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Studies of clinical and haemostatic features associated with antiphospholipid antibodies

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ABSTRACT

Background: Antiphospholipid antibodies (aPL) are associated with a heterogeneous range of clinical conditions with predisposition for thrombosis, pregnancy morbidity and other autoimmune disorders, most frequently systemic lupus erythematosus (SLE). Both the clinical manifestations themselves and their treatment may be harmful to the affected individuals and aPL testing is still controversial. There is thus an urgent need for improved aPL diagnostics and individual risk assessments. Exploring the largely unknown disease aetiology may provide clues to new biomarkers. From previous studies of other pro-thrombotic conditions, we hypothesized that fibrin clot properties, fibrinolytic function, microparticle (MP) profile and smoking could be important in aPL-related disease.

Aims: To study clinical, serological and haemostatic features of the antiphospholipid syndrome (APS) and aPL-related disorders, including SLE, in order to improve diagnostic methods and enhance understanding of disease pathogenesis.

Materials and methods: Two patient groups were cross-sectionally studied. In paper I, IV, and V, the Karolinska SLE cohort was studied; in paper I together with patients from the Swedish SLE network (n=712 patients) and in paper IV in comparison with matched population controls (n=290 patients). In paper II and III, APS patients (n=49 and 52 patients, respectively) from Karolinska University Hospital were compared both to healthy controls (paper II and III) and to non-APS thrombotic controls (paper II). Laboratory assays included: different ELISAs for aPL detection, liquid permeation technique and scanning electron microscopy (SEM) for examination of fibrin network permeability and structure, turbidimetric clotting and lysis assays, and finally MP measurement by flow cytometry.

Results: In paper I, we report only moderate agreement (Kappa-values 0.16-0.71) but similar, modest association with previous thrombotic events when comparing a new automated aPL method with standard assays in SLE patients. Antibody isotype and titer influenced the association with clinical events. Odds ratios for lupus anticoagulant (LA) for the associations with vascular events were generally higher than for the specific aPL immunoassays. In paper II, we report that fibrin clots formed in vitro in samples from APS patients have a decreased permeability compared to the clots formed in samples from healthy controls and non-APS thrombosis controls (p<0.0001 for both). SEM images visually confirmed denser fibrin structure in APS. No clear difference in fibrinolysis function between the APS patients and controls were observed (p>0.05 for two of the three investigated parameters) but APS patients with previous arterial thrombosis had prolonged clot lysis times compared to the controls (p<0.05). In paper III, we report that the number of lactadherin positive MPs, endothelial MPs, Tissue factor (TF)-positive endothelial MPs and monocyte MPs in APS samples were increased compared to healthy controls (p<0.001 for all analyses). There was no significant difference in the number of platelet MPs (p=0.13). APS patients had a high proportion of MPs positive to lactadherin. In paper IV, we report that the number of lactadherin positive MPs, endothelial MPs, platelet MPs and leukocyte MPs in SLE samples were increased compared to matched population controls (p<0.0001 for all analyses). Moreover, MPs exposing inflammation and/or activation markers such as CD40L, TF, vascular cell adhesion molecule 1 (VCAM-1), high-mobility group protein B1 (HMGB1) and C4d also were also clearly increased in the SLE patients (p<0.0001 for all analyses). However, MP number could neither be used to distinctively discriminate between SLE patients with or without APS nor between SLE patients with or without previous vascular events in multivariable models (p-values for all analyses >0.05 except for VCAM-1-positive endothelial MPs which had p-values of 0.044 and 0.047 for positive associations in the vascular event models). In paper V we report a positive association between cigarette smoking (ever smoking and, most strikingly, former smoking) and the presence of aP among patients with SLE (OR = 3 for aPL of the IgG isotype and for LA and former smoking vs. never smoking in a multivariable model). A positive interaction between ever smoking and aPL was noted for the association with previous vascular events. The additive interaction analysis demonstrated a significant interaction between ever smoking and LA (attributable proportion due to interaction, AP=0.80, 95% CI 0.5 - 1.0) and ever smoking and triple aPL positivity (AP=0.85, 95% CI 0.6 - 1.0) for the association with presence of previous vascular events.

Conclusions: Automated aPL assays are promising for facilitating aPL testing. However, it is crucial to also evaluate LA positivity, antibody titers and isotypes together with smoking status when aPL results are used for risk assessment in individual patients. Smoking, abnormal fibrin clot properties, fibrinolytic function, and MP profile may contribute to the pathogenesis of aPL-associated disease. MP formation is likely to play a role in SLE as well, which is a disorder closely related to APS. However, the exact relationship between aPL, vascular events and MPs in SLE patients needs to be further studied. Further mechanistic studies are needed to address how the different variables studied in this thesis are related to the pathogenesis of aPL-associated disease. The findings and conclusions in this thesis also need to be prospectively studied to clarify their prognostic value. With such additional studies, our observations could guide the search for new biomarkers and risk scores in aPL-related disease.
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<tr>
<td>AAE</td>
<td>Any arterial event</td>
</tr>
<tr>
<td>aAnAV</td>
<td>Anti-annexin V</td>
</tr>
<tr>
<td>aCL</td>
<td>Anticardiolipin</td>
</tr>
<tr>
<td>ACR</td>
<td>American College of Rheumatology</td>
</tr>
<tr>
<td>AM</td>
<td>Automated method</td>
</tr>
<tr>
<td>ANA</td>
<td>Antinuclear antibody</td>
</tr>
<tr>
<td>Anti-dsDNA</td>
<td>Anti-double-stranded DNA</td>
</tr>
<tr>
<td>Anti-Sm</td>
<td>Anti-Smith</td>
</tr>
<tr>
<td>anti-β₂GPI</td>
<td>Anti-β₂ glycoprotein-I</td>
</tr>
<tr>
<td>APC</td>
<td>Activated protein C</td>
</tr>
<tr>
<td>aPL</td>
<td>Antiphospholipid antibodies</td>
</tr>
<tr>
<td>APS</td>
<td>Antiphospholipid syndrome</td>
</tr>
<tr>
<td>APTT</td>
<td>Activated partial thromboplastin time</td>
</tr>
<tr>
<td>ASA</td>
<td>Acetylsalicylic acid</td>
</tr>
<tr>
<td>AVE</td>
<td>Any vascular event</td>
</tr>
<tr>
<td>CAPS</td>
<td>Catastrophic antiphospholipid syndrome</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>dRVVT</td>
<td>dilute Russell’s viper venom time</td>
</tr>
<tr>
<td>DVT</td>
<td>Deep venous thrombosis</td>
</tr>
<tr>
<td>E-selectin</td>
<td>Endothelial Selectin</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMPs</td>
<td>Endothelial microparticles</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<tr>
<td>ErMPs</td>
<td>Erythrocyte microparticles</td>
</tr>
<tr>
<td>HCs</td>
<td>Healthy controls</td>
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<tr>
<td>HMGB1</td>
<td>High-mobility group protein B1</td>
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<tr>
<td>hsCRP</td>
<td>High-sensitivity C-reactive protein</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>ICVD</td>
<td>Ischemic cerebrovascular disease</td>
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<tr>
<td>IHD</td>
<td>Ischemic heart disease</td>
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<tr>
<td>INR</td>
<td>International normalized ratio</td>
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<tr>
<td>IPVD</td>
<td>Ischemic peripheral vascular disease</td>
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<tr>
<td>LA</td>
<td>Lupus anticoagulant</td>
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<tr>
<td>LE cell</td>
<td>Lupus erythematosus cell</td>
</tr>
<tr>
<td>LMPs</td>
<td>Leukocyte microparticles</td>
</tr>
<tr>
<td>LR</td>
<td>Livedo reticularis</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MMPs</td>
<td>Monocyte microparticles</td>
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<tr>
<td>MPL/GPL</td>
<td>IgM/IgG phospholipid units</td>
</tr>
<tr>
<td>MPs</td>
<td>Microparticles</td>
</tr>
<tr>
<td>mTORC</td>
<td>Mammalian target of rapamycin complex</td>
</tr>
<tr>
<td>Non-APS TCs</td>
<td>Non-APS thrombotic controls</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PE</td>
<td>Pulmonary embolism</td>
</tr>
<tr>
<td>PMPs</td>
<td>Platelet microparticles</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PT</td>
<td>Prothrombin</td>
</tr>
<tr>
<td>RF</td>
<td>Reumatoid factor</td>
</tr>
<tr>
<td>RFL</td>
<td>Recurrent fetal loss</td>
</tr>
<tr>
<td>RM</td>
<td>Routine method</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SVT</td>
<td>Superficial vein thrombosis</td>
</tr>
<tr>
<td>TAFI</td>
<td>Thrombin-activatable fibrinolysis inhibitor</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>TIA</td>
<td>Transient ischemic attack</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VLDR</td>
<td>Venereal Disease Research Laboratory</td>
</tr>
<tr>
<td>VTE</td>
<td>Venous thromboembolism</td>
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</table>
1 BACKGROUND: WHY ARE STUDIES OF ANTIPHOSPHOLIPID ANTIBODIES (APLS) AND ANTIPHOSPHOLIPID SYNDROME (APS) URGENT?

1.1 APS is a relatively “new” disease with recently updated criteria

The antiphospholipid syndrome (APS), first described in the 1980s (1, 2), is characterized by thrombosis or obstetric morbidity (clinical criteria) in conjunction with the presence of antiphospholipid antibodies (aPL) (laboratory criteria). At least one clinical and one laboratory criterion are required for APS classification (see Figure 1). Thrombotic manifestations can affect any vessel including the arterial, venous or microvascular circulation. Obstetric morbidity includes miscarriages, premature birth, and foetal death. The laboratory criterion is fulfilled through persistent positivity (minimum of two positive tests taken at least 12 weeks apart) for either antibodies against cardiolipin of IgG or IgM isotype (IgG aCL, IgM aCL) or antibodies against the phospholipid binding cofactor β₂ glycoprotein-I of IgG or IgM isotype (IgG anti-β₂GPI, IgM anti-β₂GPI) detected by enzyme-linked immunosorbent assays (ELISA) or the functional Lupus anticoagulant (LA) test. At least 12 weeks and no more than 5 years should separate the clinical event and the positive test. Furthermore, for study purposes, it is recommended that APS patients are subclassified into one of the following categories: I, more than one laboratory criteria present; IIa, LA alone present; IIb, aCL alone present; IIc, anti-β₂GPI alone present (3).

2006 Classification criteria for Antiphospholipid syndrome (APS) 
” the Sydney criteria”

Clinical manifestations
- Vascular thrombosis
  One or more clinical episodes of arterial, venous, or small vessel thrombosis in any tissue or organ.
- Pregnancy morbidity
  a. One or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation
  b. One or more premature births before the 34th week of gestation because of eclampsia, severe pre-eclampsia or placental insufficiency
  c. Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation

Laboratory findings
- IgG or IgM aCL
  >40 MPL/GPL or 99th percentile
- IgG or IgM anti-β₂ GPI
  >99th percentile
- LA
  2 positive tests ≥12 weeks apart

1 Clinical + 1 Laboratory finding = APS

Figure 1: Classification criteria for Antiphospholipid syndrome (APS)
aCL=anticardiolipin. anti-β₂GPI= Anti-β₂ glycoprotein-I. LA=Lupus anticoagulant.
It has been argued that while it is important to have a universal definition of APS for study reasons, this concept might be misleading in the clinical context. According to this view, aPL testing in clinical practice is perhaps better regarded as part of a general risk stratification for thrombotic events, pregnancy complications etc. in certain groups of patients, rather than a diagnostic procedure (4, 5).

1.2 Knowledge of APS and aPL prevalence is limited

Data on the prevalence of this condition in the general population is meagre. Using the current cut-offs for aPL assays, at least 1% of healthy individuals will, by definition, be positive for aPL without having any clinical manifestations of APS (3). A recent literature review estimated the overall frequency of aPL positivity in different patient populations. They reported 6% for pregnancy morbidity, 13.5% for stroke, 11% for myocardial infarction (MI), and 9.5% for deep venous thrombosis (DVT). However, the authors of this review highlighted the difficulties in extracting such figures from the existing literature due to use of non-criteria cut-offs for aPL, non-criteria definitions for events, and heterogeneity in study design (6).

New studies tend to investigate the subject more according to current APS criteria. Gris et al screened 6318 patients under investigation for unexplained pregnancy loss for aPL (anti-β2GPI, aCL, and LA), and found that 8.2% were persistently positive (7). In a study of 150 young patients (<50 years) with ischaemic stroke, Renna et al diagnosed 12.7% with APS (8). In a large case control study called RATIO (Risk of Arterial Thrombosis In relation to Oral contraceptives), LA positivity was found in 17% of young women with ischaemic stroke (<50 years old) and in 3% of young women with MI (9). In a study of 443 patients with venous thromboembolism utilising the current APS criteria, the percentage of aPL-positive patients was surprisingly low: 0.7-3.1%, comparable to the control group. A similar result was observed for the proportion of patients with venous thrombosis that were aCL-positive at the 99th percentile cut-off in a large population-based nested case-cohort study (10).

APS and aPL are more common in individuals with other autoimmune disorders but the same problem as for the general population occurs when estimating the true prevalence. In a review from 2010, the prevalence of aPL among patients with systemic lupus erythematosus (SLE) was 12-44% for aCL, 10-19% for anti-β2GPI, and 15-34% for LA (11). In a group of SLE patients from mid-/southern Sweden the prevalence of aPL was 18.8% for IgG aCL, 21.8% for IgM aCL, 20.3% for anti-β2GPI, and 19.4% for LA (12). In an older retrospective study where 21 aCL-positive patients with SLE or other connective tissue diseases were followed for 10 years, 11 patients (52%) developed clinical features of APS (13). In the multicentre cohort of 1000 APS patients followed in the Euro-Phospholipid project, 36% had SLE, 5% had lupus-like syndrome, and 5% had other associated diseases (14). In a retrospective cohort study of 80 Brazilian APS patients, 14 patients (17.5%) subsequently developed SLE in an average time of 5.2+/-4.3 years (15).

Most APS patients are women; 82% of the patients in the Euro-Phospholipid cohort are female (14).
1.3 APS is a heterogeneous and potentially severe condition

The clinical features associated with aPL positivity are diverse. Apart from the established APS criteria manifestations, features include heart valve disease, livedo reticularis (LR), skin ulcerations, thrombocytopenia, and various neurological manifestations such as cognitive dysfunction, migraine, transverse myelopathy, and epilepsy (3). The most severe form of APS is known as catastrophic APS (CAPS, Asherson’s syndrome). It is characterized by the development of life-threatening multiorgan thrombosis (>2 organs) over a brief period of time (<1 week) in association with microthrombosis and persistent aPL positivity (16, 17).

Current treatment options for APS are long-term oral anticoagulation (for venous and arterial thrombosis), heparin (for obstetric APS), and acetylsalicylic acid (ASA, for certain aPL carriers, obstetric APS, and some arterial events). For CAPS, the therapeutic arsenal includes anticoagulation, steroids, intravenous immunoglobulins, and plasmapheresis/plasma exchange. This arsenal may also be used in addition to conventional treatments in high-risk APS pregnancies. Drugs such as statins, hydroxychloroquine, B-cell inhibitors, new oral anticoagulants (NOACs), older non-heparin/warfarin anticoagulants, complement inhibitors, and peptide therapies are being discussed as possible therapeutic options and tested for various APS manifestations (18-22).

Since both the disorder itself and its treatments can potentially harm the affected individual, both under- and over-treatment must be avoided. To achieve this, large randomised trials are required to estimate treatment effects on a group level. However, there is also a need to improve APS diagnostics and to identify better biomarkers to individualise care.

1.4 Laboratory testing for aPL is problematic and better prognostic tools/biomarkers are needed

1.4.1 Controversies exist regarding the strength of association between clinical manifestations and positive aPL tests

Despite the extensive body of literature published over the years on the relationship between aPL positivity and APS events, the magnitude of this association is still a matter of debate. In two systematic reviews of studies published 1988-2000, Galli et al concluded that a positive LA test is a strong risk factor for both arterial and venous thrombosis but that the odds ratios (ORs) for aCL and anti-β2GPI tests are not equally convincing. Substantial heterogeneity in study design and how the laboratory tests were performed was noted for the included studies and further harmonisation of methods was encouraged (23, 24). During the decade that has passed since these two reviews were published, the APS criteria have partly changed: anti-β2GPI assays have been added, cut-offs for aCL have been defined as >40 IgG/IgM phospholipid units (GPL/MPL) or >99th, the interval required between two positive tests increased from six to twelve weeks (3) and further studies investigating the relationship between aPL and thrombosis have been conducted. To present the findings from all of these studies is beyond the scope of this thesis but some of the most prominent publications are cited below and summarised in Table 1. The role of aPL in cardiovascular complications in SLE is also further discussed in section 1.6.

Regarding arterial manifestations, a study that prospectively followed individuals referred for aPL testing reported that aPL positivity was an independent predictor for new arterial events (25). Moreover, the previously cited RATIO study showed that LA was significantly associated
with both stroke (OR 43.1) and MI (OR 5.3), while IgG anti-β2GPI was only associated with stroke (OR 2.3) and IgG aCL was not associated with either of these two manifestations (9). However, in a case-control study of premenopausal women with MI, IgG anti-β2GPI and IgM anti-β2GPI showed clear associations with the event while the association for IgG aCL was less prominent but still statistically significant. IgM aCL showed no association (26). A report from the Hopkins Lupus Cohort published in 2010 states that both LA, anti-β2GPI, and aCL were associated with stroke but only LA was associated with MI (27). In a study from 2012 of the same cohort, LA was an independent predictor of cardiovascular events in a multivariable model (28). In our Karolinska SLE cohort and in the LUMINA (LUpus in MInorities: NAture vs. Nurture) cohort aPL positivity was an independent predictor of arterial events (29, 30). Gris et al demonstrated that during a median follow-up time of approximately 9 years persistently aPL-positive women with previous pregnancy loss (purely obstetric APS) had increased rates of stroke, DVT, pulmonary embolism (PE), and superficial vein thrombosis (SVT) compared to a group of non-thrombophilia patients with previous pregnancy losses. The mean risk rates were in general nearly twice as high in the first group. However, among the APS patients, positive LA was the only aPL test which convincingly and independently predicted thrombosis, while IgM aCL positivity seemed rather to protect against events (7). Moreover, in a study of young patients with recent ischaemic stroke/transient ischaemic attack (TIA), aPL positivity (LA and/or aCL positivity) was neither a risk factor for recurrent cerebral events nor for other thrombotic complications (31).

Concerning venous thrombosis, there are a number of contemporary and relevant studies showing a relationship between aPL positivity and clinical events. Besides the aforementioned study by Gris et al (7) there is also the 2010 publication from the Hopkins Lupus Cohort reporting a significant association between single positivity for LA, anti-β2GPI, and aCL, respectively, and (any) venous thrombosis (27). Moreover, there is a large population-based case-control study, the Leiden Thrombophilia Study, where a positive LA in the presence of anti-β2GPI or antibodies against prothrombin (PT) was associated with an increased risk for DVT corresponding to a mean OR of 10.1 (32). In line with this study, the WASP (warfarin in the antiphospholipid) study confirmed the importance of positivity in several aPL tests reporting an almost 5- to 7-fold increased risk of prospective thrombosis and abortion for LA-positive patients belonging to the upper tertile of both anti-β2GPI and anti-annexin V (aAnAV) (33). In another prospective setting, anti-β2GPI was shown to be an independent predictor of new venous thrombosis (25). As stated above, there are also, however, recent and important studies that have failed to report a convincing relationship between venous thrombosis and aPL positivity and that have investigated the association between clinical events and positivity for either aCL or LA (34) or aCL single positivity (10).

Importantly, the question of aPL positivity and risk of thrombosis in non-lupus patients has been addressed in a recent meta-analysis of studies from 1990 to 2010, including some of the studies mentioned above. Both LA, anti-β2GPI, and aCL were associated with thrombosis even though ORs varied according to the manifestation studied (35).
Table 1: Selection of recent studies (2004-2014) investigating the relationship between antiphospholipid antibodies (aPL) and thrombosis

<table>
<thead>
<tr>
<th>Study</th>
<th>Population</th>
<th>Study design</th>
<th>aPL studied</th>
<th>APS manifestation</th>
<th>Positive association aPL and manifestations:</th>
<th>Main findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toloza 2004</td>
<td>SLE-patients (n=546)</td>
<td>Prospective cohort</td>
<td>aPL, LA</td>
<td>Arterial events</td>
<td>Yes</td>
<td>aPL&lt;-&gt;arterial events</td>
</tr>
<tr>
<td>van Goor 2004</td>
<td>Patients &lt;45 years with recent TIA/stroke (n=128)</td>
<td>Prospective cohort</td>
<td>LA, aCL</td>
<td>Arterial and venous events</td>
<td>No</td>
<td>No association between aPL positivity and recurrent cerebral events/other thrombotic events</td>
</tr>
<tr>
<td>de Groot 2005</td>
<td>DVT-patients &lt;70 years (n=473) vs.controls (n=472)</td>
<td>Case-control</td>
<td>anti-β₂GPI, LA, antibodies against PT</td>
<td>DVT</td>
<td>Yes</td>
<td>anti-β₂GPI and LA&lt;-&gt;DVT</td>
</tr>
<tr>
<td>Naess 2006</td>
<td>Patients with venous thrombosis (n=508) vs.controls (n=1464)</td>
<td>Population-based nested case control study</td>
<td>aCL</td>
<td>Venous thrombosis</td>
<td>No</td>
<td>No association between aCL and venous thrombosis</td>
</tr>
<tr>
<td>Meroni 2007</td>
<td>Women &lt;45 years with first MI (n=172) vs.controls (n=172)</td>
<td>Case control</td>
<td>aCL, anti-β₂GPI</td>
<td>MI</td>
<td>Yes</td>
<td>IgG anti-β₂GPI &lt;-MI, IgG aCL&lt;-&gt;MI, IgM aCL not associated</td>
</tr>
<tr>
<td>Galli 2007</td>
<td>Patients with persistent aPL(n=112)</td>
<td>Prospective cohort</td>
<td>aCL, anti-β₂GPI, LA, antibodies against PT, aAnAv, aPS</td>
<td>Thrombosis and obstetric events</td>
<td>Yes</td>
<td>Prominently increased risk of thrombosis and abortion for LA-positive patients belonging to the upper tertile of anti-β₂GPI and aAnAV</td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Population</td>
<td>aPL</td>
<td>Events</td>
<td>Results</td>
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<tr>
<td>Neville 2009</td>
<td>Individuals referred for aPL testing and full blood count respectively (n=414)</td>
<td>Prospective cohort</td>
<td>aCL, anti-β₂GPI, LA</td>
<td>Arterial and venous events</td>
<td>Yes</td>
<td>aPL ↔ arterial events, anti-β₂GPI ↔ venous events</td>
</tr>
<tr>
<td>Urbanus 2009</td>
<td>Women &lt;50 years with stroke (n=175) or MI (n=203) vs. controls (n=628)</td>
<td>Population-based case control</td>
<td>aCL, anti-β₂GPI, LA, antibodies against PT</td>
<td>Stroke and MI</td>
<td>Yes</td>
<td>LA ↔ stroke, MI anti-IgG β₂GPI ↔ stroke. IgG aCL and antibodies against PT not associated</td>
</tr>
<tr>
<td>Gustafsson 2009</td>
<td>SLE patients (n=182)</td>
<td>Prospective cohort</td>
<td>aCL, anti-β₂GPI, LA</td>
<td>Arterial events</td>
<td>Yes</td>
<td>aPL ↔ arterial events</td>
</tr>
<tr>
<td>Petri 2010</td>
<td>SLE patients (n not reported)</td>
<td>Cohort</td>
<td>aCL, anti-β₂GPI, LA</td>
<td>Arterial and venous events</td>
<td>Yes</td>
<td>LA, anti-β₂GPI and aCL ↔ stroke, LA ↔ MI</td>
</tr>
<tr>
<td>Magder 2012</td>
<td>SLE patients (n=1874)</td>
<td>Cohort</td>
<td>LA</td>
<td>Cardiovascular events (MI, stroke, angina, PCI, coronary bypass, claudication)</td>
<td>Yes</td>
<td>LA ↔ cardiovascular events</td>
</tr>
<tr>
<td>Gris 2012</td>
<td>Women with previous pregnancy loss (n=1592)</td>
<td>Prospective cohort</td>
<td>aCL, anti-β₂GPI, LA</td>
<td>Stroke, DVT, PE and superficial vein thrombosis</td>
<td>Yes</td>
<td>aPL ↔ Stroke, DVT, PE and superficial vein thrombosis</td>
</tr>
<tr>
<td>Grifoni 2012</td>
<td>DVT patients (n=443) vs. controls (n=304)</td>
<td>Case-control</td>
<td>aCL, LA</td>
<td>DVT</td>
<td>No</td>
<td>No difference between cases and controls regarding aPL positivity</td>
</tr>
</tbody>
</table>

*aCL=Anticardiolipin, anti-β₂GPI=Anti-β₂ glycoprotein-I, LA=Lupus anticoagulant, SLE=Systemic lupus erythematosus, TIA=Transient ischemic attack, DVT=Deep venous thrombosis, MI=Myocardial infarction, PT=Prothrombin, AnAV=Anti-annexin V, aPS=Anti-phosphatidylerine, PE=Pulmonary embolism, PCI=Percutaneous coronary intervention, n=number, ↔=associated with.*
Obstetric morbidity was not included in the reviews by Galli et al. For women without autoimmune disease, thorough meta-analysis show that LA and aCL are convincingly associated with recurrent foetal loss (RFL) (36) and aCL with preeclampsia (37). Moreover, the relationship between positive aPL and adverse pregnancy outcome has been corroborated in prospective settings (38, 39). For pregnancies complicated by SLE, a recent meta-analysis confirms an association between aPL and hypertension as well as premature birth (40) and many but not all studies report a clear relationship between aPL and pregnancy loss (41-48).

1.4.2 aPL testing includes a number of complex procedures

ELISA tests for aCL and anti-β₂GPI should be conducted according to the international guidelines from 2012, updated in 2014, which describe important preanalytical, analytical, and postanalytical issues to be considered. For example, it is important to use a complex of cardiolipin and β₂GPI (human) in aCL assays, to properly quantify and report the results, to demonstrate acceptable precision (coefficient of variation=CV preferably below 10%, maximum 20%), and to evaluate the possible IgM-rheumatoid factor (RF) interference. Universal units of measurement for the aCL-tests, GPL for IgG, and MPL for IgM, exist for quantification. Traditionally, aCL tests are calibrated using calibrators from Louisville APL Diagnostics which can be traced back to the original Harris standards, but there are also monoclonal calibrators. The cut-off for positivity is set at 40 GPL/MPL or the 99th percentile of normal controls. For anti-β₂GPI, no universal measurement units exist, so the results are reported in arbitrary units. The cut-off for positivity is set at the 99th percentile of healthy controls (49, 50).

The functional LA test is based on indirect evidence of the presence of LA acting on phospholipid-dependent clotting tests; quenching the phospholipids required for coagulation so that clotting time is prolonged. The test is traditionally performed in three steps: screening, mixing, and confirmation. In the screening test, LA is suspected if the clotting time is prolonged. In the mixing test, positive plasma from the screening step is retested using the same procedure as in the previous step but this time with the addition of normal plasma in a 1:1 ratio with patient plasma. If clotting time is still prolonged (and not corrected as would be the case if the prolonged clotting time was due to naturally occurring or induced coagulation deficiencies) LA is still suspected. In the confirmation test, LA is verified if the prolonged clotting time is normalised after addition of increasing amounts of phospholipids and thus identifying the inhibitor of coagulation as phospholipid-dependent. As described in the current international guidelines published 2009, two different tests based on different principles should be used for screening; preferably dilute Russell’s viper venom time (dRVVT), where coagulation is activated by a viper venom, and a sensitive activated partial thromboplastin time (APTT) test using silica as an activator and low phospholipid concentration. The screening test is positive if the clotting time is longer than the local cut-off, preferably set at the 99th percentile of healthy blood donors. The mixing test is positive if clotting time is longer than the local cut-off and the confirmation test is positive if the clotting time is shorter than the local cut-off value. LA is considered present if at least one complete testing procedure is positive (performed with either dRVVT or APPT). Problems with LA detection include: loss of low-titer LA if platelets are not completely removed before freezing and thawing, interpretation during ongoing antithrombotic treatment/acute thrombotic event/presence of coagulation factor inhibitors, as well as generally low specificity. For heparins, the recommendation is to postpone testing until treatment is discontinued, if possible, but many low molecular weight heparins do not in fact affect the testing. For oral anticoagulants, there are different procedures to overcome the effect of the drug (like dilution with normal plasma in case of vitamin K antagonists and international normalized ratio; INR, 1.5-3), but the results are recommended to be interpreted with caution and ideally,
LA testing should be postponed until treatment is stopped or INR<1.5. Detection of LA in plasmas containing the new direct oral anticoagulants is not possible with regular assays (51, 52) (53). The correlation between clinical events and a positive LA can be affected by which aPL that is responsible for the observed prolongation of clotting time in vitro (54).

1.4.3 Assays need to be more standardised

An important explanation for the conflicting results in the clinical studies of APS is the continuing lack of standardisation of how aPL assays are conducted. Consequently, there is significant interlaboratory variation (3, 55, 56).

For LA, the lack of an available “golden standard” laboratory test and the high rate of both false-negative and false-positive detections are major issues. Most laboratories can correctly identify clearly positive and clearly negative samples, but problems arise when LA is weak. There may also be a risk of falsely classifying a very strongly positive sample as negative if the mixing test is not used (57). There is an on-going debate concerning the need to routinely perform the mixing test. Some argue that current guidelines should be changed and dilution with normal plasma only used under some special circumstances (58).

For ELISA tests for aCL and anti-β2GPI, the lack of reference material and uniform units of measurement are important problems. It has been suggested that many problems stemming from lack of standardisation could be eliminated if a new common, international primary standard is established. The use of monoclonal antibodies has been proposed. Until this goal is reached, efforts should be made to improve the existing methods and to standardise cut-offs, procedures and materials (53, 59, 60).

1.4.4 Cut-offs are crucial and have been much discussed

Over the years many studies, including prospective such, have shown that higher levels of aPL are more strongly associated with clinical events than lower levels (61-68). This was also the conclusion drawn for aCL when studies on thrombosis were summarised in the systematic reviews by Galli et al, although for anti-β2GPI, it was stated that lack of reference material for quantification made this type of assessments impossible (23, 24). Moreover, titer affects the probability of being positive in more than one aPL test (68). However, it is important but difficult to establish the exact threshold that confers a clinically (and not only statistically) significant increased risk for events. Moreover, this threshold could vary depending on the clinical manifestation studied (68). The current cut-offs for positivity are based on the best available evidence in 2006 when the APS criteria were modified from the previous Sapporo criteria after an international meeting in Sydney (3, 4, 69). There are, however, reports that titers below criteria cut-off can also be important. This could be true for obstetric APS in particular (30, 68, 70-72).

1.4.5 The role of aPL IgM has been questioned

The current APS criteria include positivity in both the IgG and IgM isotypes for aCL and anti-β2GPI (3). However, the rationale for continuing to test for IgM as part of clinical risk stratification has been questioned since associations with events for aPL of IgM isotype seem to be generally weaker than for IgG isotype (73).

The systematic reviews of aPL and thrombosis by Galli et al (23, 24) reported that IgM was less strongly associated with thrombosis in both the aCL and the anti-β2GPI-assays, but the authors stated that the subject could only partially be addressed as IgM antibodies were rarely studied.
separately. After these systematic reviews, a number of prospective studies have confirmed that the clinical association between IgM isotype and thrombosis is dubious (7, 33, 74, 75). However, results are conflicting if other study designs are also included. Some of these studies present findings that could justify continuing IgM use, sometimes demonstrating isotype-specific patterns of associations (76-81), while others report results that argue against further use of IgM aPL (65, 82-84).

For obstetric manifestations, a metaanalysis of RFL among women without additional autoimmune disease showed a convincing association for IgM aCL as well as for IgG aCL, but the relationship with anti-β2GPI, regardless of isotype, was more uncertain (36). For SLE patients, studies reporting association with obstetric events for the two criteria-isotypes separately are hard to find. Julkunen et al studied the association between different aPL tests and a history of foetal loss in 107 SLE women and found that IgM aCL was the only individual test with a statistically significant OR: 3.0 (1.1-7.8) (42).

1.4.6 The concept of multiple positivity and risk scores — the only way to go?

Through all the controversies and difficulties in aPL testing, APS diagnostics and aPL-related risk stratification, a new concept with increasing popularity has emerged. Despite the weaknesses and dubious clinical association of the individual aPL tests, combining the results of multiple testing could provide a good strategy for risk estimation. The idea is that triple positivity in the criteria aPL assays, but not single positivity, confers a substantially increased risk for APS manifestations. The value of isolated positivity in the ELISA assays (aCL, anti-β2GPI) has been particularly questioned (23, 24, 55, 73, 85-90). It should be noted that many of the clinical studies described in section 1.4.1 were not designed for/did not use the statistical method required to evaluate the risk associated with a single positive aPL test. However, the concept of multiple positivity is very important and has probably paved the way for attempts to develop new aPL risk scores (91-93). Ideally, such scores could guide treatment which in itself is not without risk (55). It is recognised that in this context, non-aPL factors also need to be taken into consideration (30, 94). In addition, there is an on-going debate about the value of antibody avidity (95) as well as of “non-criteria” aPL such as IgA aPL, antibodies against PT or phosphatidylerine-PT complex, and anti-β2GPI against domain I (50, 53, 96). An association with triple positivity has been reported for anti-β2GPI against domain I (further discussed in section 1.5.1) (97).

1.4.7 New methods in the pipeline?

As stated in a recent review on the future of antiphospholipid antibody testing by de Groot and Urbanus: “Not only is there a need for better assay standardization, there is also a need for better assays” (55).

More automated assays for aPL testing are being developed. Hopes for these methods are that they will be less time- and manpower-consuming, have improved reproducibility, and have reduced interlaboratory variation. A few years ago, the publications on the performance of these assays were scarce, but the body of literature on this subject is growing. The concept seems promising even though obstacles like the lack of a golden standard and uniform measurement units complicate the evaluation of the assays (98-105).
1.5 Exploring the largely unknown APS aetiology could provide clues to new biomarkers

A better understanding of the still enigmatic APS pathophysiology can be regarded as a prerequisite for the development of better methods for risk assessment in APS (55). It could also guide more targeted therapies.

The body of knowledge of APS aetiology is currently growing, supported by new important pieces of information from clinical APS studies as well as experimental aPL studies and through studies of conditions with similar clinical manifestations as APS.

1.5.1 Many factors have been proposed to contribute to APS pathogenesis

Five recent reviews of APS pathogenesis summarise prevalent hypotheses of APS pathogenesis (54, 106-109) as they emerge from a number of important original studies (some of which are cited below):

- **β2GPI is important.** The physiological role of this plasma protein is unclear, but it has been proposed to act as a “vacuum cleaner,” involved in binding and clearance of injured and apoptotic cells, microparticles (MPs), and bacterial products (110-112). There are speculations that naturally occurring antibodies against this molecule are involved in its regulation and that the increased thrombotic risk observed in APS could be related to increased titer and avidity of such naturally occurring antibodies and/or their prolonged presence in the circulation. This hypothesis, put forward in one of the above mentioned reviews (108), is based on the finding that healthy individuals without APS can have memory B-cells that produce aPL (113). Moreover, molecular mimicry has been suggested as a mechanism for aPL production (114).

- **The B-cell epitope in domain I of the β2GPI molecule is especially interesting.** Patients that are aPL triple positive have high titers of antibodies against this domain (97) and these antibodies confer LA activity associated with high risk of thrombosis (115). It has thus been suggested that APS is an autoimmune disease with a single type of autoantibody which can give rise to positive results in both aCL/anti-β2 GPI ELISAs and the LA test (97).

- **The structure and function of β2GPI can be affected by external factors.** Infections, smoking, and pregnancy can cause increased oxidative stress which seem to modify the β2GPI molecule (116), affecting domain I with its important B-cell epitope (117). Moreover, β2GPI can exist in two different configurations with different exposure of the domain I epitope, depending on whether it is bound to an anionic surface or not (118). Conformational changes have recently been shown to occur after binding of β2GPI to bacterial proteins as well (119). Conformational changes affect the epitope recognition of anti-β2GPI domain I antibodies (120).

- **aPL are most likely pathogenic.** Antibodies from patients with APS can potentiate the development of thrombosis when they are infused into mice, but this effect is lost when the anti-β2GPI component is eliminated (121) (122).

- **aPL presumably confer their effect through activation of specific receptors on target cells** (see Figure 2). The binding of aPL to the target cells is mediated by their main autoantigen β2GPI. It is the complex of the antibody and the antigen that activates the cells. Target cells include endothelial cells (123), monocytes (124), neutrophils (125),
and platelets (126). aPL most likely upregulates target cell expression of pro-adhesive and pro-coagulant molecules like intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), endothelial Selectin (E-Selectin), and tissue factor (TF) (125, 127-130). However, the mere presence of aPL or aPL complexes does not appear to be sufficient to cause thrombosis. In agreement with the proposed “two hit” hypothesis, there seems to be a need for priming or stimulation of the endothelium (122, 131). Oxidative stress can upregulate the expression of annexin II (132) which is an important endothelial cell surface receptor for β2GPI (133).

- **Endothelial nitric oxide synthase (eNos) is probably a key player.** There are indications that aPL promote leukocyte-endothelial cell adhesion and thrombosis by antagonising eNOS (134). eNOS is upregulated by statins (135), drugs that prevent anti-β2GPI-mediated endothelial activation (136).

- **Several other pathogenic mechanisms for aPL/APS have been proposed.** Suggested mechanisms include aberrations related to coagulation factors such as FXI (137), aPL interference with the protective annexin V shield (138, 139) and with the activity of anticoagulants such as Activated Protein C (APC) (140, 141) and Antithrombin III (142), complement activation (leading to activation of neutrophils and TF expression) (125), other disturbances of the innate immunity (143), and finally various abnormalities in the fibrinolytic systems (see section 1.5.3).

- **Pathogenic mechanisms behind obstetric and thrombotic APS are only partly overlapping.** The main mechanism for the obstetric manifestations of APS was initially believed to be thrombosis, to some extent supported by histopathological findings in placenta from patients with intrauterine foetal death and aPL (144). However, further studies have suggested the importance of additional mechanisms such as defective trophoblast proliferation/invasion and decidual transformation in early pregnancy (145, 146) as well as placental injury caused by local inflammatory events including complement activation (147, 148).

A recent study by Canaud et al proposes that the mammalian target of rapamycin complex (mTORC) pathway is important in vascular lesions associated with APS and that this could have important therapeutic implications (149).
Figure 2: Schematic overview of potential antiphospholipid (aPL) mechanisms of action on coagulation

a. Activation of endothelium, platelets, monocytes, and neutrophils resulting in expression of pro-adhesive and pro-coagulant molecules like tissue factor (TF). Exposure of pro-coagulant phospholipid (PL) phosphatidylserine (PS) through disruption of the protective annexin V shield

b. Initiation of coagulation cascade by TF on PL-expressing cell surfaces onto which the calcium (Ca)-dependent coagulation factors can bind

c. Aberrations related to coagulation factors and anticoagulants (Protein C, Antithrombin III) determining thrombin formation

d. Disruption of the fibrinolytic system (fibrinogen/fibrin, fibrin network, plasminogen/plasmin, tissue plasminogen activator=tPA, plasminogen activator inhibitor-1=PAI-1)
1.5.2 Is smoking important?

Mechanistic studies have suggested that APS patients could be more sensitive to oxidative stress from exogenous sources such as smoking (54). A joint effect of aPL and smoking on the occurrence of cardiovascular events has been reported (9). There are few studies on the association between smoking status and aPL positivity, however.

1.5.3 Are fibrin network characteristics and fibrinolysis relevant?

Emerging evidence suggest that the formation of thrombi characterised by abnormal architecture (high fiber density, thin fibers, small pores) and decreased fibrin permeability demonstrated in in vitro assays is a feature of many conditions with increased risk for cardiovascular and thrombotic events (150-159). Such thrombi are associated with an increased resistance to fibrinolysis due to, for example, decreased permeability of fibrinolytic factors through the fibrin clot (160-163). Hypofibrinolysis is discussed as a risk factor for both venous and arterial thrombosis (164).

The structure of fibrin formed in vitro can be visualised using different microscopic techniques. Fibrin permeability can be studied using a liquid permeation technique, originally developed by Blomback et al (165, 166) and later subject to some modifications to facilitate its use (167). In normal haemostasis, conversion of fibrinogen to fibrin monomers is catalysed by thrombin, this is followed by polymerisation into protofibrils that in the presence of thrombin-activated FXIII subsequently form a cross-linked fibrin network/clot. The conditions prevailing during this process determines fibrin structure and function, such as permeability of the fibrin network. Factors of potential importance include: concentrations and variations of fibrinogen, fibrin(o)gen) modulators (albumin, fibronectin, factor XIII, and cells present during coagulation), thrombin/thrombin generating factors, calcium and polyphosphate as well as pH, ionic strength, and local blood flow (159, 165, 168, 169). Moreover, MPs have been shown to bind fibrin (170) and to affect fibrin characteristics (171). Finally, fibrin permeability is increased by common cardiovascular therapies such as statins (171), dalteparin (172), ASA (173, 174), and warfarin (175). In the experimental models developed to study fibrin permeability (discussed above) where calcium and thrombin are added to plasma samples to start clot formation, some of the factors contributing to fibrin gel porosity are kept constant. Fibrin permeability thus depends mainly on the sample fibrinogen, an acute phase reactant that in itself is well known to be associated with cardiovascular disease (CVD) risk, but may also be affected by modulators in the investigated samples (166, 167, 176, 177).

The fibrinolytic system is the final phase of normal haemostasis, when the fibrin clot is removed and the damaged vessel wall restored. It is activated and regulated by several proteins affecting the generation of the fibrin clot-degrading enzyme plasmin from plasminogen. The most important plasminogen activator in plasma and the main regulator of fibrinolysis is tissue plasminogen activator (tPA), which is produced by stimulated endothelial cells. When bound to fibrin, tPA is protected from its principal inhibitor in plasma: plasminogen activator inhibitor-1 (PAI-1) which is produced by different cell types including endothelial cells, platelets and hepatic cells. α2-antiplasmin, the primary physiological inhibitor of plasmin, as well as FXIII and thrombin-activatable fibrinolysis inhibitor (TAFI) are other key players of the fibrinolytic system (164, 178, 179). Fibrinolysis can generally be investigated through assays measuring antigen or activity levels of various plasma components of the fibrinolytic system or by more global assays (180). Antigen levels of tPA, PAI-1, and tPA-PAI-1 complexes are important for CVD risk (181, 182). In global assays, overall hypofibrinolysis is associated with both arterial
and venous thrombosis (164). In turbidimetric assays, clot formation and lysis characteristics of plasma samples can be examined in vitro through addition of thrombin, calcium, and tPA (183).

Though seldom studied, there are some reports that impaired fibrinolysis contributes to the pro-coagulant state in APS (109). Hypofibrinolysis has been demonstrated using global in vitro assays in both obstetric (184) and thrombotic APS populations (185), but the underlying mechanisms are unclear. Antibodies blocking the ability of β2GPI to stimulate clot lysis have been suggested as a mechanism, while increased fibrinogen concentration as well as enhanced activity of factor XIII have been demonstrated in APS. Moreover, increased levels and activity of PAI-1 have been reported in patients with connective tissue diseases (109, 186-189). Clot architecture is another possible factor, which may affect fibrinolysis and coagulation equilibrium, but its role had not yet been investigated in patients with APS at the time when the studies presented here were conducted.

1.5.4: Do microparticles play a role?

Changes in the number or function of circulating MPs are associated with many disorders with arterial or venous thrombosis (190, 191) and pregnancy morbidity (pre-eclampsia and RFL) (192, 193) as well as various rheumatic diseases (194). It is thus reasonable to suspect that MPs have implications also for APS.

![Figure 3: Schematic presentation of a microparticle (MP)](image)

PS= Phosphatidylserine

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MPs are small vesicles (0.1-1 µm) that upon activation or apoptosis are released from membranes of different cell types in a way that usually causes the anionic phospholipid phosphatidylserine (PS) to be exposed to the circulation, see Figure 3. Under normal conditions, platelet-derived MPs (PMPs) are by far the most abundant MPs in the blood. MPs are most often measured using flow cytometry. They are commonly identified using size and exposure of PS. PS exposure is frequently detected through annexin V binding (194) but can also be identified using lactadherin (195), a milk-fat protein that that binds to PS regardless of calcium levels. PS serves as a red flag for macrophage recognition and lactadherin is involved in the phagocytic elimination of activated or apoptotic cells (196) (197), and perhaps also of MPs. A mechanistic study suggests that binding of β2GPI to PS-expressing pro-coagulant PMPs may promote their clearance by phagocytosis and that autoantibodies to β2GPI may induce a pro-coagulant state by inhibiting this process (198). The number of circulating MPs is probably dependent on a dynamic balance between release and clearance (191). MPs are more prone to escape phagocytic clearance than for example the larger apoptotic bodies, and they express different surface, cytoplasmic, and nuclear molecules. Their surface and content depend on the parent cell type and the conditions during their release. MPs can thus be regarded as tiny messengers of previous or occurring cellular events. However, one must keep in mind that MP structure may be partly dynamic, as binding of for example immunoglobulins and complement proteins can take place not only before but also after detachment from the parent cells. MPs may provide information about tissues, like the endothelium, that otherwise could only be obtained through invasive biopsies. Furthermore, MPs can themselves have biological effects through signalling at both local and distant sites. Their pro-thrombotic potential is well-known. They provide an enlarged catalytic surface with negatively charged PS for the assembly of calcium-dependent procoagulant enzyme complexes. They can also express pro-coagulant entities such as TF (194). Furthermore, there are indications that MP could affect fibrinolysis. However, some MPs might also be pro-fibrinolytic depending on protein exposure (191).

MPs have a pro-inflammatory potential that can be of importance in many pathological conditions. Moreover, important contributions to disease pathogenesis can be made through MPs taking part in the formation of immune complexes. Particles generated through apoptosis can contain nuclear components, which in this way become more accessible to the immune system for example for antibody binding (194). Additionally, MPs can stimulate apoptosis, providing a positive feedback loop which amplifies the initial response (191, 198).

There are to date relatively few previous studies of MP in aPL-associated disorders including SLE (studies are summarised in Table 2 and Table 3). Moreover, the majority of existing studies are small, seldom include obstetric patients, use non-uniform measurement-methods, and report inconsistent findings (198-213). Many studies demonstrate an increased number of different MPs in these conditions (199-202, 204-206, 209, 214), but a recent Danish study found an overall decreased number of annexin V positive MPs in SLE as compared to healthy controls. However, the authors reported the SLE patients did have an increased number of annexin V negative MPs, followed by a suggestion that this could have implications for phagocytic recognition (208). There are also indications that the number of circulating MPs could correlate (positively or negatively) with SLE disease activity (214, 215). It is, however, likely that not only the mere number of circulating MPs but also their composition and function is of importance in these contexts (216).

In a review by Pericleous et al (198) it was suggested that aPL positivity could be enough to confer elevated levels of endothelial MPs (EMPs), but the properties of these EMPs may differ between the aPL carriers that are affected by thrombosis and those who stay
thrombosis-free. Consistent with this hypothesis, APS patients were recently reported to have higher MP-TF activity than asymptomatic aPL carriers (217).

Table 2: Previous studies of microparticles in antiphospholipid Syndrome (APS)

<table>
<thead>
<tr>
<th>STUDY</th>
<th>PATIENT POPULATION</th>
<th>NUMBER OF APS-PATIENTS/APL-POSITIVE</th>
<th>TYPE OF MICROPARTICLES</th>
<th>CHANGE IN NUMBER OF MP: APS VS. HEALTHY CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combes et al 1999</td>
<td>LA-positive</td>
<td>30 (8 APS)</td>
<td>EMPs</td>
<td>↑</td>
</tr>
<tr>
<td>Joseph et al 2001</td>
<td>APS (SLE)</td>
<td>20</td>
<td>PMPs</td>
<td>→</td>
</tr>
<tr>
<td>Dignant-George et al 2004</td>
<td>APS, SLE+ aPL</td>
<td>111 (35 APS)</td>
<td>EMPs</td>
<td>↑</td>
</tr>
<tr>
<td>Nagahama et al 2003</td>
<td>APS</td>
<td>37</td>
<td>MMPs/PMPs</td>
<td>↑ (for all MPs)</td>
</tr>
<tr>
<td>Jy et al 2007</td>
<td>Persistently aPL-positive</td>
<td>88 (60 with thrombosis)</td>
<td>EMPs/PMPs</td>
<td>↑/→</td>
</tr>
<tr>
<td>Flores-Nascimento et al 2009</td>
<td>APS (DVT, FVL-carriers)</td>
<td>11</td>
<td>PMPs, EMPs, MMPs, ErMPs, LMPs</td>
<td>→ (for all MPs)</td>
</tr>
<tr>
<td>Antwi-Baffour et al 2010</td>
<td>aPL-positive, (SLE)</td>
<td>13</td>
<td>Total MPs</td>
<td>↑</td>
</tr>
</tbody>
</table>

Table 3: Previous studies of microparticles in Systemic Lupus Erythematosus (SLE)

<table>
<thead>
<tr>
<th>STUDY</th>
<th>PATIENT POPULATION</th>
<th>NUMBER OF SLE-PATIENTS</th>
<th>TYPE OF MICROPARTICLES</th>
<th>CHANGE IN NUMBER OF MP SLE VS. HEALTHY CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Joseph et al 2001</td>
<td>SLE (APS)</td>
<td>30</td>
<td>PMPs</td>
<td>→</td>
</tr>
<tr>
<td>Nauta et al 2002</td>
<td>SLE</td>
<td>16</td>
<td>Total MPs</td>
<td>→</td>
</tr>
<tr>
<td>Pereira et al 2006</td>
<td>SLE</td>
<td>30</td>
<td>Total MPs/LMPs/EMP</td>
<td>↑/↑/↑/→</td>
</tr>
<tr>
<td>Sellam et al 2009</td>
<td>SLE (Sjögren, RA)</td>
<td>20</td>
<td>Total MPs/LMPs/EMP</td>
<td>↑/↑/→</td>
</tr>
<tr>
<td>Duval et al 2010</td>
<td>SLE</td>
<td>27</td>
<td>EMPs</td>
<td>↑</td>
</tr>
<tr>
<td>Antwi-Baffour et al 2010</td>
<td>SLE (APS)</td>
<td>20</td>
<td>Total MPs</td>
<td>↑</td>
</tr>
<tr>
<td>Oyabu et al 2011</td>
<td>Connective tissue disease</td>
<td>88</td>
<td>PMPs</td>
<td>→</td>
</tr>
<tr>
<td>Ullal et al 2011</td>
<td>SLE</td>
<td>21</td>
<td>Total MPs</td>
<td>→</td>
</tr>
<tr>
<td>Nielsen et al 2011</td>
<td>SLE</td>
<td>70</td>
<td>a. Total Annexin+ MPs/LMPs/PMPs, EMPs</td>
<td>a. ↓/↓/↓/→ b. ↑</td>
</tr>
<tr>
<td>Parker et al, 2013</td>
<td>SLE (active disease)</td>
<td>27</td>
<td>EMPs</td>
<td>↑</td>
</tr>
<tr>
<td>Crookston et al 2013</td>
<td>SLE</td>
<td>51</td>
<td>Total MPs, PMPs, EMPs</td>
<td>→ (for all MPs)</td>
</tr>
</tbody>
</table>


1.6 aPL are important for vascular disease in SLE

As described in section 1.2, there is a close relationship and an overlap between APS and SLE. The overlap is clinical as well as immunological and genetic (218, 219). Like APS, SLE is in itself a heterogeneous and unpredictable disorder with incompletely understood, most likely multifactorial pathogenesis. An excess of apoptotic material in the circulation due to increased production and/or defective clearance is thought to be an important feature. This exposes nuclear components to immune cells, enabling production of autoantibodies and,
in genetically susceptible individuals, persistent autoimmunity (220). aPL has been suggested to play a role in these processes as a mediator of autoimmunity against dying cells (221). Whatever the exact aetiology, the production of a various autoantibodies is a hallmark of SLE, which together with a set of defined symptoms is used to distinguish SLE from other conditions (222). The first criteria for SLE were published in 1971 and criteria revisions were then proposed in 1982, 1997, and 2012. The 1982 criteria, which are used in this thesis, are presented in Table 4. Generally, all sets of classification criteria rely on a number of clinical and/or immunological manifestations. Clinical symptoms include serositis, haematological disorders, and manifestations from skin/mucosa, joints, the nervous system, and kidneys. Positive antinuclear antibodies (ANA), anti-double-stranded DNA (anti-dsDNA) and anti-Smith (anti-Sm) are important immunological criteria which in the updates have been accompanied by positive aPL (including aCL and anti-β2GPI of IgA isotype), low complement, and direct Coombs test in the absence of haemolytic anaemia (222-224). However, these criteria were designed to enable consistency between research studies and no specific diagnostic criteria exists (225). SLE is a rare disease with reported incidence ranging from 2.2 to 7.6 cases per 100 000 persons per year (226). Women are affected on average 6-8 times more frequently than men (225). Current treatment options include anti-malarials, steroids, and various immunosuppressive and biological agents (227). Even though morbidity and mortality have improved over the years, there is still a pronounced increase in overall mortality compared to the general population (226).

Table 4: The 1982 American College of Rheumatology criteria for classification of SLE

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Malar rash</td>
<td>Fixed erythema over the malar eminences</td>
</tr>
<tr>
<td>2. Discoid rash</td>
<td>Erythematous raised patches with adherent keratotic scaling and follicular plugging</td>
</tr>
<tr>
<td>3. Photosensitivity</td>
<td>Skin rash caused by unusual reaction to sunlight</td>
</tr>
<tr>
<td>4. Oral ulcers</td>
<td>Oral and nasopharyngeal</td>
</tr>
<tr>
<td>5. Arthritis</td>
<td>Non-erosive</td>
</tr>
<tr>
<td>6. Serositis</td>
<td>Pleuritis or pericarditis</td>
</tr>
<tr>
<td>7. Renal disorder</td>
<td>Persistent proteinuria or cellular casts</td>
</tr>
<tr>
<td>8. Neurologic disorder</td>
<td>Seizures or psychosis</td>
</tr>
<tr>
<td>9. Haematologic disorder</td>
<td>Haemolytic anaemia or leukopenia or lymphopenia or thrombocytopenia</td>
</tr>
<tr>
<td>10. Immunologic disorder</td>
<td>Positive LE cell preparation or anti-dsDNA or anti-Sm antibodies or false positive VLDR</td>
</tr>
<tr>
<td>11. Antinuclear antibodies</td>
<td>An abnormal titer of ANA by immunofluorescence or an equivalent assay</td>
</tr>
</tbody>
</table>

If four of these criteria are present at any time during the cause of the disease, a diagnosis of SLE can be made. Sm = the Smith antigen. LE cell = lupus erythematosus cell. VLDR = Venereal Disease Research Laboratory. ANA = antinuclear antibodies. anti dsDNA = anti-double stranded DNA.

Cardiovascular and thrombotic complications are responsible for a large part of the total morbidity and mortality in SLE. Traditional atherosclerotic risk factors, partly affected by immunosuppressive therapy, does not fully account for the increased vascular burden. Instead,
factors related to the chronic inflammatory disorder itself have proven to be of major importance (228-233). One such factor is aPL, which is a positive predictor of vascular events in several prospective studies (29, 30, 232, 234). An increased understanding of how these auto-antibodies confer increased cardiovascular risk is hoped to aid the development of better biomarkers and methods for individual risk assessment and treatment in SLE patients, which are much needed (55).
2 AIMS

To study clinical, serological and haemostatic features of APS and aPL-related disorders, including SLE, in order to improve and individualise patient care. Major objectives are:

- To improve diagnostic methods for APS/aPL-related disease and identify new biomarkers
- To enhance understanding of the pathogenesis behind APS/aPL-related disease with focus on the role of fibrin clot properties, fibrinolytic function, MP profile, and smoking
3 MATERIAL AND METHODS

3.1 Study design and population

3.1.1 Paper I

This is a cross-sectional study including 712 patients clinically diagnosed with SLE during 1984-2010 from the rheumatology clinics at Karolinska, Uppsala, and Lund University Hospitals (n=381, n=139, and n=192, respectively). For a clinical diagnosis of SLE, we required characteristic SLE serology in combination with a minimum of two typical organ manifestations and absence of more plausible explanations for the condition (235). The majority of the patients also fulfilled at least four of the 1982 revised classification criteria for SLE according to the American College of Rheumatology (ACR), but 25 patients fulfilled less than four criteria (222). At inclusion, clinical information was collected by interviewing the patients and reviewing medical records. Blood samples were drawn.

To calculate a cut-off level corresponding to the 99th and 90th percentile, we used samples from 280 controls from the general population without any history of thrombosis or obstetric morbidity, as defined in the APS criteria (3). These individuals were matched to Karolinska patients for age, sex, and place of residence.

Definition of clinical events

The clinical APS manifestations studied were the following objectively verified vascular events:

1) Ischaemic heart disease (IHD): MI confirmed by electrocardiography and a rise in plasma creatine kinase, muscle and brain fraction (CK-MB), or troponin T and/or angina pectoris confirmed by exercise stress test and/or coronary bypass intervention.

2) Ischaemic cerebrovascular disease (ICVD): Stroke including cerebral infarction confirmed by computer tomography or magnetic resonance imaging and/or TIA, defined as transient focal symptoms from the brain or retina with a maximum duration of 24 hours.

3) Ischaemic peripheral vascular disease (IPVD): Intermittent claudication and/or peripheral arterial thrombosis or embolus confirmed by angiogram or Doppler flow studies and/or surgical intervention of peripheral vascular disease.

4) Venous thromboembolism (VTE): DVT, confirmed by venography or ultrasonography and/or PE, confirmed by pulmonary perfusion scintigram, angiogram, or spiral computer tomography.

The term “any arterial event” (AAE) refers to the occurrence of one or more of events 1-3. The term “any vascular event” (AVE) refers to the occurrence of one or more of the events 1-4.

We also tabulated obstetric morbidity, defined according to established APS criteria (i.e. miscarriages, premature birth, foetal death) (3), and thrombocytopenia, defined according to ACR criteria (222).

3.1.2 Paper II and III

Paper II and III are cross-sectional studies where patients fulfilling the APS criteria (3) from the Rheumatology, Haematology and Women’s and Children’s Health clinics at Karolinska University Hospital were asked to participate during a 1-year inclusion period starting January
2009. At inclusion, patients filled out a questionnaire supervised by a physician about symptoms and medications. Blood samples were drawn. Initially, 54 patients consented to participation but all were not included in the two studies as errors during sample handling and transport prevented all blood samples from being used. In study II, 49 of the APS patients were included, and in study III, 52 patients were included.

According to criteria (3) aPL positivity was defined as at least two positive tests of medium/high titer for IgM aCL/IgG aCL, a titer >99th percentile for IgG anti-β2GPI, or a positive LA test. 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. In both studies, 12 of the patients had secondary APS but the majority were primary APS patients (37 patients in paper II and 40 patients in study III).

In paper II, we compared the APS patients with two different control groups. The primary control group consisted of 19 healthy individuals recruited through our laboratory. All of them were instructed to avoid non-steroidal anti-inflammatory drugs (NSAID) or ASA at least two weeks prior to the study. The second control group comprised 39 non-APS thrombosis patients, all treated with warfarin at our coagulation unit. These control groups will be referred to in this thesis as healthy controls (HCs) and non-APS thrombotic controls (non-APS TCs), respectively.

In paper III, 52 healthy, non-NSAID/ASA-treated individuals recruited as in study II served as the control group. The control groups in study II and III were partly but not completely overlapping.

3.1.3 Paper IV and V

The participants in study IV and V are from the Karolinska SLE cohort. All patients in this cohort fulfilled at least four of the 1982 revised ACR criteria for SLE (222). The study population in these two cross-sectional studies thus overlaps with the participants in study I and we used the same definitions for clinical events as in study I.

In study IV, we only included patients from whom MP data was available for both patients and their respective matched controls. Two hundred ninety SLE patients who received care at the Department of Rheumatology, Karolinska University Hospital in Solna in 2004-2011 were included. As controls, 290 “SLE-free” individuals identified through the Swedish national population registry and matched to the SLE patients for age, sex and place of residence were included.

In study V, 367 SLE patients who received care at the Department of Rheumatology, Karolinska University Hospital in Solna in 2004-2010 were included.
3.2 Laboratory investigations

3.2.1 ELISA methods for detection of aPL and LA test

In this thesis, testing for specific aPL, i.e. aCL and anti-β2GPI, was generally carried out according to clinical standard, using indirect ELISAs. In brief, the principles of these assays (see Figure 5) are that the antigens (β2GPI for the anti-β2GPI test or CL+β2GPI for the aCL test) are bound to wells in microtiter plates and non-specific binding sites are blocked, after which and patient samples are added. If aPL is present in the patient sample, it will bind to the solid phase. The plate is then washed to remove unspecific serum and plasma components and an antihuman IgG/M-antibody labelled with an enzyme is added. Unbound reagent is removed by washing and the substrate for the bound enzyme is added. If a reaction subsequently occurs, a colour change is yielded and the bound antibodies can be detected spectrophotometrically (236, 237).

In paper I, IgG aCL and IgM aCL as well as IgG anti-β2GPI were analysed by a routinely performed ELISA (Orgentec, Mainz, Germany) and a new fully automated fluorescence enzyme immunoassay method (Elia Cardiolipin IgG, Elia Cardiolipin IgM, and Elia β2Glycoprotein I IgG performed on Phadia 250, Phadia AB, now Thermo Fisher Scientific,
Germany), according to the manufacturer’s descriptions. With the latter method, IgM anti-β2GPI was also analysed (Elia β2-Glycoprotein I). The two methods are, when compared in this thesis, referred to as the routine ELISA method (RM) and the automated method (AM).

In paper II, III, IV and V, IgG aCL, IgM aCL and IgG anti-β2GPI were analysed by the routinely performed ELISA (Orgentec, Mainz, Germany) as described above.

Anti-β2GPI IgM was determined in paper IV and V and IgA aCL and IgA anti-β2GPI were determined in paper V with a multiplex immunoassay, Bioplex 2200 APLS IgG, IgM, and IgA (Bio-Rad Laboratories, Hercules, CA, USA).

Figure 5: Principles of antiphospholipid (aPL) ELISA testing

The primary method for LA determination in this thesis was a dRVVT method. Reagents from Biopool, Umeå, Sweden and Gradipore, North Ryde, Australia were used in accordance with standard procedure. Patient samples were diluted 1:1 with normal plasma and clotting time was measured with addition of the LA reagent and compared to the clotting time for normal plasma. As a confirmation test, excess phospholipids were added. LA was considered positive if a normalisation of the clotting time was achieved according to the local cut-off. In February 2010, we began to routinely use a new testing method at our clinic. This method uses two integrated tests (one dRVVT and one APTT) but skips the routine mixing with normal plasma. However, since most samples were analysed before February 2010, only a very small percentage of our patients’ samples were analysed according to this new method.

3.2.2 Fibrin network permeability

In this work, the fibrin permeability was examined using a liquid permeation technique originally described by Blombäck et al (165, 166) and later modified according to He et al in 2005 (167). In brief, plasma samples are dialysed against a dialysing buffer to ensure constant ionic strength in all examined samples. The buffer also contains the fibrinolysis inhibitor aprotinin. Subsequently, CaCl$_2$ and thrombin, the latter in relatively high concentration, are added. The mixture is left in plastic cylinders overnight so that clots can be formed. A buffer is then passed through the clots at different pressures and the amount of fluid that has permeated the fibrin gels at a given time is recorded and used to calculate a specific permeability constant (Darcy constant, Ks). A low Ks value indicates less permeability and a tighter fibrin clot (166, 167).
In study II, blood samples from the 49 APS patients and 19 HCs were centrifuged at room temperature to receive platelet-poor plasma (for 20 min at 2570 and 2000 g, respectively) which was then dispensed into aliquots and stored at -70°C to -80°C. Blood samples from the 39 non-APS TCs were also centrifuged (15 min at 2000 g) prior to storage at -70°C to -80°C. The APS-patient plasma samples, samples from HCs, and samples from non-APS TCs (total volume 500 µL) were dialysed at 4°C against dialysing buffer (pH 7.4, 0.05 mol/L Tris, 0.1 mol/L NaCl (VWR, number: 16404-1), 1 mmol/L ethylenediamine tetraacetic acid (EDTA) (Fisher Scientific, Stockholm, Sweden), 5 KIU/mL aprotinin (standard drug ordered from Swedish pharmacy)) for 3 hours, with change of outer fluid at 1-hour intervals. Subsequently, 200 µL of dialysed plasma were transferred into plastic test tubes and 10 µL of CaCl₂ (440 mmol/L) and 10 µL of thrombin (Sigma Aldrich, St. Louis, MO, USA) (4.5 IU/mL) were added. Plasma was mixed and immediately transferred into small plastic cylinders where the fibrin clots were formed. The final concentrations of CaCl₂ and thrombin were 20 mmol/L and 0.2 IU/mL, respectively. The clots were left in a moist atmosphere overnight in order to allow clot formation in all samples. Thereafter, percolating buffer (pH 7.4, 0.02 mol/L Tris, 0.02 mol/L imidazole (Fisher Scientific, Stockholm, Sweden), 0.1 mol/L NaCl) was allowed to pass through the clots at 5 different hydrostatic pressures and the volume of collected eluate was measured after an indicated time (167). Kₛ (in cm²) was calculated from the equation given by Carr et al in 1977. As seen in Figure 6, the parameters determining the constant are: the volume (Q, in cm³) that passed through over time (t, in seconds), viscosity of the liquid (H, in dyne x seconds), gel length (L, in cm), internal area of the cylinder (A, in cm²), and hydrostatic pressure (ΔP, in dyne) (238, 239). For 49 APS patients, 19 HCs, and 38 non-APS TCs, Kₛ could be calculated and used in the subsequent analysis.

**Permeability coefficient - Kₛ:**

\[
Kₛ = \frac{Q \times L \times h}{(t \times A \times ΔP)} \text{ (cm}^²\times 10^{-9})
\]

- Q: elution volume
- T: percolation time
- H: viscosity
- L: gel length
- A: internal area of the cylinder
- ΔP: differ. of hydrostatic pressure

**Figure 6: Principles of fibrin permeability assay (240)**
3.2.3 Turbidimetric clotting and lysis assays

In this thesis, fibrin clot density and overall fibrinolysis function were analysed by a turbidimetric clotting and lysis assay previously described by Carter et al (183).

In study II, 49 APS patients, 19 HCs and 39 non-APS TCs were investigated with this assay. Two APS patients (one with a history of arterial thrombosis, both treated with dalteparin) were excluded from the analysis since no clotting reaction was initiated in the analysis of their samples. Plasma samples from 47 APS patients, 19 HCs, and 39 non-APS TCs were eventually included in the analyses.

For the turbidimetric clotting assay, 75 µl of assay buffer (pH 7.4, 0.05 mol/L Tris-hydrochloric acid, 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 each well of a microplate and the absorbance at 340 nm was read every 18 sec (240 cycles for each sample). The turbidimetric lysis assay was carried out in the same way as the clotting assay, but with the addition of recombinant, single-chain tPA (activity at least 400 000U/mg, Technoclone GmbH, Vienna, Austria) to 75 µl of the assay buffer (final concentration of recombinant tPA 83 ng/mL). Both assays were run simultaneously in the same plate.

From the turbidimetric clotting assay, the following variables, based on previous studies (183), were measured:

1) Lag time C ($\text{Lag}_C$): the time at which an exponential increase in absorbance occurred.
2) $\Delta$ Maximum absorbance 50 ($\Delta \text{MaxAbs 50}_C$): the highest absorbance value minus the absorbance at the baseline level. This variable was determined at the plateau phase reached at 50 min for all patients.

From the turbidimetric lysis assay, the following variables were measured:

1) $\text{Lag time}_L$ ($\text{Lag}_L$): the time at which an exponential increase in absorbance occurred.
2) $\text{Lys}_{50T}$: the time from the addition of thrombin and calcium to the time at which 50% fall in absorbance from $\Delta$ Maximum Absorbance (Maximum Absorbance minus baseline) in the lysis assay ($\Delta \text{MaxAbs}_L$) is achieved.
3) $\text{Lys}_{50C}$: the time between a 50% increase and a 50% decrease in $\Delta \text{MaxAbs}_L$.
4) $\text{Lys}_{50MA}$: the time from $\Delta \text{MaxAbs}_L$ to the time at which 50% reduction in absorbance has occurred.

3.2.4 Scanning electron microscopy

In study II, the structure of the fibrin network was studied using scanning electron microscopy (SEM). Electron microscopy is a microscopy technique that uses electrons instead of light for the imaging of objects, allowing much smaller structural details to be visualised. Two main variants exists: transmission electron microscopy (TEM) and scanning electron microscopy (SEM). In SEM, an image is formed point by point using a focused electron beam to scan the surface of a solid specimen (241).
In our study, three clots formed in assays from APS patient plasma and one clot formed in assay from HC plasma were fixed in 2% glutaraldehyde in HEPES-buffered saline (HBS) (Medicago, Uppsala, Sweden) for 60 min at room temperature and then stored at 4°C. The specimens were briefly rinsed in distilled water and placed in 70% ethanol for 10 min, 95% ethanol for 10 min, absolute ethanol for 15 min, and pure acetone for 10 min at room temperature and then transferred to tetramethylsilane (Merck, Darmstadt, Germany) for 10 min and air-dried. After drying, the specimens were mounted on an aluminium stub and coated with carbon (Bal-Tec MED 010, Balzers, Lichtenstein). The specimens were studied in an Ultra 55 field emission scanning electron microscope (Carl Zeiss, Oberkochen, Germany) at 3 kV(242).

3.2.5 Determination of MPs by flow cytometry

In study III and IV, MPs were studied using a Beckman Coulter Gallios flow cytometer as described previously by Mobarrez et al (243, 244) (see Figure 7). The overall principle of MP determination by flow cytometry is that particles in samples incubated with fluorescent antibodies are passed through a laser beam while the light scattered and emitted from each particle is recorded. Through this procedure, particles can be characterised by size, complexity, and phenotype (220, 245).

In paper III, we defined MPs as particles <1.0 µm in size, negative to phalloidin-Alexa 660 (a cell-fragment marker, which binds to intracellular actin; Invitrogen, Paisley, United Kingdom), positive to lactadherin-FITC (binds to PS; Haematologic Technologies, VT, USA), and positive to either CD42a-PE (PMPs; BD, NJ, USA), CD14-PC7 (monocyte MP=MMPs; Beckman Coulter, CA, USA), or CD144-APC (EMPs; AH diagnostics, Stockholm, Sweden). Moreover, exposure of CD142-PE (TF; BD, NJ, USA) was measured on EMPs. Conjugate isotype-matched immunoglobulins (IgG1-FITC, IgG1-PE, IgG1-PC7 and IgG1-APC) with no reactivity against human antigens were used as negative controls. In the present study, results are presented as absolute numbers of MPs: (MPs counted * standard beads added/L)/standard beads counted (FlowCount, Beckman Coulter, CA, USA). The MP gate was set using Megamix beads (0.5 µm, 0.9 µm, and 3.0 µm; BioCytex, Marseille, France). Analysis were conducted on thawed citrate plasma from patients and controls that had previously been drawn, centrifuged (for 20 min at 2570 and 2000 g, respectively) and stored at -70°C to -80°C. Prior to flow cytometry analysis, the samples were again centrifuged at 2000 g for 20 min and the supernatant was then re-centrifuged at 13 000 g for 2 minutes at room temperature. The supernatant was then used for the MP analysis.

In paper IV, the method for MP detection was similar to the method used in paper III with the same markers for cell fragments, PS exposure, EMPs, and TF. As a marker for platelets, CD41-PE (Abcam, Cambridge, United Kingdom) was used. CD45-PC7 (Beckman Coulter, Brea, CA, USA) was used for leukocytes, CD154-APC (BD, San Jose, CA, USA) for CD40 ligand (CD40L), CD106-PE (AH diagnostics, Stockholm, Sweden) for VCAM-1, and anti-C4d-FITC (ALPCO Diagnostics, Salem, NH, USA) for C4d. High-mobility group protein B1 (HMGB1) (anti-HMGB1-PE; R&D Systems, Minneapolis, MN, USA) was also measured. Measurement of C4d was not performed in the presence of lactadherin, as they are both conjugated with FITC dye. Thus, both PS+ and PS- MPs were included when C4d-expressing MPs were counted.
3.2.6 Other laboratory measurements

For the 49 APS patients in paper II, fibrinogen concentrations in plasma were measured using a BN Prospec nephelometer (Dade Behring, Deerfield, IL, USA) with reagents from the same manufacturer. For the 39 non-APS TCs and 17 HCs, fibrinogen concentrations in plasma were analysed using a Sysmex® CS2100i (Sysmex, Kobe, Japan) with reagent Dade Thrombin from Siemens Healthcare Diagnostics Inc (Tarrytown, NY, USA). High-sensitivity C-reactive protein (hsCRP) was measured using a Beckman Coulter Synchron LX system Chemistry Analyser with reagents and calibrators supplied by Beckman Coulter Inc. (Sydney, NSW, Australia).

3.3 Statistical analyses

All statistical analyses were performed using JMP software (SAS Institute, Carey, NC, USA) or Statistical Analysis System (SAS). A p-value <0.05 was considered statistically significant.

The Kappa coefficient was used for analyses of agreement between aPL tests. Calculations of OR with 95% confidence interval (CI) were used to compare the performance of the aPL tests in a clinical setting. Chi-square test or Fisher’s exact test were used for comparisons of categorical variables. For continuous variables, comparisons were made using t-tests/ANOVA in the case of normally distributed data and Mann-Whitney U test (Wilcoxon rank sum test) in the case of non-normal distributions. Spearman’s correlation coefficient (r) was used as a nonparametric measure of the correlation between continuous variables. Multivariable logistic regression
models were performed to analyse any association between a dependent dichotomous variable and multiple predictor variables.

In paper IV, data was log transformed, if necessary, to obtain a normal distribution. Patients and matched controls were then compared with paired tests; McNemar’s test or paired t-test depending on data type. Association between MPs and clinical and laboratory characteristics were analysed with linear regression or t-test.

In paper V, restriction was used as a method to assess possible effect modification. Stratified analyses and sensitivity analyses were performed to investigate the possibility of reverse causation explaining the associations between former smoking and aPL. Interaction between ever smoking and aPL for the risk of events was assessed with departure from additivity of effects as the criterion of interaction (246). The attributable proportion due to interaction (AP) was computed with 95% CI (247).

Some of the studies in this thesis include a rather high number of conducted analysis. Since we tested a number of predetermined hypothesis no correction for multiple comparisons was made. Therefore it is justified to treat associations that are borderline statistically significant with some caution.
4 RESULTS

4.1 Paper I

4.1.1 Agreement between the two methods for aPL testing

The cut-offs recommended by the manufacturer were generally higher than the 90\textsuperscript{th} percentile cut-off but lower than the 99\textsuperscript{th} percentile cut-off of our controls.

We used data from the 331 patients with continuous values to calculate the Kappa coefficients for cut-offs corresponding to the 99\textsuperscript{th} and 90\textsuperscript{th} percentile, while all 712 patients were used to calculate the Kappa coefficient for the cut-offs corresponding to the manufacturer’s recommendation. Kappa values ranged from 0.16-0.71. For IgG aCL, IgM aCL, and IgG anti-\(\beta_2\)GPI, agreement between the two methods were best using the manufacturer’s recommended cut-off.

4.1.2 Association between different aPLs and vascular events and the impact of different cut-offs for positivity

ORs for the associations with previous vascular events using cut-offs corresponding to the 99\textsuperscript{th} and 90\textsuperscript{th} percentile (n=331) and the manufacturer’s recommendation (n=712) for single-positive aPL-tests as well as for double positivity are presented in Table 5.
## Table 5: Associations between the aPL tests and thrombosis, using different cut-offs

<table>
<thead>
<tr>
<th>Test</th>
<th>Any thrombosis</th>
<th>Arterial thrombosis</th>
<th>Ischaemic heart Disease (IHD)</th>
<th>Ischaemic cerebrovascular disease (ICVD)</th>
<th>Venous thrombosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG aCL (RM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99th percentile</td>
<td>1.9 (1.03-3.4)</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td>3.1 (1.6-6.0)</td>
</tr>
<tr>
<td>90th percentile</td>
<td>2.3 (1.4-3.8)</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td>2.9 (1.6-5.1)</td>
</tr>
<tr>
<td>Manufacture</td>
<td>2.3 (1.6-3.5)</td>
<td>1.6 (1.01-2.5)</td>
<td>Ns</td>
<td>2.1 (1.2-3.6)</td>
<td>1.6 (1.7-4.1)</td>
</tr>
<tr>
<td>IgG aCL (AM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99th percentile</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>90th percentile</td>
<td>1.8 (1.1-3.1)</td>
<td>Ns</td>
<td>Ns</td>
<td>3.1 (1.7-5.6)</td>
<td></td>
</tr>
<tr>
<td>Manufacture</td>
<td>2.2 (1.4-3.2)</td>
<td>1.6 (1.02-2.5)</td>
<td>Ns</td>
<td>2.2 (1.3-3.9)</td>
<td>2.7 (1.7-4.2)</td>
</tr>
<tr>
<td>IgG anti-β2GPI (RM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99th percentile</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td>2.9 (1.1-7.7)</td>
<td>2.6 (1.1-6.1)</td>
</tr>
<tr>
<td>90th percentile</td>
<td>2.0 (1.2-3.3)</td>
<td>Ns</td>
<td>Ns</td>
<td>2.7 (1.5-4.8)</td>
<td></td>
</tr>
<tr>
<td>Manufacture</td>
<td>2.2 (1.5-3.2)</td>
<td>1.7 (1.1-2.5)</td>
<td>Ns</td>
<td>2.1 (1.2-3.5)</td>
<td>2.6 (1.7-4.0)</td>
</tr>
<tr>
<td>IgG anti-β2GPI (AM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99th percentile</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>90th percentile</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td>2.5 (1.4-4.4)</td>
<td></td>
</tr>
<tr>
<td>Manufacture</td>
<td>2.1 (1.4-3.1)</td>
<td>1.6 (1.02-2.5)</td>
<td>Ns</td>
<td>2.6 (1.5-4.5)</td>
<td>2.1 (1.3-3.4)</td>
</tr>
<tr>
<td>IgM aCL (RM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99th percentile</td>
<td>1.8 (1.03-3.2)</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>90th percentile</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>Manufacture</td>
<td>1.7 (1.2-2.5)</td>
<td>1.7 (1.1-2.6)</td>
<td>1.7 (1.02-3.0)</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>IgM aCL (AM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99th percentile</td>
<td>Ns</td>
<td>2.4 (1.05-5.3)</td>
<td>2.7 (1.002-7.2)</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>90th percentile</td>
<td>Ns</td>
<td>Ns</td>
<td>2.3 (1.1-4.7)</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>Manufacture</td>
<td>Ns</td>
<td>1.6 (1.03-2.4)</td>
<td>Ns</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>IgM anti-β2GPI (AM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99th percentile</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>90th percentile</td>
<td>2.0 (1.3-3.2)</td>
<td>2.1 (1.2-3.5)</td>
<td>4.8 (2.2-10.3)</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>Manufacture</td>
<td>1.6 (1.06-2.5)</td>
<td>1.7 (1.05-2.7)</td>
<td>Ns</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>Double positivity (RM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99th percentile</td>
<td>2.0 (1.01-4.0)</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td>2.7 (1.3-5.6)</td>
</tr>
<tr>
<td>90th percentile</td>
<td>2.4 (1.5-3.9)</td>
<td>1.8 (1.1-3.1)</td>
<td>Ns</td>
<td>2.6 (1.5-4.6)</td>
<td></td>
</tr>
<tr>
<td>Manufacture</td>
<td>2.5 (1.7-3.7)</td>
<td>1.8 (1.2-2.8)</td>
<td>Ns</td>
<td>2.4 (1.4-4.1)</td>
<td>2.9 (1.8-4.5)</td>
</tr>
<tr>
<td>Double positivity (AM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99th percentile</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td>Ns</td>
<td></td>
</tr>
<tr>
<td>90th percentile</td>
<td>2.2 (1.3-3.7)</td>
<td>Ns</td>
<td>Ns</td>
<td>2.5 (1.4-4.6)</td>
<td></td>
</tr>
<tr>
<td>Manufacture</td>
<td>2.6 (1.7-4.0)</td>
<td>2.0 (1.3-3.3)</td>
<td>Ns</td>
<td>3.0 (1.7-5.3)</td>
<td>2.6 (1.6-4.3)</td>
</tr>
<tr>
<td>LA</td>
<td>5.4 (3.1-9.4)</td>
<td>3.1 (1.7-5.6)</td>
<td>Ns</td>
<td>5.0 (2.5-10.0)</td>
<td>4.3 (2.3-8.0)</td>
</tr>
</tbody>
</table>

OR (95% CI) for thrombotic manifestations using the Routine method (RM) and Automated method (AM) respectively with cut-offs corresponding to the 99th percentile and 90th percentile of controls (n=731) and manufactures recommendation (n=712) as well as OR for Lupus anticoagulant (LA) (n=180). aPL = Antiphospholipid antibody. aCL = Anticardiolipin. anti-β2GPI = Anti-β2 glycoprotein-I. IgG = Immunoglobulin. Ns = Number. Ns = Non-significant.
4.1.3. Comparison between the specific aPL tests and the LA test regarding association with vascular events

For comparison, OR for LA, using data from the 380 Karolinska patients, are also shown in Table 5. When we compared a positive LA test to double positivity with any of the two specific aPL methods using the manufacturer’s recommended cut-off, OR for any vascular event was approximately twice as high.

4.1.4. Associations between aPL tests and other clinical features of APS

Data on obstetric morbidity as defined in the APS criteria (3) were available for 296 female patients from Karolinska: 50 patients (17%) had experienced such events. Of the aPLs measured, merely IgG aCL (RM) and LA were associated with obstetric APS manifestations: OR 2.3 (95% CI 1.2-4.7) and 2.2 (1.1-4.4), respectively.

A substantial proportion of the SLE patients (142 out of 712) had presented with thrombocytopenia. IgG aCL (RM), IgG anti-β2GPI (RM), IgG anti-β2GPI (AM), and LA were associated with thrombocytopenia: ORs 1.8 (1.2-2.8), 1.6 (1.0-2.5), 1.8 (1.1-2.8) and 2.4 (1.4-4.4), respectively.

4.2 Paper II

4.2.1 Fibrin porosity and concentration of fibrinogen

Ks values for the APS samples were lower compared to both HC samples and non-APS TCs samples (p<0.0001 for both), see Table 6.

Table 6: Fibrin network porosity and turbidimetric results in patients vs. controls

<table>
<thead>
<tr>
<th>Parameter, unit</th>
<th>APS Patients</th>
<th>Healthy controls (HCs)</th>
<th>NonAPS thrombosis controls (non-APS TCs)</th>
<th>P-value APS vs. HC</th>
<th>P-value APS vs. non-APS TCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ks, cm²x10⁻⁹ (n=49+19+38)</td>
<td>6.3 (4.7-7.5)</td>
<td>10.2 (7.4-12.1)</td>
<td>11.7 (8.6-15.2)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ΔMaxAbs 50°C, Absorbance Units (n=47+19+39)</td>
<td>0.41 (0.36-0.47)</td>
<td>0.34 (0.32-0.38)</td>
<td>0.50 (0.44-0.56)</td>
<td>0.006</td>
<td>0.0003</td>
</tr>
<tr>
<td>Lys₅₀₀ₐ, seconds (n=47+19+39)</td>
<td>1596(1170-1850)</td>
<td>1207 (1124-1494)</td>
<td>1412 (1301-1583)</td>
<td>0.002</td>
<td>0.01</td>
</tr>
<tr>
<td>Lys₅₀₀ₐ, seconds (n=47+19+39)</td>
<td>1456 (1170-1850)</td>
<td>1207 (1124-1494)</td>
<td>1412 (1301-1583)</td>
<td>0.002</td>
<td>0.01</td>
</tr>
<tr>
<td>Lys₅₀₀ₐ, seconds (n=47+19+39)</td>
<td>967 (765-1195)</td>
<td>847 (779-1042)</td>
<td>1000 (863-1113)</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Lys₅₀₀ₐ, seconds (n=47+19+39)</td>
<td>374 (330-409)</td>
<td>280 (246-297)</td>
<td>246 (208-272)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lys₅₀₀ₐ, seconds (n=47+19+39)</td>
<td>381 (333-445)</td>
<td>276 (252-306)</td>
<td>257 (229-294)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

P-value<0.05 using Mann-Whitney U test is interpreted as a significant difference between the groups. Values are given as median (interquartile range). Ns=Non-signficiant difference between the groups. ΔMaxAbs 50°C = Maximum Absorbance at 50 minutes minus baseline level. Lys₅₀₀ₐ =the time from the addition of thrombin and calcium to the time at which 50% fall in absorbance from ΔMaximum Absorbance in the turbidimetric lysis assay (ΔMaxAbs₅₀°C) occurred. Lys₅₀₀₀ₐ = the time between a 50% increase and fall in ΔMaxAbs₅₀°C, in the turbidimetric lysis assay. Lys₅₀₀ₐ = the time from ΔMaxAbs₅₀°C, to the time at which 50% reduction in absorbance occurred in the turbidimetric lysis assay. Lag₅₀°C = the time at which an exponential increase in absorbance occurred in the turbidimetric clotting assay. Lag₅₀₀ₐ =the time at which an exponential increase in absorbance occurred in the turbidimetric lysis assay.
Plasma fibrinogen concentrations were higher in APS patients as compared to both HC and non-APS TCs (p<0.0001 for both). However, both fibrinogen and Ks values remained independently associated with group assignment in a multivariable adjusted analysis (p=0.005 for Ks in the first model comparing APS vs. HC, p=0.001 for Ks in the second model comparing APS vs. non-APS TCs).

Within the APS group, IgM aCL and dalteparin treatment were inversely associated with fibrin network tightness (p=0.007 and p=0.049, respectively). Apart from these two associations, the Ks values did not vary depending on differences in treatment, clinical APS manifestations, or the aPL test supporting the diagnosis at inclusion. The Ks values also did not correlate with age (r= 0.02, p>0.05, n=49), CRP (r=−0.18, p>0.05, n=45), fibrinogen concentration (r=−0.28, p>0.05, n=49), antibody titer (IgM aCL : r=0.15, n=46; IgG aCL r=0.13, n=47; IgG anti-β2GPI , r=0.16, n=47, p>0.05 for all), or number of MPs (r=0.15, 0.04, 0.02, -0.06 and 0.10 for MPs positive for lactadherin, Cd42a, Cd144, Cd14, and TF+Cd144 respectively, n=49, p>0.05 for all).

4.2. SEM

The fibrin structure in the SEM images from the APS samples was more compact with smaller intrinsic pores when visually compared to the image from the control sample. Moreover, the fibres in the APS samples were thinner with increased branch-points. The SEM image from an APS sample and the image from the control sample are shown with the same magnification (200 nm) in Figure 8.

A.  

B.  

Figure 8: Scanning electron microscopy images (SEM) of fibrin network in one healthy control sample (A) and one representative sample from a patient with antiphospholipid syndrome (APS) (B)
4.2.3 Turbidimetric clotting and lysis assays

Results from the turbidimetric clotting and lysis assays are shown in Table 6. In the turbidimetric lysis assay, the results of the various assessments of lysis of fibrin fibrils (Lys50_t0, Lys50_C, Lys 50_MA) were not uniform. For Lys50_C and Lys 50_MA, there was no difference between APS patients and the two control groups, respectively.

Within the APS group, longer clot lysis times were observed in the subgroup with previous arterial events compared to the rest of the patients (p=0.01, 0.006, and 0.008 for Lys50_t0, Lys50_C, and Lys 50_MA, respectively). To further investigate this, we compared the APS subgroup with history of an arterial thrombosis (n=15) to the HCs and the non-APS TCs. We then found a more distinct difference with longer clot lysis time in the arterial APS subgroup as compared to both control groups. For all three lysis parameters, a difference between APS patients with arterial events and the two control groups were found (p<0.05 for all analyses).

4.3 Paper III

4.3.1 Microparticle measurement

The number of total lactadherin-positive MPs, EMPs, TF-positive EMPs, and MMPs in samples from APS patients were increased compared to healthy controls (p<0.001 for all analyses). There was no significant difference between the number of PMPs in APS samples compared to control samples (p=0.13).

APS patients also had a higher proportion of MPs negative to lactadherin as compared to healthy controls (p<0.001).

4.3.2 Microparticles and clinical associations

Neither the number of lactadherin-positive nor the proportion of lactadherin-negative MPs differed between different clinical manifestations or treatments (p>0.05 for all analyses).

4.4 Paper IV

4.4.1 Microparticle measurement

All PS’ MPs were more frequent in SLE patients compared to matched controls (p<0.0001); numbers were roughly three times higher in the SLE patients. Platelet-derived MPs, endothelial-derived MPs, and leukocyte-derived MPs were 2-3 times more frequent in patients compared to controls (p<0.0001, Figure 9 and Table 7). The activation and inflammation markers CD40L, VCAM-1, TF, and HMGB1 expressed on MPs were also higher in SLE patients (p<0.0001). C4d-expressing PMPs and EMPs were nearly 10 and 5 times higher, respectively, in patients as compared to controls.
Figure 9. Number of total PS$^+$ microparticles (MPs), platelet microparticles (PMPs), leukocyte microparticles (LMPs) and endothelial MPs (EMPs) in SLE patients and controls.

Microparticles were measured on a flow cytometer and phenotyped according to cellular origin. The figure shows numbers of all PS$^+$ MPs, platelet MPs (PMPs), leukocyte MPs (LMPs) and endothelial MPs (EMPs) in SLE patients and controls. Line indicates median value. Log transformation was performed, when needed, to ensure normally distributed data, after which a Paired T-test was used to compare groups. *** = p<0.0001.
Table 7: Number of microparticles in 290 SLE patients vs. 290 matched non-SLE controls

<table>
<thead>
<tr>
<th>Microparticles</th>
<th>SLE</th>
<th>Controls</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>median, (IQR)</td>
<td>median, (IQR)</td>
<td></td>
</tr>
<tr>
<td>PS+ MPs</td>
<td>25 487 (18 898-34 016)</td>
<td>8 613 (5 218-11 535)</td>
<td>2.9x10^{-80}</td>
</tr>
<tr>
<td>PMPs#</td>
<td>12 845 (9 342-17 504)</td>
<td>6 042 (3 519-8 716)</td>
<td>8.3x10^{-55}</td>
</tr>
<tr>
<td>PMPs + CD40L</td>
<td>1 196 (842-1 588)</td>
<td>328 (163-508)</td>
<td>1.2 x 10^{-76}</td>
</tr>
<tr>
<td>PMPs + C4d</td>
<td>4 576 (2 250-7 239)</td>
<td>467 (341-637)</td>
<td>1.7x10^{-44}</td>
</tr>
<tr>
<td>EMPs#</td>
<td>2 306 (1 638-3 506)</td>
<td>633 (376-883)</td>
<td>9.8x10^{-75}</td>
</tr>
<tr>
<td>EMPs + VCAM-1#</td>
<td>300 (168-548)</td>
<td>138 (88-200)</td>
<td>4.1x10^{-33}</td>
</tr>
<tr>
<td>EMPs + C4d</td>
<td>1 083 (672-1 477)</td>
<td>270 (122-575)</td>
<td>4.2x10^{-43}</td>
</tr>
<tr>
<td>EMPs + TF</td>
<td>778 (453-1253)</td>
<td>171 (106-322)</td>
<td>5.9x10^{-33}</td>
</tr>
<tr>
<td>LMPs#</td>
<td>1 678 (1 166-2 568)</td>
<td>636 (337-983)</td>
<td>2.2x10^{-45}</td>
</tr>
<tr>
<td>LMPs + TF#</td>
<td>583 (356-1 038)</td>
<td>344 (197-530)</td>
<td>1.6x10^{-14}</td>
</tr>
<tr>
<td>MMPs+ HMGB1</td>
<td>1 529 (914-2 188)</td>
<td>500 (232-862)</td>
<td>1.1x10^{-47}</td>
</tr>
</tbody>
</table>

#Calculations performed on values that were logarithmically transformed in order to obtain a normal distribution. Paired t-test was used to compare groups. PS = Phosphatidylserine positive. MP = Microparticle. PMPs = Platelet microparticles. EMPs = Endothelial microparticles. LMPs = Leukocyte microparticles. MMPs = Monocyte microparticles. TF = Tissue factor. CD40L = CD40 ligand. VCAM-1 = Vascular cell adhesion molecule 1. HMGB1 = High-mobility group protein B1. IQR = Interquartile range: 25th percentile-75th percentile. N = number.

4.4.2 Microparticles and clinical association

Based on the MP definition in this study, we examined if MP profile could be used to distinguish between SLE patients with or without APS or between SLE patients with or without AVE. We performed multivariable analyses with APS and AVE as outcome. We presented two models for each outcome. In the first model, we adjusted for disease duration. In the second model, we adjusted for age, sex, and current smoking. We found associations between previous vascular events and EMPs expressing VCAM-1 (p=0.044 and p=0.047 for the two models, respectively). We also noted a non-significant trend between PMPs positive for CD40L and APS (p=0.06 and p=0.08).
4.5 Paper V

4.5.1 Association between smoking status and aPL positivity

First, we compared ever vs. never smokers, and found that ever smokers were more prone to be positive for IgG aCL, IgG anti-β2GPI, and LA (data not shown). Ever smokers were then divided into former and current smokers.

In a model adjusted for age, sex, and age at disease onset, former smoking was associated with IgG aCL, IgG anti-β2GPI, LA, and triple aPL positivity (see Table 8).

Table 8: Association between current/former vs. never smoking and positive aPL (in comparison with negativity for the respective antibody), adjusted for age, sex, and age at disease onset, presented as odds ratio (95% CI)

<table>
<thead>
<tr>
<th></th>
<th>IgG aCL</th>
<th>IgM aCL</th>
<th>IgA aCL</th>
<th>IgG anti-β2GPI</th>
<th>IgM anti-β2GPI</th>
<th>IgA anti-β2GPI</th>
<th>LA</th>
<th>Triple positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.01(0.99-1.04)</td>
<td>0.99(0.96-1.02)</td>
<td>0.99(0.97-1.82)</td>
<td>0.98(0.96-1.02)</td>
<td>0.98(0.96-1.01)</td>
<td>1.00(0.97-1.02)</td>
<td>1.00(0.96-1.03)</td>
<td></td>
</tr>
<tr>
<td>Age at SLE onset</td>
<td>0.97(0.94-0.98)*</td>
<td>1.00(0.98-1.04)</td>
<td>0.98(0.95-1.01)</td>
<td>1.01(0.98-1.05)</td>
<td>1.02(0.99-1.04)</td>
<td>1.00(0.97-1.05)</td>
<td>1.00(0.95-1.03)</td>
<td></td>
</tr>
<tr>
<td>Sex (male)</td>
<td>0.7(0.2-1.6)</td>
<td>0.6(0.2-1.4)</td>
<td>0.7(0.3-1.7)</td>
<td>0.6(0.2-1.5)</td>
<td>0.5(0.07-1.7)</td>
<td>0.8(0.3-1.9)</td>
<td>1.2(0.5-2.5)</td>
<td>1.1(0.5-3.0)</td>
</tr>
<tr>
<td>Current smoking</td>
<td>1.3(0.5-2.9)</td>
<td>2.1(0.97-4.3)</td>
<td>2.0(0.9-4.1)</td>
<td>1.4(0.6-3.2)</td>
<td>2.2(0.9-5.5)</td>
<td>1.8(0.8-3.9)</td>
<td>2.1(0.9-4.5)</td>
<td>2.7(0.9-7.8)</td>
</tr>
<tr>
<td>Former smoking</td>
<td>3.0(1.6-5.8)*</td>
<td>1.1(0.5-2.2)</td>
<td>1.5(0.8-2.9)</td>
<td>3.1(1.6-6.1)*</td>
<td>0.4(0.2-1.7)</td>
<td>1.8(0.9-3.6)</td>
<td>3.1(1.6-5.9)*</td>
<td>4.0(1.7-10.0)*</td>
</tr>
</tbody>
</table>

*p<0.05 in the multivariable model. aCL= Anti-cardiolipin, anti-β2GPI= anti-β2 glycoprotein I. LA=Lupus anticoagulant.
4.5.2 Associations between smoking, aPL and AVE

Due to a lack of information on the temporal relationship between smoking and AVE and to increase the power, we used ever smoking in the main analyses of the association between smoking, aPL, and AVEs.

In never smokers, aPL positivity was not associated with a history of AVE. In aPL-negative patients, ever smoking was moderately associated with arterial events but the combination of ever smoking and aPL was more strongly associated with both arterial and venous events. These associations were significant for IgG/A aCL, IgG/A anti-β2GPI, LA, and triple positivity (data not shown). The additive interaction analysis showed a significant interaction between ever smoking and LA (attributable proportion due to interaction, AP=0.80, 95% CI 0.5 - 1.0) (see Figure 10), and ever smoking and triple positivity (AP=0.85, 95% CI 0.6 - 1.0) concerning the association with a history of AVE.

Figure 10. Ever smoking and Lupus anticoagulant (LA) in relation to previous vascular events

Art/ven = Any arterial/venous event. IHD= Ischemic heart disease. ICVD= Ischemic cerebrovascular disease. VTE= Venous thromboembolism.
AP=Attributable proportion due to interaction
5 SUMMARY AND DISCUSSION

5.1 Our findings contribute to increased knowledge of aPL testing and improved APS diagnosis and clinical risk assessment

5.1.1 Results from a new automated aPL assay had similar associations to clinical events as the routine ELISA

In study I, we report only moderate agreement but similar associations with previous thrombotic events when comparing a new automated aPL method with standard assays in a large group of Swedish SLE patients. Overall, the associations with thrombosis were modest. This was also true for the associations with obstetric morbidity and thrombocytopenia investigated as complementary analyses.

With this study, we were one of the first groups to conduct a comparative, clinical study between a new, fully automated aPL method and currently used routine ELISAs. Since the levels of performance were mostly comparable for the two assays, our results agree with the currently growing body of literature presenting more automated aPL assays as a promising strategy for facilitating aPL testing. These new assays may also improve reproducibility and reduce interlaboratory variation (98-105). If this is achieved, it would be a step forward in aPL diagnostics.

5.1.2 aPL of IgG and IgM isotype show different associations with clinical events

In study I, we noticed that IgG antibodies were primarily associated with VTE and ICVD while IHD was only associated with aPL of the IgM isotype. Our study thus gives some support to keeping both IgG and IgM aPL tests in the “standard investigation panel.” This is noteworthy since the rationale for keeping the IgM tests has been questioned as many (7, 23, 24, 33, 65, 74, 75, 82-84), though not all (36, 42, 76-81), earlier studies have indicated poor clinical utility. Moreover, the information from our study is interesting since it indicates that isotype-specific patterns of APS manifestations may exist. These patterns should be further explored in prospective settings.

5.1.3 Antibody titer is important when results from aPL testing are used for individual risk assessment

From our results in study I, we conclude that associations with clinical events differ according to aPL titer. This is in accordance with previously well-established knowledge. Many earlier studies have reported that higher levels of aPL are more strongly associated with clinical events than lower levels (61-68) and the current cut-offs for positivity were based on the best available evidence 2006 when the APS criteria were last modified (3). In our study, we examined how three different cut-offs performed in relation to previous thrombotic events. Surprisingly, we noticed that aPL titers below the criteria cut-off were also important, i.e. there were positive associations between such titers and clinical events. We have previously reported that low aPL titers are predictive of the first arterial event in a prospective study of SLE patients (30) and others have proposed that low titer aPL might be included in the diagnosis of obstetric APS (71). It is possible that different aPL cut-offs should be used for different clinical entities and situations, modified according to the purpose of aPL testing and background risk. This type of flexible approach could perhaps result in a more true estimate of the individual risk.

5.1.4 LA and other factors such as smoking are also important when results from aPL testing are used for individual risk assessment

The equivalent performance but modest agreement between the two assays in study I might appear paradoxical. An important reason for this inconsistency is probably that associations between thrombotic events and a single-positive aPL ELISA test are generally weak. These associations are clearly less strong than with LA positivity, which in our study performed better overall than both the specific aPL immunoassays. Similar observations have been published by other groups and have led to increased
Other vascular risk factors (30, 94) such as oral contraceptives and smoking are also important, however, and seem to interact with aPL and impact the overall risk for individual SLE patients (9). In study V, we report that despite the individual associations between smoking and aPL with AVE being rather modest, the combination of the two variables lead to a distinctive increase of the ORs. A significant interaction was found between ever smoking and LA as well as aPL triple positivity for the association with AVE. These results are in line with the findings by Urbanus et al who reported that the combination of aPL and smoking was associated with a several-fold enhanced risk for the occurrence of MI and stroke in young women from the general population (9). We believe that this knowledge is important to account for in risk assessment of individual patients. In everyday practice, it implies that aPL-positive SLE patients who have never smoked are in a different risk category compared to aPL-positive former/current smokers. Based on this information, it would perhaps be wise to include smoking status in the APS risk scores that are currently being developed (91-93).

In summary, we agree with Roube (4) that aPL testing should in most situations be regarded as part of a general risk stratification for specific clinical events in certain groups of patients rather than a diagnostic procedure. A reliable risk estimate is crucial in the clinical situation since it is mandatory for planning surveillance and treatment of the individual patient. In this context, the risk for thrombosis or other APS manifestations must of course be compared to the risk of developing side effects from primary/secondary prophylaxis (18).

5.2 Our findings contribute to the understanding of the pathogenesis of aPL-related disease and give clues to the search for new biomarkers

5.2.1 Abnormal clot properties is a feature of APS

In study II, we report for the first time that fibrin clots formed in vitro in plasma samples from APS patients have a decreased permeability compared to the clots formed in samples from healthy controls and from non-APS thrombosis controls. In addition, SEM images visually confirm denser fibrin structure in the samples from APS patients as compared to a control sample. Since tighter fibrin networks are more resistant to fibrinolysis (160-163) and impaired fibrinolytic capacity has been associated with APS (109, 184, 185, 187), we conducted additional turbidimetric lysis analyses. On a group level there was no clear difference in overall fibrinolysis function between the APS patients and the two control groups. However, fibrinolysis properties differed between APS subgroups. We observed that the APS patients with a history of arterial thrombosis had prolonged clot lysis time as compared to our two non-APS control groups.

Our study adds APS to the growing list of conditions with increased cardiovascular and thrombotic risk where clots with abnormal architecture and decreased permeability are formed in in vitro assays (150-159). This is interesting since the tendency to form tight fibrin networks in vitro could reflect an in vivo situation with disturbed fibrinolysis, due to for example decreased permeability of fibrinolytic factors through the clot (160-163), even though we could in study II only demonstrate hypofibrinolysis in the arterial subgroup of our patients. Moreover, efforts to explain our finding point to different factors of possible pathogenic importance. Under our experimental conditions, some factors known to contribute to fibrin gel porosity are kept constant (see section 1.5.3). Fibrin permeability thus depends mainly on the quantity and quality of sample fibrinogen but may also be affected by possible modulators in the investigated samples (166, 167). In agreement with previous studies (189), the APS patients in our study...
indeed had high concentrations of fibrinogen. A high fibrinogen concentration is in itself a risk factor for CVD (176) and links between fibrinogen and activation/maintenance of autoimmune responses have been suggested (179). However, Ks values remained independently associated with group assignment after adjusting for fibrinogen, and fibrinogen hence does not seem to be the only factor of importance. Raised plasma levels of PAI-1, previously reported in patients with connective tissue disease (187), could play a role, given the known association between PAI-1 activity and fibrin permeability (150). APS patients have also been reported to have enhanced activity of FXIII, which correlates to both aPL, PAI, and fibrinogen (186) and which is probably relevant for clot structure and permeability (168). Altered levels of fibrinogen binding proteins could theoretically affect fibrin structure and so could cellular activity (168), possibly mediated by MPs. Previous studies demonstrate that MPs can bind to fibrin and affect its structure (170) (171). However, in our study, the numbers of different MPs in the investigated samples did not correlate with fibrin permeability. If abnormalities in circulating MPs are important for our current in vitro findings of atypical fibrin structure/function in APS, these MP abnormalities could be functional rather than quantitative in nature. However, if the primary aim of our study had been to explore the relationship between circulating MPs and fibrin permeability in APS, a modified version of the fibrin permeability assay would probably have been more suitable. In the assay we used for study II, large amounts of thrombin were added to initiate fibrin formation. Thus, the possible effect of TF, FXII, or even PS expression on MPs was bypassed, since thrombin is the common factor of the coagulation cascade (both extrinsic and intrinsic pathway). It is possible that if we had used a low dose of TF instead of thrombin as a reaction trigger, an approach probably better reflecting the in vivo processes (249) there would have been a correlation between numbers of MPs (which can for example express TF and expose PS, see discussion in section 5.2.2) and fibrin permeability.

We detected a clear difference in fibrin permeability between patients and controls, but clinical subsets of APS patients could not (with a few exceptions) be separated. The latter finding was in some ways surprising given previous knowledge and literature in the field (171, 173-175), but our population size and design may not have been optimal for subgroup analyses. The main findings in our work regarding fibrin permeability and fibrin structure as visualised with SEM were verified in a study published by Celinska-Lowenhoff soon after ours (250). This study comprised a larger group of APS patients (n=126) none of whom were taking oral anticoagulants at the time of blood sampling and a control group (n=105) of patients frequency-matched for age, sex, current smoking, and type of prior thromboembolic event, in whom APS was excluded. Besides confirming our finding of increased fibrin permeability in APS, this study also demonstrated longer clot lysis times in the whole APS group compared to controls. The intergroup differences remained after adjusting for both fibrinogen and PA-I antigen. In a more recent publication, the same group reported an association between these clot properties and intima-media thickness in APS patients (251). Like us, Celinska-Lowenhoff et al reported that APS patients with arterial events had more pronounced clot abnormalities than other APS patients (250). Taken together, clot characteristics thus seem important for APS phenotype and prospective studies will reveal potential prognostic importance. Standardisation of the assay is critical in this context (252). Why the arterial subset displays more distinct clot abnormalities than the rest of the APS patients is not clear. Differences in levels or function of fibrinogen, fibrinolysis activators, and fibrinolysis inhibitors are possible explanations. The method we used to investigate fibrinolysis is known to be sensitive to concentration of PAI-I and fibrinogen (183) so group differences in these two variables, known to be associated with CVD risk in the general population (176, 178), are good candidates. Fibrinogen levels were indeed higher in the APS patients with arterial thrombosis compared to the APS patients without arterial events in our study (p=0.02), but PA-I and other factors of possible importance were not measured. Larger groups of patients need to be investigated in order to construct multivariable models where all the variables of interest may be accounted for.
5.2.2 Increased number of MPs is a feature of APS and SLE but the exact relationship between MPs, aPL, and vascular events is unclear

With study III, we were the first group to report that TF-expressing EMPs are much more frequent in samples from APS patients than in samples from healthy controls. We also demonstrated that total number of lactadherin-positive MPs, EMPs, and MMPs in APS patients were increased as compared to healthy controls, which is in agreement with most (199-202, 209) but not all (203) previous studies. However, the number of PMPs did not differ between APS patients and controls, a finding that is also supported by some previous studies (201, 203, 211) but in conflict with others (200). Recently, Chaturvedi et al published a study where they replicated our finding of increased number of total PS-expressing MPs in APS as well as increased numbers of EMPs and TF-positive MPs compared to healthy controls. However, in contrast to our study, they reported an increase in PMPs but not MMPs (253). Our study also revealed that APS patients a had higher proportion of MPs negative to the PS marker lactadherin compared to healthy controls. Finally, the MP pattern did not discriminate between major clinical APS subsets such as “obstetric” and “thrombotic” APS.

In study IV, a large study conducted to examine MPs in SLE patients and matched controls, we found a striking elevation of the total number of circulating MPs including platelet, endothelial, and leukocyte MPs in SLE patients. This is in agreement with some (204-206, 209, 214) but partially conflicting with other (207, 208, 210-212, 215) previous, smaller studies on similar clinical populations. Moreover, all investigations of MPs exposing inflammation and/or activation markers such as CD40L, TF, VCAM-1, HMGB1, and C4d also demonstrate clearly increased numbers in the SLE patients compared to the controls. These findings are, as far as we know, novel and they support and expand previous theories that ongoing cell activation, apoptosis and/or impaired clearance of cellular remnants is a general feature of SLE pathogenesis (254). Based on our findings in study III, our a priori hypothesis was that the MP profile could be used to distinguish SLE subgroups with APS and vascular events, but we found only borderline associations between such features and MPs.

Some of the discrepancies between our findings and the findings in other MP studies in APS and SLE may be explained by methodological differences as there are many such factors that could influence the results (255). These factors are associated with different steps of the pre-analytical handling, centrifugation protocol, and the specific method used for MP detection.

Overall, the data from study III and IV support or give rise to several hypotheses about the pathogenesis of aPL-related disease: APS and SLE.

One hypothesis on the role of MPs in APS pathogenesis, presented in a review by Pericleous et al (198), suggests, based on several separate studies (199, 201, 202), that aPL positivity is sufficient to stimulate production of elevated EMPs. However, the properties of these EMPs could be different in patients with clinical APS compared to carriers of nonpathogenic aPL since it has been shown that only plasma from the former group can stimulate production of procoagulant EMPs (199) and that PMPs are not raised in aPL-positive subjects unless they had a previous thrombosis (201). Partly consistent with this hypothesis, the result from study III could imply that aPL positivity in itself is enough to cause chronic endothelial and monocyte activation associated with increased EMP and MMP count, but additional factors are required for platelet activation, PMP release, and APS events to subsequently occur. The aPL-positive patients in our study had more circulating EMPs and MMPs as compared to healthy controls. The fact that PMP count did not differ from controls might be due to the fact the samples were not obtained at the time of a clinical event but at a point in time when the patients’ disease was well-treated, i.e. associated with lower risk of new events. The MP abnormalities that we detected in the APS samples indicate nevertheless that the total surface covered with negatively charged PS is increased and exposed to the coagulation system also when APS patients are in a tranquil phase of their disease. This observation is consistent with the presence of a chronic pro-coagulant state. Additional triggers, as described by the
second hit hypothesis of APS pathogenesis (19, 54), may in this setting more easily initiate clinical events. The increased number of TF-exposing EMPs is particularly interesting. TF is pivotal for coagulation initiation, TF has previously been proposed to play a role in APS pathogenesis through aPL-mediated upregulation on different target cells (54), and TF activity from MPs of undetermined cellular origin is increased in APS patients compared to aPL carriers (217). However, to better assess the relevance of our findings, it would be of pivotal interest to measure the pro-coagulant activity of TF expressed on the EMPs. TF in the circulation may occur in different truncated forms, including an “active” and a “non-active” form (256). Various factors have been proposed to regulate TF activity such as the characteristics of the PS-positive surfaces (activated cells or MPs) where the coagulation reactions occur, factor VIIa binding, and tissue factor pathway inhibitor (257, 258). These variables could influence the extent to which the increase of TF-expressing EMPs that we detected confers a true increase in TF activity in APS patients.

Contrary to our findings in APS patients, we found increased levels of PMPs in SLE patients. PMPs exposing CD40L were also higher than in matched controls. These MPs probably contribute to autoantibody production and disease pathogenesis. Platelets and platelet activation are known to be important in SLE (259). CD40L was originally described on different immune cells, but more recent studies have revealed that platelets are in fact the main source of this inflammatory mediator, producing more than 90% of soluble CD40L (sCD40L). Platelet-derived sCD40L acts as a trigger for endothelial activation and inflammation, for example through increasing expression of VCAM-1. Levels of CD40L on immune cells as well as soluble sCD40L are increased in SLE, and a link to disease activity and B-cell activation has been demonstrated (260-263). Platelets from SLE patients can activate mesangial cells through CD40/CD40L interactions leading to glomerular injury associated with nephritis (264). There are also indications that platelet CD40L contributes to activation of the type I IFN system in SLE (265). PMPs may act as a vector for CD40L (CD154) signalling by removing CD40L from the site of platelet activation and thereby making it possible for CD40L to encounter and activate its receptor on target cells. If the target cells are B-cells, this can ultimately lead to production of an antigen-specific response (266).

The increased levels of MPs expressing the complement degradation product C4d in SLE patients is consistent with previous findings of increased levels of platelet-bound C4d in SLE. Platelet-bound C4d has in fact been proposed as a useful lupus biomarker, associated with all-cause mortality, vascular events, and aPL (267-272). Contrary to our expectations, we did not, however, find an association between C4d-exposing MPs and vascular events or aPL/APS. It is important to note that measurement of C4d on MPs was performed in the absence of lactadherin, as they both are conjugated with the same dye (FITC). Thus, C4d was measured on “all” MPs regardless of whether they exposed PS or not. By not initially differentiating MPs by PS expression, we found ten times more C4d⁺ PMPs and five times more C4d⁻ EMPs in patients than controls.

The high number of EMPs as well as EMPs positive for the adhesion molecule VCAM-1, and coagulation initiator TF in the SLE samples indicate a general endothelial dysfunction and activation similar to what we hypothesised for APS in the paragraph above. VCAM-1 is reported to have a regulatory role in peripheral tissue inflammation in several diseases such as infections, chronic inflammatory conditions, and cardiovascular diseases (273). Previous SLE studies have shown increased soluble (s) VCAM-1 levels in patients compared to controls (274, 275) and we reported previously that sVCAM-1 levels were predictive of cardiovascular events and of cardiovascular mortality (30, 231). However, study IV is the first to demonstrate an increased number of MPs expressing VCAM-1 in SLE. Surprisingly, we found only borderline association with VCAM-1-positive MPs and vascular events in this population.

Apart from a study by Nojima et al, where increased TF expression on monocytes was found to be associated with thrombosis and aPL in SLE patients (276), little is known about the role of TF in SLE.
Study IV is the first to investigate numbers of TF-expressing MPs in a large group of SLE patients. Despite the important role that TF-positive MPs seem to play in vascular disease (277), including APS as reported in study III, and their ability to increase thrombin formation in vitro previously demonstrated by us (278), we did not find any association between these MPs and vascular events/secondary APS in SLE. This is not easy to explain. There seem to be factors in SLE that lead to signs of endothelial activation/dysfunction even in the absence of aPL and overt vascular disease. Whether they are the same or different from the factors causing endothelial activation/dysfunction in primary APS, a condition emerging as a systemic autoimmune disease with close links to SLE rather than a simple autoimmune coagulopathy (218), remains to be elucidated. As discussed previously, it is however important to remember that the level of activity of the TF expressed by the MPs was not measured in our studies. This could in fact be the variable that differentiates SLE patients with vascular phenotype from the rest of the SLE population.

The increased number of MMPs exposing HMGB1 found in our SLE patients could constitute a source of extracellular HMGB1 with potential to trigger autoantibodies. HMGB1 is an intranuclear protein that under normal physiological circumstances binds to DNA and helps to control gene transcription. When it is translocated to the extracellular space during for example apoptosis, HMGB1 can stimulate pathogenic mechanisms in inflammatory and autoimmune diseases. High titers of anti-HMGB1 autoantibodies as well as increased levels of circulating HMGB1 have been detected in SLE patients (279). Levels of HMGB1 are related to disease activity (280). Moreover, the ability of HMGB1 to form stable complexes with other nuclear components from apoptotic cells is probably crucial for the loss of tolerance to auto-antigens and inflammatory response in SLE. However, HMGB1 can, like TF, undergo post-translational modifications that alter its activity (279). Our flow cytometric results demonstrate high expression of HMGB1, but we cannot assess its functional activity.

Alternative hypothesis about mechanisms in aPL-related diseases can be generated by our finding that APS patients had a large proportion of MPs negative for the PS exposure marker lactadherin. To our knowledge, this is a new finding in APS, but a recent Danish study report similar results in SLE patients. In this study, the patient samples contained increased numbers of MPs negative to the PS exposure marker annexin V, and it was suggested that this could have implications for phagocyte recognition (208). As pointed out by the Danish group, it is possible that this is caused by true lack of surface PS exposure or enzymatic degradation of PS. Blockage by endogenous ligands/immunoglobulins is another plausible explanation (208). β2GPI or β2GPI/anti-β2GPI complexes could possibly block a significant amount of the PS on MPs in patients with aPL-associated disease. Both antiβ2GPI antibodies and the circulating levels of their antigen β2GPI are increased in APS patients compared to controls (116) and it has been demonstrated that β2GPI binds to PS-expressing PMPs (112). In SLE, increased MP content of both β2GPI (216) and IgG (281) as compared to MPs from controls has been reported. Hypothetically, a substantial amount of the lactadherin-negative MPs we detected in our APS patients could be PMPs where PS was blocked by β2GPI/antiβ2GPI. With a different method for MP detection, not dependent on PS for MP definition, an increase of PMPs in APS would perhaps have been detected. Such an increase could, at least partly, be caused by antiβ2GPI interfering with β2GPI-mediated phagocytic clearance and contribute to the pro-coagulant state of APS (112). In APS, several factors such as smoking and infections may lead to increased release of circulating MPs and to increased oxidative stress (244, 282). β2GPI is upregulated by oxidative stress (283). One could speculate that the upregulation of the this “vacuum cleaner” (108) constitutes a sort of feedback mechanisms to mediate increased clearance in certain situations, but if anti-β2GPI levels also become augmented, phagocytosis is inhibited and levels of MPs with pro-coagulant potential remain elevated. Strategies to test the above hypothesis of APS-associated PMP increase masked by β2GPI/anti-β2GPI binding could be to examine the share of β2GPI/anti-β2GPI-positive MPs or to examine numbers of “PS-negative” MPs positive for platelet surface markers. Recently, an excess of serum lactadherin was reported in SLE-patients and suggested to
be associated with decreased phagocytic clearance and disease activity (284). Hence, this molecule could affect our analyses and disease pathogenesis in a way similar to what was described for β2GPI/anti-β2GPI above.

Indeed, the high number of lactadherin-negative MPs in our APS study and the striking rise of C4d-positive lactadherin-negative MPs in our SLE study as well as the results of the Danish SLE study (208) make it reasonable to believe that measuring MPs regardless of PS exposure is important in both APS and SLE. The “classical” way of defining MPs (i.e. size and PS expression) may not be suitable for such patient groups. Hypothetically, clinical subsets like SLE patients with vascular events and aPL as well as “obstetric” and “thrombotic” APS patients may be more clearly distinguished by MP profile if “all” MPs (i.e. also PS-negative MPs), were measured and further phenotyped. New associations with other parameters such as serological profile and fibrin permeability may emerge with such an approach as well.

Despite the methodological concerns that remain, we would like to underline that with our current laboratory method for MP detection, we have addressed many of the analytical and pre-analytical concerns associated with MP flow cytometry (213, 255). We used a flow cytometer with relatively good sensitivity for detection of MPs (245), lactadherin which is a more sensitive marker than annexin V for PS exposure (196), and phalloidin as a “sample quality control” to identify cell fragmentation which could occur as a consequence of freezing/thawing or other types of pre-analytic handling (245).

Finally, when discussing MPs as biomarkers for APS and SLE, it is of course important to recognise the need for prospective studies. It is possible that our future follow-up of these patients will provide us with insights on how MP profile (analysed with our current method or a modified approach) could be used as predictor of clinical outcome.

5.2.3 Smoking is important for aPL positivity and aPL-related thrombosis

In study V, we report an association between cigarette smoking (ever smoking and in particular former smoking) and aPL positivity in SLE patients. Moreover, an interaction between ever smoking and aPL was noted for the association with previous vascular events.

Our results are interesting since previous experimental studies indicate that smoking plays a role in the pathogenesis of aPL-related disease. Smoking is associated with increased oxidative stress which can affect a number of factors of importance for the development of aPL-associated events (54, 285) such as the endothelial surface receptors (132) and the conformation of the β2GPI-molecule. Patients with APS have a larger proportion of oxidized β2GPI than controls (116) which probably has implications for antibody binding (120). Smoking is also associated with increased levels of circulating MPs, which (as discussed in section 5.2.2) is another factor through which smoking could mediate increased thrombotic risk. Theoretically, there are thus a number of plausible mechanisms through which smoking in combination with other environmental and/or genetic factors could lead to immunological and vascular changes that are critical for clinical APS manifestations to occur.

It is intriguing that it is mainly former and not current smoking that is associated with aPL positivity in our study. This association was primary found for the antibodies of IgG isotype and for LA positivity. Hypothetically, smoking discontinuation could change the immunological balance and trigger either an aPL “class switch” from IgM to IgG or a rebound IgG production, as proposed by Rubin et al. In their study, autoimmune-prone mice exposed to smoke displayed a lower spontaneous production of IgG auto-antibodies than control mice, but after smoking was discontinued, IgG autoantibody production was augmented. They supported their findings in the murine model by investigating SLE patients and reported that, compared to non-smokers, patients who smoked at the time of diagnosis had lower levels of IgG anti-dsDNA while former smokers had higher levels (286). Even though larger clinical studies in
SLE patients, including our own, have reported partly conflicting results regarding the association between anti-dsDNA and former smoking (287, 288) the hypothesis put forward in the paper by Rubin et al is interesting. Through this mechanism or other pathways, smoking may cause a rise in IgG autoantibodies such as aPL of such magnitude that it could constitute a “triggering event” in individuals that are previously positive for aPL in lower titers.

5.4 Limitations

The five studies conducted are cross-sectional with the inherent limitations related to such a study design; they can generate hypotheses about associations between variables but not establish temporal and/or causal relationship. In the case of APS, it is known that aPL titers can decrease or increase over time, for example due to infections (289). This is important to bear in mind when judging the “snapshot” that these cross-sectional studies provide of the association between aPL and other variables like clinical APS manifestations. Moreover, since aPL positivity is predictive of cardiovascular mortality (231), there is a risk for selective survival bias. In other words, there is a risk that some aPL-positive patients die before study entry and are thus not included in our analyses. It is also important to mention that in study V there is, due to the study design, a risk that reverse causation explains our main finding regarding the association between former smoking and aPL.

It is difficult to determine how relevant our findings of abnormal fibrin clot properties, fibrinolytic function, and MP profile are for the in vivo situation in APS and SLE patients. Regarding MPs, we have found striking differences in the number of MPs between patients and controls but we have not investigated the activity of the proteins they express or their overall effect on biological processes. Moreover, further method development and standardisation may be needed, as discussed in 5.2.2. Concerning the in vitro methods we used to study fibrin network characteristics and fibrinolysis, one must keep in mind that these methods test only some of the factors that could be important for the situation in vivo. Factors of possible in vivo relevance but not fully taken into account by our methods include cell activity, thrombin generation and local blood flow (168).
6 CONCLUSIONS

- Antiphospholipid determinations by a new automated assay show associations to previous thrombotic events similar to the routine ELISA.

- Lupus anticoagulant positivity, antiphospholipid titer, and isotype are, together with smoking status, important for vascular risk assessment in patients with systemic lupus erythematosus.

- Lupus anticoagulant is a more powerful marker of thrombotic history than anticardiolipin and anti-β2 glycoprotein I.

- Abnormal fibrin clot properties, fibrinolytic function, microparticle profile, and smoking may contribute to the pathogenesis of antiphospholipid-associated disease.

- Microparticles are more abundant in patients with antiphospholipid syndrome and in patients with systemic lupus erythematosus than in controls.

- The exact relationship between antiphospholipid antibodies, vascular events, and microparticles in patients with systemic lupus erythematosus have to be further studied and we believe that it is important to examine phosphatidylserine -negative microparticles more closely.

- The findings in this thesis merits further prospective and mechanistic investigation to evaluate their importance for the development of new biomarkers for antiphospholipid-related disease.

Fig 11: A summary of factors relevant to pathogenesis and diagnosis of aPL-associated disease studied in this thesis
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