coronary events: a prospective study of 1150 patients. Thrombogenic Factors, and Recurrent Coronary Events Investigators. *Circulation* 2000; **102**(11): 1258.
256. Morton K, Krilis S, Baron D, et al. CORONARY ARTERY BYPASS GRAFT FAILURE—AN AUTOIMMUNE PHENOMENON? *The Lancet* 1986; **328**(8520): 1353-7.
257. Meroni PL, Peyvandi F, Foco L, et al. Anti-beta 2 glycoprotein I antibodies and the risk of myocardial infarction in young premenopausal women. *Journal of Thrombosis and Haemostasis* 2007; **5**(12): 2421-8.
258. Davies JOJ, Hunt BJ. Myocardial infarction in young patients without coronary atherosclerosis: assume primary antiphospholipid syndrome until proved otherwise. *Int J Clin Pract* 2007; **61**(3): 379-84.
259. Urbanus RT, Siegerink B, Roest M, Rosendaal FR, de Groot PG, Algra A. Antiphospholipid antibodies and risk of myocardial infarction and ischaemic stroke in young women in the RATIO study: a case-control study. *Lancet Neurol* 2009; **8**(11): 998-1005.
260. Mattila K, Vaarala O, Palosuo T, et al. Serologic response against cardiolipin and enterobacterial common antigen in young patients with acute myocardial infarction. *Clinical Immunology and Immunopathology* 1989; **51**(3): 414-8.
261. Edwards T, Thomas RD, McHugh N. Anticardiolipin antibodies in ischaemic heart disease. 1993. p. 989-.

262. Adler Y, Finkelstein Y, Zandeman-Goddard G, et al. The presence of antiphospholipid antibodies in acute myocardial infarction. *Lupus* 1995; **4**(4): 309-13.
263. Zuckerman E, Toubi E, Shiran A, et al. Anticardiolipin antibodies and acute myocardial infarction in non-systemic lupus erythmatosus patients: A controlled prospective study. *The American Journal of Medicine* 1996; **101**(4): 381-6.
264. Dropinski J, Szczeklik W, Rubis P, Sydor WJ. Anti-phospholipid antibodies and carotid-artery intima-media thickness in young survivors of myocardial infarction. *Med Sci Monit* 2003; **9**(4): BR105-9.
265. Veres K, Lakos G, Kerényi A, et al. Antiphospholipid antibodies in acute coronary syndrome. *Lupus* 2004; **13**(6): 423-7.
266. Giorgia G, Natalie S, Barbro K, et al. Antiphospholipid antibodies in patients with Myocardial Infarction. *Annals of Internal Medicine* 2018; **169**(8).
267. Grosso G, Sippl N, Kjellström B, et al. Antiphospholipid Antibodies in Patients With Myocardial Infarction. *Annals of Internal Medicine* 2019; **170**(4): 277-80.
268. Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. *British journal of haematology* 2005; **129**(3): 307-21.
269. Lachmann P. Complement before molecular biology. *Molecular immunology* 2006; **43**(6): 496-508.
270. Esmon CT. The interactions between inflammation and coagulation. *Br J Haematol* 2005; **131**(4): 417-30.
271. Esmon CT. Inflammation and thrombosis. *Journal of Thrombosis and Haemostasis* 2003; **1**(7): 1343-8.
272. Amara U, Flierl MA, Rittirsch D, et al. Molecular Intercommunication between the Complement and Coagulation Systems. *The Journal of immunology (1950)* 2010; **185**(9): 5628-36.
273. Amara U, Rittirsch D, Flierl M, et al. Interaction between the coagulation and complement system. *Adv Exp Med Biol* 2008; **632**: 71.
274. Ritis K, Doumas M, Mastellos D, et al. A Novel C5a Receptor-Tissue Factor Cross-Talk in Neutrophils Links Innate Immunity to Coagulation Pathways. *The Journal of Immunology* 2006; **177**(7): 4794-802.
275. Girardi G, Mackman N. Tissue factor in antiphospholipid antibody-induced pregnancy loss: a pro-inflammatory molecule. *Lupus* 2008; **17**(10): 931.
276. Huber-Lang M, Sarma JV, Zetoune FS, et al. Generation of C5a in the absence of C3: a new complement activation pathway. *Nat Med* 2006; **12**(6): 682-7.
277. Mollnes TE, Garred P, Bergseth G. Effect of time, temperature and anticoagulants on in vitro complement activation: consequences for collection and preservation of samples to be examined for complement activation. *Clin Exp Immunol* 1988; **73**(3): 484.
278. Cavazzana I, Nebuloni M, Cetin I, et al. Complement activation in anti-phospholipid syndrome: a clue for an inflammatory process? *J Autoimmun* 2007; **28**(2-3): 160-4.
279. Fischetti F, Durigutto P, Pellis V, et al. Thrombus formation induced by antibodies to beta2-glycoprotein I is complement dependent and requires a priming factor. *Blood* 2005; **106**(7): 2340-6.
280. Girardi G, Berman J, Redecha P, et al. Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *Journal of Clinical Investigation* 2003; **112**(11): 1644-54.
281. Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody–induced fetal loss by inhibiting complement activation. *Nature Medicine* 2004; **10**(11): 1222.
282. Holers VM, Girardi G, Mo L, et al. Complement C3 Activation Is Required for Antiphospholipid Antibody-induced Fetal Loss. *The Journal of Experimental Medicine* 2002; **195**(2): 211-20.
283. Pierangeli SS, Girardi G, Vega-Ostertag M, Liu X, Espinola RG, Salmon J. Requirement of activation of complement C3 and C5 for antiphospholipid antibody-mediated thrombophilia. *Arthritis Rheum* 2005; **52**(7): 2120-4.

284. Pierangeli SS, Vega-ostertag M, Liu X, Girardi G. Complement Activation: A Novel Pathogenic Mechanism in the Antiphospholipid Syndrome. *Annals of the New York Academy of Sciences* 2005; **1051**(1): 413-20.
285. Salmon J. SP0011 Complement Activation and Antiphospholipid Syndrome. *Annals of the Rheumatic Diseases* 2014; **72**(Suppl 3): A4-A.
286. Stewart M EW, Gordon P. Antiphospholipid antibody-dependent C5b-9 formation. *Br J Haematol* 1997; **96**: 451-7.
287. Breen KA, Seed P, Parmar K, Moore GW, Stuart-Smith SE, Hunt BJ. Complement activation in patients with isolated antiphospholipid antibodies or primary antiphospholipid syndrome. *Thrombosis and haemostasis* 2012; **107**(3): 423.
288. Cohen D, Buurma A, Goemaere NN, et al. Classical complement activation as a footprint for murine and human antiphospholipid antibody-induced fetal loss. *J Pathol* 2011; **225**(4): 502-11.
289. Devreese KMJ, Hoylaerts MF. Is there an association between complement activation and antiphospholipid antibody-related thrombosis? *Thrombosis and Haemostasis* 2010; **104**(6): 1279-81.
290. Leffler J, Bengtsson AA, Blom AM. The complement system in systemic lupus erythematosus: an update. *Ann Rheum Dis* 2014; **73**(9): 1601-6.
291. Lim W. Complement and the antiphospholipid syndrome. *Curr Opin Hematol* 2011; **18**(5): 361-5.
292. Oku K, Amengual O, Hisada R, et al. Autoantibodies against a complement component 1 q subcomponent contribute to complement activation and recurrent thrombosis/pregnancy morbidity in anti-phospholipid syndrome. *Rheumatology* 2016; **55**(8): 1403-11.
293. Oku K, Atsumi T, Bohgaki M, et al. Complement activation in patients with primary antiphospholipid syndrome. *Ann Rheum Dis* 2009; **68**(6): 1030-5.
294. Oku K, Nakamura H, Kono M, et al. Complement and thrombosis in the antiphospholipid syndrome. *Autoimmunity Reviews* 2016.
295. Rand JH, Wu XX, Wolgast LR, Lei V, Conway EM. A novel 2-stage approach that detects complement activation in patients with antiphospholipid antibody syndrome. *Thromb Res* 2017; **156**: 119-25.
296. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol* 2010; **11**(9): 785-97.
297. Samarkos M, Mylona E, Kapsimali V. The role of complement in the antiphospholipid syndrome: a novel mechanism for pregnancy morbidity. *Semin Arthritis Rheum* 2012; **42**(1): 66-9.
298. Tedesco F, Borghi MO, Gerosa M, et al. Pathogenic Role of Complement in Antiphospholipid Syndrome and Therapeutic Implications. *Front Immunol* 2018; **9**: 1388.
299. Davis WD, Brey RL. Antiphospholipid antibodies and complement activation in patients with cerebral ischemia. *Clin Exp Rheumatol* 1992; **10**(5): 455-60.
300. Wig S, Chan M, Thachil J, Bruce I, Barnes T. A case of relapsing and refractory catastrophic anti-phospholipid syndrome successfully managed with eculizumab, a complement 5 inhibitor. *Rheumatology* 2016; **55**(2): 382-4.
301. Zhou H, Wolberg AS, Roubey RAS. Characterization of monocyte tissue factor activity induced by IgG antiphospholipid antibodies and inhibition by dilazep. *Blood* 2004; **104**(8): 2353.
302. Peerschke EIB, Yin W, Alpert DR, Roubey RAS, Salmon JE, Ghebrehiwet B. Serum complement activation on heterologous platelets is associated with arterial thrombosis in patients with systemic lupus erythematosus and antiphospholipid antibodies. *Lupus* 2009; **18**(6): 530-8.
303. Navratil JS, Manzi S, Kao AH, et al. Platelet C4d is highly specific for systemic lupus erythematosus. *Arthritis & Rheumatism* 2006; **54**(2): 670-4.
304. Svenungsson E, Gustafsson JT, Grosso G, et al. Complement deposition, C4d, on platelets is associated with vascular events in systemic lupus erythematosus. *Rheumatology (Oxford)* 2020; **59**(11): 3264-74.

305. Svenungsson E, Gustafsson JT, Grosso G, et al. Complement deposition, C4d, on platelets is associated with vascular events in systemic lupus erythematosus. *Rheumatology (Oxford)* 2020.
306. Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. *Nat Rev Immunol* 2009; **9**(10): 729-40.
307. Kim DD, Song W-C. Membrane complement regulatory proteins. *Clinical Immunology* 2006; **118**(2): 127-36.
308. Sjoberg AP, Trouw LA, Blom AM. Complement activation and inhibition: a delicate balance. *Trends Immunol* 2009; **30**(2): 83-90.
309. Hammond A, Rudge AC, Loizou S, Bowcock SJ, Walport MJ. Reduced numbers of complement receptor type 1 on erythrocytes are associated with increased levels of anticardiolipin antibodies. Findings in patients with systemic lupus erythematosus and the antiphospholipid syndrome. *Arthritis & Rheumatism* 1989; **32**(3): 259-64.
310. Xu C, Mao D, Holers VM, Palanca B, Cheng AM, Molina H. A Critical Role for Murine Complement Regulator Crry in Fetomaternal Tolerance. *Science* 2000; **287**(5452): 498-501.
311. Dahlbäck B. C4b-binding protein: a forgotten factor in thrombosis and hemostasis. *Seminars in thrombosis and hemostasis* 2011; **37**(4): 355.
312. Blom AM, Villoutreix BO, Dahlbäck B. Complement inhibitor C4b-binding protein—friend or foe in the innate immune system? *Molecular Immunology* 2004; **40**(18): 1333-46.
313. Dahlbäck B. Protein S and C4b-binding protein: components involved in the regulation of the protein C anticoagulant system. *Thrombosis and haemostasis* 1991; **66**(1): 49.
314. Ermert D, Blom AM. C4b-binding protein: The good, the bad and the deadly. Novel functions of an old friend. *Immunology Letters* 2016; **169**: 82-92.
315. García de Frutos P, Alim RI, Härdig Y, Zöller B, Dahlbäck B. Differential regulation of alpha and beta chains of C4b-binding protein during acute-phase response resulting in stable plasma levels of free anticoagulant protein S. *Blood* 1994; **84**(3): 815.
316. Hessing M. The interaction between complement component C4b-binding protein and the vitamin K-dependent protein S forms a link between blood coagulation and the complement system. *The Biochemical journal* 1991; **277 (Pt 3)**: 581.
317. Hessing M, Vlooswijk RA, Hackeng TM, Kanters D, Bouma BN. The localization of heparin-binding fragments on human C4b-binding protein. *Journal of immunology (Baltimore, Md : 1950)* 1990; **144**(1): 204.
318. Nishioka J, Suzuki K. Inhibition of cofactor activity of protein S by a complex of protein S and C4b-binding protein. Evidence for inactive ternary complex formation between protein S, C4b-binding protein, and activated protein C. *The Journal of biological chemistry* 1990; **265**(16): 9072.
319. Schwalbe R, Dahlbäck B, Hillarp A, Nelsestuen G. Assembly of protein S and C4b-binding protein on membranes. *The Journal of biological chemistry* 1990; **265**(27): 16074.
320. van de Poel RHL, Meijers JCM, Rosing J, Tans G, Bouma BN. C4b-Binding Protein Protects Coagulation Factor Va from Inactivation by Activated Protein C †. *Biochemistry* 2000; **39**(47): 14543-8.
321. Webb JH, Blom AM, Dahlbäck B. Vitamin K-dependent protein S localizing complement regulator C4b-binding protein to the surface of apoptotic cells. *Journal of immunology (Baltimore, Md : 1950)* 2002; **169**(5): 2580.
322. Buil A, Trégouët D-A, Souto JC, et al. C4BPB/C4BPA is a new susceptibility locus for venous thrombosis with unknown protein S-independent mechanism: results from genome-wide association and gene expression analyses followed by case-control studies. *Blood* 2010; **115**(23): 4644.
323. Maurissen LFA, Thomassen MCLGD, Nicolaes GAF, et al. Re-evaluation of the role of the protein S-C4b binding protein complex in activated protein C-catalyzed factor Va-inactivation. *Blood* 2008; **111**(6): 3034.
324. Trouw LA, Bengtsson AA, Gelderman KA, Dahlbäck B, Sturfelt G, Blom AM. C4b-binding protein and factor H compensate for the loss of membrane-bound complement inhibitors

to protect apoptotic cells against excessive complement attack. *The Journal of biological chemistry* 2007; **282**(39): 28540.
325. Trouw LA, Nilsson SC, Gonçalves I, Landberg G, Blom AM. C4b-binding protein binds to necrotic cells and DNA, limiting DNA release and inhibiting complement activation. *The Journal of Experimental Medicine* 2005; **201**(12): 1937-48.
326. Carroll MV, Sim RB. Complement in health and disease. *Adv Drug Deliv Rev* 2011; **63**(12): 965-75.
327. Nelson RM, Long GL. Binding of protein S to C4b-binding protein. *The Journal of biological chemistry* 1992; **267**(12): 8140-5.
328. Mohlin FC, Mercier E, Fremeaux-Bacchi V, et al. Analysis of genes coding for CD46, CD55, and C4b-binding protein in patients with idiopathic, recurrent, spontaneous pregnancy loss. *European journal of immunology* 2013; **43**(6): 1617.
329. Idborg H, Zandian A, Sandberg A-S, et al. Two subgroups in systemic lupus erythematosus with features of antiphospholipid or Sjögren's syndrome differ in molecular signatures and treatment perspectives. *Arthritis research & therapy* 2019; **21**(1): 62.
330. Barnum SR, Dahlbäck B. C4b-Binding Protein, a Regulatory Component of the Classical Pathway of Complement, Is an Acute-Phase Protein and Is Elevated in Systemic Lupus Erythematosus. *Complement Inflamm* 1990; **7**(2): 71-7.
331. Forastiero RR, Kordich L, Basilotta E, Carreras LO. Differences in protein S and C4b-binding protein levels in different groups of patients with antiphospholipid antibodies. *Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis* 1994; **5**(4): 609.
332. Gropp K, Weber N, Reuter M, et al. β_2-glycoprotein I, the major target in antiphospholipid syndrome, is a special human complement regulator. *Blood* 2011; **118**(10): 2774.
333. Agar C, de Groot PG, Morgelin M, et al. beta(2)-glycoprotein I: a novel component of innate immunity. *Blood* 2011; **117**(25): 6939-47.
334. Nimpf J, Wurm H, Kostner GM. β 2-glycoprotein-I (apo-H) inhibits the release reaction of human platelets during ADP-induced aggregation. *Atherosclerosis* 1987; **63**(2): 109-14.
335. Schousboe I. beta 2-Glycoprotein I: a plasma inhibitor of the contact activation of the intrinsic blood coagulation pathway. *Blood* 1985; **66**(5): 1086.
336. Yasuda S, Atsumi T, Ieko M, et al. Nicked beta2-glycoprotein I: a marker of cerebral infarct and a novel role in the negative feedback pathway of extrinsic fibrinolysis. *Blood* 2004; **103**(10): 3766-72.
337. Brighton TA, Hogg PJ, Dai YP, Murray BH, Chong BH, Chesterman CN. Beta 2 -glycoprotein I in thrombosis: evidence for a role as a natural anticoagulant. *British Journal of Haematology* 1996; **93**(1): 185-94.
338. Atsumi T, Khamashta M, Ames P, Ichikawa K, Koike T, Hughes G. Effect of beta 2-glycoprotein I and human monoclonal anticardiolipin antibody on the protein S/C4b-binding protein system. *Lupus* 1997; **6**(4): 358-64.
339. Merrill J, T., Zhang HW, Shen C, et al. Enhancement of Protein S Anticoagulant Function by beta 2-glycoprotein I, a Major Target Antigen of Antiphospholipid Antibodies. *Thrombosis and Haemostasis* 1999; **81**(5): 748-57.
340. Guerin J SR, Feighery C, Jackson J. Antibody recognition of complement regulatory proteins, factor H, CR1 and C4BP in antiphospholipid syndrome patients. *Lupus* 1998; **A031:S180**.
341. Parke AL, Weinstein RE, Bona RD, Maier DB, Walker FJ. The thrombotic diathesis associated with the presence of phospholipid antibodies may be due to low levels of free protein S. *The American Journal of Medicine* 1992; **93**(1): 49-56.
342. Bertina R, van Wijngaarden A, Reinalda-Poot J, Poort S, Bom V. Determination of plasma protein S: the protein cofactor of activated protein C. *Thromb Haemost* 1985; **53**(2): 268-72.
343. Rossi E, Gatti L, Cossu MM, Preda L, Finotto E, Gianotti GA. Protein S in patients with antiphospholipid antibodies syndrome. 1991. p. 67-.
344. Ruíz-Argüelles GJ, Ruíz-Argüelles A, Alarcón-Segovia D, et al. Natural anticoagulants in systemic lupus erythematosus. Deficiency of protein S bound to C4bp associates with recent

history of venous thromboses, antiphospholipid antibodies, and the antiphospholipid syndrome. *The Journal of rheumatology* 1991; **18**(4): 552.
345. Crowther MA, Johnston M, Weitz J, Ginsberg JS. Free protein S deficiency may be found in patients with antiphospholipid antibodies who do not have systemic lupus erythematosus. *Thrombosis and haemostasis* 1996; **76**(5): 689.
346. Ginsberg JS, Demers C, Brill-Edwards P, et al. Acquired free protein S deficiency is associated with antiphospholipid antibodies and increased thrombin generation in patients with systemic lupus erythematosus. *The American Journal of Medicine* 1995; **98**(4): 379-83.
347. D'Angelo A, Vigano-D'Angelo S, Esmon CT, Comp PC. Acquired deficiencies of protein S. Protein S activity during oral anticoagulation, in liver disease, and in disseminated intravascular coagulation. *The Journal of clinical investigation* 1988; **81**(5): 1445.
348. Takahashi H, Tatewaki W, Wada K, Shibata A. Plasma protein S in disseminated intravascular coagulation, liver disease, collagen disease, diabetes mellitus, and under oral anticoagulant therapy. *Clin Chim Acta* 1989; **182**(2): 195-208.
349. Takahashi H, Wada K, Hayashi S, Hanano M, Tatewaki W, Shibata A. Behavior of protein S during long-term oral anticoagulant therapy. *Thrombosis Research* 1988; **51**(3): 241-9.
350. Fair DS, Revak DJ. Quantitation of human Protein S in the plasma of normal and warfarin-treated individuals by radioimmunoassay. *Thrombosis Research* 1984; **36**(6): 527-35.
351. Weiss P, Soff GA, Halkin H, Seligsohn U. Decline of proteins C and S and factors II, VII, IX and X during the initiation of warfarin therapy. *Thrombosis Research* 1987; **45**(6): 783-90.
352. Oosting JD, 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**(10): 2618.
353. Vermylen J, Arnout J. Is the antiphospholipid syndrome caused by antibodies directed against physiologically relevant phospholipid-protein complexes ? *The Journal of laboratory and clinical medicine* 1992; **120**(1): 10-2.
354. Rossetto V, Spiezia L, Franz F, et al. The role of antiphospholipid antibodies toward the protein C/protein S system in venous thromboembolic disease. *Am J Hematol* 2009; **84**(9): 594-6.
355. Nutescu EA, Burnett A, Fanikos J, Spinler S, Wittkowsky A. Erratum to: Pharmacology of anticoagulants used in the treatment of venous thromboembolism. *J Thromb Thrombolysis* 2016; **42**(2): 296-311.
356. Gross P, Ecker EE. Anticomplementary Action of Purified Heparin. *Proc Soc Exp Biol Med* 1929; **26**(6): 508-9.
357. Kazatchkine MD, Fearon DT, Metcalfe DD, Rosenberg RD, Austen KF. Structural determinants of the capacity of heparin to inhibit the formation of the human amplification C3 convertase. *The Journal of clinical investigation* 1981; **67**(1): 223.
358. Weiler JM, Edens RE, Linhardt RJ, Kapelanski DP. Heparin and modified heparin inhibit complement activation in vivo. *Journal of immunology (Baltimore, Md : 1950)* 1992; **148**(10): 3210.
359. Hansson K, Stenflo J. Post-translational modifications in proteins involved in blood coagulation. *Journal of thrombosis and haemostasis* 2005; **3**(12): 2633-48.
360. Zöller B, García de Frutos P, Dahlbäck B. Evaluation of the relationship between protein S and C4b-binding protein isoforms in hereditary protein S deficiency demonstrating type I and type III deficiencies to be phenotypic variants of the same genetic disease. *Blood* 1995; **85**(12): 3524.
361. Arachchillage DR, Mackie IJ, Efthymiou M, et al. Rivaroxaban limits complement activation compared with warfarin in antiphospholipid syndrome patients with venous thromboembolism. *J Thromb Haemost* 2016.
362. Tektonidou MG, Andreoli L, Limper M, et al. EULAR recommendations for the management of antiphospholipid syndrome in adults. *Ann Rheum Dis* 2019; **78**(10): 1296-304.
363. Napoleone E, Santo AD, Camera M, Tremoli E, Lorenzet R. Angiotensin-Converting Enzyme Inhibitors Downregulate Tissue Factor Synthesis in Monocytes. *Circulation Research* 2000; **86**(2): 139-43.

364. Ferrara DE, Swerlick R, Casper K, et al. Fluvastatin inhibits up-regulation of tissue factor expression by antiphospholipid antibodies on endothelial cells. *Journal of Thrombosis and Haemostasis* 2004; **2**(9): 1558-63.
365. Meroni PL, Raschi E, Testoni C, et al. Statins prevent endothelial cell activation induced by antiphospholipid (anti–β 2 -glycoprotein I) antibodies: Effect on the proadhesive and proinflammatory phenotype. *Arthritis & Rheumatism* 2001; **44**(12): 2870-8.
366. Steiner S, Speidl WS, Pleiner J, et al. Simvastatin blunts endotoxin-induced tissue factor in vivo. *Circulation* 2005; **111**(14): 1841-6.
367. Tripodi A, de Laat B, Wahl D, Ageno W, Cosmi B, Crowther M. Monitoring patients with the lupus anticoagulant while treated with vitamin K antagonists: communication from the SSC of the ISTH. *Journal of thrombosis and haemostasis* 2016; **14**(11): 2304-7.
368. Pengo V, Denas G, Zoppellaro G, et al. Rivaroxaban vs warfarin in high-risk patients with antiphospholipid syndrome. *Blood* 2018; **132**(13): 1365-71.
369. Ordi-Ros J, Saez-Comet L, Perez-Conesa M, et al. Rivaroxaban Versus Vitamin K Antagonist in Antiphospholipid Syndrome: A Randomized Noninferiority Trial. *Ann Intern Med* 2019.
370. Haładyj E, Olesińska M. Rivaroxaban - a safe therapeutic option in patients with antiphospholipid syndrome? Our experience in 23 cases. *Reumatologia* 2016; **54**(3): 146-9.
371. Malec K, Góralczyk T, Undas A. The use of direct oral anticoagulants in 56 patients with antiphospholipid syndrome. *Thrombosis research* 2017; **152**: 93-7.
372. Signorelli F, Nogueira F, Domingues V, Mariz HA, Levy RA. Thrombotic events in patients with antiphospholipid syndrome treated with rivaroxaban: a series of eight cases. *Clin Rheumatol* 2016; **35**(3): 801-5.
373. Dufrost V, Wahl D, Zuily S. Direct oral anticoagulants in antiphospholipid syndrome: Meta-analysis of randomized controlled trials. *Autoimmunity reviews* 2021; **20**(1): 102711.
374. Crowley MP, Cuadrado MJ, Hunt BJ. Catastrophic antiphospholipid syndrome on switching from warfarin to rivaroxaban. *Thrombosis research* 2017; **153**: 37-9.
375. Cohen H. Rivaroxaban versus warfarin to treat patients with thrombotic antiphospholipid syndrome. Dr. Hannah Cohen about the results of the RAPS trial (Lancet Haematol 2016; 3: e426-36). *Rheumatology (Oxford)* 2017; **56**(9): e23-e.
376. Cohen H, Doré CJ, Clawson S, et al. Rivaroxaban in antiphospholipid syndrome (RAPS) protocol: a prospective, randomized controlled phase II/III clinical trial of rivaroxaban versus warfarin in patients with thrombotic antiphospholipid syndrome, with or without SLE. *Lupus* 2015; **24**(10): 1087-94.
377. Cohen H, Hunt BJ, Efthymiou M, et al. Rivaroxaban versus warfarin to treat patients with thrombotic antiphospholipid syndrome, with or without systemic lupus erythematosus (RAPS): a randomised, controlled, open-label, phase 2/3, non-inferiority trial. *The Lancet Haematology* 2016; **3**(9): e426-e36.
378. Cohen H, Hunt BJ, Efthymiou M, Mackie IJ, Khamashta M, Isenberg DA. Direct Oral Anticoagulants Use in Antiphospholipid Syndrome: Are These Drugs an Effective and Safe Alternative to Warfarin? A Systematic Review of the Literature: Comment. *Current Rheumatology Reports* 2017; **19**(8): 1-2.
379. Giles I, Khamashta M, D'Cruz D, Cohen H. A new dawn of anticoagulation for patients with antiphospholipid syndrome? *Lupus* 2012; **21**(12): 1263-5.
380. Pengo V, Banzato A, Bison E, Zoppellaro G, Padayattil Jose S, Denas G. Efficacy and safety of rivaroxaban vs warfarin in high-risk patients with antiphospholipid syndrome: Rationale and design of the Trial on Rivaroxaban in AntiPhospholipid Syndrome (TRAPS) trial. *Lupus* 2016; **25**(3): 301-6.
381. Pengo V, Hoxha A, Andreoli L, et al. Trial of Rivaroxaban in AntiPhospholipid Syndrome (TRAPS): Two-year outcomes after the study closure. *Journal of thrombosis and haemostasis* 2021; **19**(2): 531-5.
382. Andreoli L, Bertsias GK, Agmon-Levin N, et al. EULAR recommendations for women's health and the management of family planning, assisted reproduction, pregnancy and menopause

in patients with systemic lupus erythematosus and/or antiphospholipid syndrome. *Annals of the Rheumatic Diseases* 2017; **76**(3): 476-85.
383. Vargas-Hitos JA, Ateka-Barrutia O, Sangle S, Khamashta MA. Efficacy and safety of long-term low molecular weight heparin in patients with antiphospholipid syndrome. *Ann Rheum Dis* 2011; **70**(9): 1652-4.
384. Rodriguez P, Santacreu, Cervera, Espinosa. What is the best strategy in treating catastrophic antiphospholipid syndrome? *International journal of clinical rheumatology* 2015; **10**(4): 245-55.
385. Lonze BE, Zachary AA, Magro CM, et al. Eculizumab prevents recurrent antiphospholipid antibody syndrome and enables successful renal transplantation. *Am J Transplant* 2014; **14**(2): 459-65.
386. Lonze BE SA, Montgomery RA. Eculizumab and renal transplantation in a patient with CAPS. *N Engl J Med* 2010; **362**: 1744-5.
387. Shapira I, Andrade D, Allen SL, Salmon JE. Brief report: induction of sustained remission in recurrent catastrophic antiphospholipid syndrome via inhibition of terminal complement with eculizumab. *Arthritis Rheum* 2012; **64**(8): 2719-23.
388. Strakhan M, Hurtado-Sbordoni M, Galeas N, Bakirhan K, Alexis K, Elrafei T. 36-year-old female with catastrophic antiphospholipid syndrome treated with eculizumab: a case report and review of literature. *Case Rep Hematol* 2014; **2014**: 704371.
389. Zikos TA, Sokolove J, Ahuja N, Berube C. Eculizumab Induces Sustained Remission in a Patient With Refractory Primary Catastrophic Antiphospholipid Syndrome. *Journal of clinical rheumatology : practical reports on rheumatic & musculoskeletal diseases* 2015; **21**(6): 311-3.
390. Erkan D, Salmon JE. The Role of Complement Inhibition in Thrombotic Angiopathies and Antiphospholipid Syndrome. *Turkish journal of haematology : official journal of Turkish Society of Haematology* 2016; **33**(1): 1.
391. Tinti MG, Carnevale V, Inglese M, et al. Eculizumab in refractory catastrophic antiphospholipid syndrome: a case report and systematic review of the literature. *Clin Exp Med* 2019; **19**(3): 281-8.
392. Ryden L, Buhlin K, Ekstrand E, et al. Periodontitis Increases the Risk of a First Myocardial Infarction: A Report From the PAROKRANK Study. *Circulation* 2016; **133**(6): 576-83.
393. Hay EM, Bacon PA, Gordon C, et al. The BILAG index : a reliable and valid instrument for measuring clinical disease activity in systemic lupus erythematosus. *Q J Med* 1993; **86**(7): 447-58.
394. He S, Cao H, Antovic A, Blombäck M. Modifications of flow measurement to determine fibrin gel permeability and the preliminary use in research and clinical materials. *Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis* 2005; **16**(1): 61.
395. Antovic A. The overall hemostasis potential: a laboratory tool for the investigation of global hemostasis. *Semin Thromb Hemost* 2010; **36**(7): 772-9.
396. Carr ME, Shen LL, Hermans J. Mass–length ratio of fibrin fibers from gel permeation and light scattering. *Biopolymers* 1977; **16**(1): 1-15.
397. Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006; **4**(2): 295-306.
398. Gustafsson JT, Herlitz Lindberg M, Gunnarsson I, et al. Excess atherosclerosis in systemic lupus erythematosus,-A matter of renal involvement: Case control study of 281 SLE patients and 281 individually matched population controls. *PLoS One* 2017; **12**(4): e0174572.
399. Sandholm K, Persson B, Skattum L, et al. Evaluation of a Novel Immunoassay for Quantification of C1q for Clinical Diagnostic Use.(Report). *Frontiers in Immunology* 2019; **10**.
400. Johnson U, Truedsson L, Gustavii B. Complement components in 100 newborns and their mothers determined by electroimmunoassay. *Acta pathologica, microbiologica et immunologica Scandinavica Section C Immunology* 1983; **91**(2): 147.
401. Hemker HC, Giesen P, AlDieri R, et al. The Calibrated Automated Thrombogram (CAT): a universal routine test for hyper- and hypocoagulability. *Pathophysiology of Haemostasis and Thrombosis* 2002; **32**(5–6): 249-53.

402. Ekdahl KN, Nilsson B, Pekna M, Nilsson UR. Generation of iC3 at the Interface between Blood and Gas. *Scand J Immunol* 1992; **35**(1): 85-91.
403. Vanderweele TJ, Vansteelandt S. Conceptual issues concerning mediation, interventions and composition. *Statistics and Its Interface* 2009; **2**(4): 457-68.
404. Weiler H, Isermann BH. Thrombomodulin. Oxford, UK; 2003. p. 1515-24.
405. Kelchtermans H, Pelkmans L, de Laat B, Devreese KM. IgG/IgM antiphospholipid antibodies present in the classification criteria for the antiphospholipid syndrome: a critical review of their association with thrombosis. *J Thromb Haemost* 2016; **14**(8): 1530-48.
406. Vikerfors A, Johansson A-B, Gustafsson JT, et al. Clinical manifestations and anti-phospholipid antibodies in 712 patients with systemic lupus erythematosus: evaluation of two diagnostic assays. 2013. p. 345.
407. Grosso G, Sandholm K, Antovic A, et al. The Complex Relationship between C4b-Binding Protein, Warfarin, and Antiphospholipid Antibodies. *Thrombosis and haemostasis* 2021.
408. Hamsten A, Norberg R, Bjorkholm M, de Faire U, Holm G. Antibodies to cardiolipin in young survivors of myocardial infarction: an association with recurrent cardiovascular events. *Lancet* 1986; **1**(8473): 113-6.
409. Vaarala O, Mänttäri M, Manninen V, et al. Anti-Cardiolipin Antibodies and Risk of Myocardial Infarction in a Prospective Cohort of Middle-Aged Men. *Circulation* 1995; **91**: 23-7.
410. Zuckerman E, Toubi E, Shiran A, et al. Anticardiolipin antibodies and acute myocardial infarction in non-systemic lupus erythmatosus patients: a controlled prospective study. *Am J Med* 1996; **101**(4): 381-6.
411. Dropinski J, Szczeklik W, Rubis P, Sydor WJ. Anti-phospholipid antibodies and carotid-artery intima-media thickness in young survivors of myocardial infarction. *Med Sci Monit* 2003; **9**(4): BR105-9.