University of Udine

PhD course in Clinical Sciences and Technologies (XXVIII cycle)

Department of Medical and Biological Sciences

To investigate in vitro the pathogenic mechanism of anti-PS/PT antibodies to better define their role in the diagnosis of APS syndrome

Supervisor:

Prof. Francesco Curcio

Assistant supervisor:

Dott. Martina Fabris

PhD student:

Adriana Cifù

Year Of The Final Exam: 2017

Sommario

1. ABSTRACT 1
2. ANTIPHOSPHOLIPID SYNDROME (APS) 3
2.1 HISTORICAL BACKGROUND 3
2.2 CLASSIFICATION CRITERIA AND DIAGNOSIS 4
2.3 HAEMOSTASIS 7
2.3.1 CELL-BASED MODEL OF HAEMOSTASIS 8
2.4 ANTIGENIC TARGETS OF ANTIPHOSPHOLIPID AUTOANTIBODIES (aPL) 10
2.4.1 β2-GLYCOPROTEIN I 10
2.4.2 PROTHROMBIN 12
2.5 APS AND THROMBOSIS 13
2.5.1 THE “TWO-HIT” HYPOTHESIS 13
2.5.1.1 CELLULAR COMPONENTS 13
2.5.1.2 HUMORAL FACTORS 15
2.5.1.3 INFLAMMATION 16
2.6 APS AND PREGNANCY 17
2.6.1 aPL AND INFLAMMASOMES 19
2.7 RISK FACTORS OTHER THAN aPL IN APS PATIENTS 20
2.7.1 PLASMATIC PLATELET-ACTIVATING FACTOR ACETYLHYDROLASE ACTIVITY (PAF-AH) 21
3. AIM OF THE STUDY 23
4. MATERIAL AND METHODS 23
4.1 PATIENTS 23
4.2 ANTIBODY DETERMINATION AND ANTIGENIC SPECIFICITY 24
4.2.1 LA 24
4.2.2 aCL and aβ2GpI 25
4.2.3 aPS/PT 26
4.3 MEASUREMENT OF PAF-AH ACTIVITY 26
4.4 ISOLATION OF IgG 27
4.5 CELL CULTURE 29
4.5.1 ISOLATION OF MONOCYTES 29
4.5.2 HUVEC 29
4.6 PROCOAGULANT CELL TREATMENT 30
4.7 RNA ISOLATION AND REAL-TIME PCR 31
4.8 MEASUREMENT OF NITRIC OXIDE (NO) PRODUCTION 32
4.9 QUANTIFICATION OF CYTOKINES AND CHEMOKINES 33
4.10 STATISTICAL ANALYSIS 34
5. RESULTS 35
5.1 IgG PURIFICATION 35
5.2 SETTING OF PROCOAGULANT TREATMENT 36

5.3 EFFECT OF IgG FROM BLOOD DONORS' SERUM ON MONOCYTES AND HUVECs 36
5.4 EFFECT OF IgG FROM APS PATIENTS' SERUM ON MONOCYTES 38
5.5 TF EXPRESSION IN HUVECs TREATED WITH IgG OBTAINED FROM APS PATIENTS' SERUM 41
5.6 THE EFFECT OF IgG OBTAINED FROM APS PATIENTS' SERUM ON NITRIC OXIDE (NO) PRODUCTION IN HUVECs 42
5.7 SOLUBLE FACTOR RELEASED BY HUVECs AFTER PROCOAGULANT TREATMENT: PRELIMINARY DATA 42
5.8 PAF-AH 44
5.8.1 PAF-AH PLASMATIC ACTIVITY IN PATIENTS AND CONTROLS: CORRELATION WITH LIPOD METABOLIC MARKERS 44
5.8.2 PAF-AH PLASMATIC ACTIVITY IN PATIENTS DISCLOSING DISTINCT PATTERN OF aPL POSITIVITY 45
6. DISCUSSION 47
7. CONCLUSIONS 52
8. PUBLISHED ABSTRACTS 53
9. PUBLICATIONS 56
10. REFERENCES 57

1. ABSTRACT

Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by vascular thrombosis (venous or arterial) and/or adverse obstetric outcomes accompanied by persistent and elevated levels of antiphospholipid (aPL) antibodies. According to the 2006 revised international classification criteria, the presence of one among anti-beta2 glycoprotein I (aβ2GPI) IgG or IgM, anti-cardiolipin (aCL) IgG or IgM and the lupus anticoagulant (LA) is indicated for a definite diagnosis of APS. However, not infrequently, none of the "criteria" antibodies can be demonstrated. Only recently the so-called "seronegative APS" was definitely recognized as a distinctive setting, or better re-defined by the demonstration of new classes of aPL antibodies, such as the autoantibodies directed against prothrombin (aPT and aPS/PT). In the next future, these autoantibodies, particularly aPS/PT, could become additional serological classification criteria for APS especially to recognized patients negative for classical aPL. The combination of aβ2GPI, aPS/PT and LA demonstrates the best diagnostic accuracy for APS and aPS/PT were recently recommended as a surrogate of LA when specific inhibitors and/or analytical variables may affect its interpretation. Despite these recommendations, very few clinical laboratories include aPS/PT in routine analyzes so far. Moreover, no definite recommendations are available to guide the therapeutic approach in patients positive only for aPS/PT antibodies. To clarify their role in APS diagnosis and treatment, a better comprehension of its pathogenic mechanisms is needed. Thus, the principal aim of this thesis is to investigate the pathogenic mechanism underlying the thrombotic manifestations associated to the presence of aPS/PT. To address this issue, the biological effects sustained in vitro by aPS/PT were compared to those sustained by aβ2GpI, the most studied and recognized player in APS, by developing an experimental model able to investigate the thrombotic effect of these autoantibodies on monocytes and endothelial cells. Beside this principal study, to improve the risk management of APS patients, the plasmatic activity

of the PAF-AH (Platelet Activating Factor Acetylhydrolase) was investigated as a new potential prognostic biomarker. PAF-AH is a specific marker of vascular inflammation dependent to common lipid metabolism markers (i.e. LDL) which is involved in the atherosclerotic plaque instability.

Obtained data on the TF mRNA expression and Nitric Oxide production (colorimetric assay), confirmed that aPS/PT and aβ2GpI exert similar pro-thrombotic effects on monocytes and endothelial cells. On the contrary, the different effect of aβ2GpI and aPS/PT on mRNA expression of IL1β and NLRP3, and the different impact on PAF-AH activity (colorimetric assay), may suggest that these classes of antibodies probably activate different metabolic pathways. Moreover, plasmatic PAF-AH activity in patients with positive aPL antibodies appeared to be independent to common lipid metabolism markers (i.e. LDL). Based on these results, PAF-AH plasmatic activity may represent a new prognostic biomarker also in the context of aPL antibodies, to identify patients at major risk and favouring more tailored therapeutic interventions. Further prospective studies on selected patients are ongoing.

2. ANTIPHOSPHOLIPID SYNDROME (APS)

Antiphospholipid Syndrome (APS) is a systemic autoimmune disease characterized by vascular thrombosis (venous or arterial) and/or adverse obstetric outcomes, accompanied by persistent and elevated levels of antiphospholipid (aPL) antibodies, namely lupus anticoagulant (LA), anticardiolipin antibodies (aCL) or anti-β2 glycoprotein I antibodies (aβ2GpI) (Harper, 2011; Gomez-Puerta, 2014).

2.1 HISTORICAL BACKGROUND

The antiphospholipid antibody story begins in 1906 when Wasserman developed a serological test for syphilis (Arachchillage, 2014). The Wasserman reagin test was attributed to antibody reactivity against antigens derived from Treponema Pallidum, the causative organism of this infection (Hanly, 2003). In 1941 Pangborn demonstrated that isolated cardiolipin from bovine heart, was the antigenic component of reagin test. The use of purified cardiolipin together with lecithin and cholesterol formed the basis for more efficient tests as the Venereal Disease Research Laboratory (VDRL) microflocculation assay. In 1952 Moore and Mohr identified two circumstances in which biological false positive for syphilis test could occurs:

- transient positivity during acute viral infection or after vaccination;
- persistent positivity (>6 months) associated with autoimmune disorders like Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA) and Sjogren's Syndrome (SjS).

At same time Conley and Hartmann (1951) wrote a case report of two patients with SLE, biological false positive for syphilis and with a "peculiar hemorrhagic disorder" (prolongation of prothrombin time). This was the initial description of "Lupus Anticoagulant" (LA) (Arachchillage, 2014; Hanly, 2003).

LA is a biological paradox: in vivo causes thrombotic effects, but in vitro there is a prolongation of a phospholipid dependent coagulation test that is not due to a specific inhibitor of coagulation factor (Watson, 2012).

In the 60's, the association of LA phenomenon with thrombosis, recurrent fetal losses and thrombocytopenia was observed (Gomez-Puerta, 2014).

The development of more sensitive assay for anticardiolipin antibody (such as enzyme-linked immunosorbent assay -ELISA-), facilitated clinical and epidemiological studies and description of the APS (Hanly, 2003).

2.2 CLASSIFICATION CRITERIA AND DIAGNOSIS

The international criteria for the classification of patients with definite APS were defined in 1998 (the so-called "Sapporo criteria") and then revised in 2004 during the 11° International Congress on aPL in Sydney. APS is diagnosed if at least one of clinical criteria and one of laboratory criteria are both present (Table 1) (Wilson, 1999; Miyakis,2006).

However, there are different features associated with APS, but not included in the revised criteria (Table 2) (Miyakis,2006). Only recently APS patients so-called "seronegative APS" (negative for LA, aCL and aβ2GpI) was definitely recognized as a distinctive setting, or better re-defined by the demonstration of new classes of aPL antibodies, such as the autoantibodies directed against prothrombin (aPT and aPS/PT): in the next future these autoantibodies, particularly aPS/PT, could become additional serological classification criteria for APS especially to recognized patients negative for classical aPL (Sciascia and Khamashta, 2014).

TABLE 1. REVISED CLASSIFICATION CRITERIA FOR THE ANTIPHOSPHOLIPID SYNDROME

CLINICAL CRITERIA *

1. Vascular thrombosis: one or more objectively confirmed episodes of arterial, venous or small vessel thrombosis occurring in any tissue or organ
2. Pregnancy morbidity:

a. one or more unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation; or
b. one or more premature births of a morphologically normal neonate before the 34th week of gestation because of eclampsia, pre-eclampsia or placental insufficiency; or
c. three or more unexplained consecutive spontaneous abortions before the 10th week of gestation.

LABORATORY CRITERIA **

1. Lupus Anticoagulant, detected according the guidelines of International Society on Thrombosis and Hemostasis (ISTH)
2. Anticardiolipin Antibody of IgG and IgM isotype, present in medium or high titer (greater than 40 GPL o MPL, or greater than 99th percentile), measured by a standardized ELISA
3. Anti-β2-glycoprotein-1 Antibody of IgG and IgM isotype, present in titer greater than 99th percentile, measured by a standardized ELISA

*one or more; ** one or more, present on 2 or more occasions at least 12 weeks apart using recommended procedures.

TABLE 2. FEATURES ASSOCIATED WITH APS, BUT NOT INCLUDED IN THE REVISED CRITERIA

CLINICAL CRITERIA	LABORATORY CRITERIA
1. Heart valve disease;	1. IgA aCL and aβ2GpI;
2. Livedo reticularis;	2. Antiphosphatidylserine antibodies (aPS);
3. Thrombocytopenia	3. Antiphosphatidylethanolamine antibodies (aPE);
4. Nephropathy;	4. Antiprothrombin antibodies (aPT);
5. Neurological manifestation.	5. Antiphosphatidylserine-prothrombin antibodies (aPS/PT).

The APS could present itself either in a primary form (PAPS), where patients have no evidence of other disease; or in a secondary form (SAPS), thus associated with another autoimmune disorder among which systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are the most frequent (Gomez-Puerta, 2014).

In APS, the most frequent site of venous thrombosis is lower limb venous system, frequently associated with pulmonary embolism (PE), while cerebral vessels are most commonly involved in arterial thrombosis (Watson,2012).

Different from PAPS and SAPS is the catastrophic APS (CAPS), this is the most severe form of APS and is characterized by multiple organ failure usually associated with microthrombosis (Aguiar, 2013).

Prevalence of aPL in the general population ranges between 1 and 5%. However, only a minority of these individuals develop the APS (Gomez-Puerta, 2013).

2.3 HAEMOSTASIS

Haemostasis is a tightly regulated homeostatic mechanism that ensures the maintenance of blood flow under physiological conditions, but also permits rapid, localized coagulation in the event of tissue damage (Allford, 2007; Norris, 2003; Panteleev, 2015). A delicate balance exists between four major components: vascular endothelium, platelets, the coagulation pathway and fibrinolysis (Allford, 2007).

The traditional concept of coagulation was based on two main pathways that were mutually exclusive and of equal importance: the intrinsic (or contact) pathway and the extrinsic (or tissue factor) pathway (Allford, 2007; Norris, 2003). This model (figure 1) described the coagulation as a “cascade” of reactions involving activation of several clotting factors resulting in the production of a large amount of thrombin and subsequent formation of a fibrin clot (Hoffman,2003). However, this cascade paradigm was useful in vitro for diagnostic purposes, but failed to explain in vivo phenomena (Allford, 2007). In 90’s Mann proposed a cell-based model of haemostasis in which was emphasized the interaction of clotting factors with specific surfaces, explaining the unresolved in vivo phenomena (Mann, 1991; Hoffman, 2003).

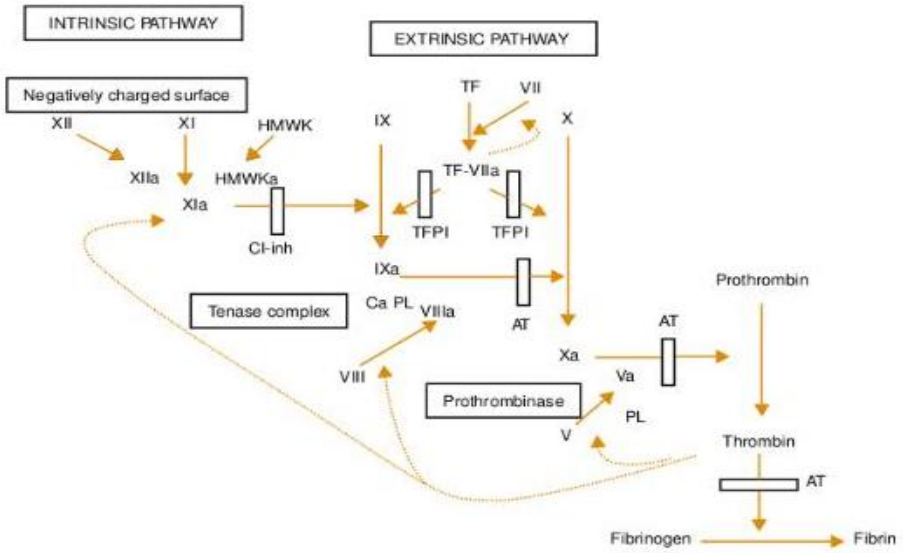

Figure 1: The Coagulation "Cascade" (Norris, 2003).

2.3.1 CELL-BASED MODEL OF HAEMOSTASIS

The cell-based model has three phases: initiation, amplification and propagation phase.

The initiation phase starts when TF (also called thromboplastin or FIII), comes in contact with circulating factor VII activated (VIIa), this complex allows the generation of small amount of thrombin (McMichael, 2012). TF is an integral membrane protein synthesized and expressed by different types of cells, including stromal fibroblasts, mononuclear cells, macrophages and endothelial cells (Hoffman, 2003). The majority of TF is not present within the bloodstream, only a small amount can be detected in plasma (McMichael, 2012).

Haemostasis begins with the formation of the complex between VIIa and TF that activates factor IX (IXa) and factor X (Xa). In this way, in vivo, the intrinsic and extrinsic pathways are integrated (figure 2). Once assembled, VIIa-TF complex activates limited amounts of membrane-bound IX and X. The initial Xa produced by this mechanism generates sufficient thrombin to induce local platelet aggregation and activation of the critical cofactors V and VIII

(amplification phase). However, it is insufficient to sustain haemostasis because of rapid Xa-dependent inactivation of the complex VIIa-TF by TF pathway inhibitor (TFPI). Instead, during propagation phase a marked expansion is achieved via the action of IXa and VIIIa. XIa may be required to produce additional IXa if insufficient quantities are generated by the VIIa-TF complex or fibrinolysis is particularly active. The prothrombinase complex (Xa–Va-calcium-phospholipids) rapidly converts prothrombin (FII) to thrombin. Thrombin hydrolyses the arginine–glycine bonds of fibrinogen to form fibrin monomers and activates factor XIII (XIIIa), which stabilizes the fibrin clot by cross-linkage. It also has a positive feedback role, promoting activation of factor XI and the cofactors V and VIII, and thereby ensuring rapid coagulation (Hoffman, 2003; Allford, 2007; McMichael, 2012).

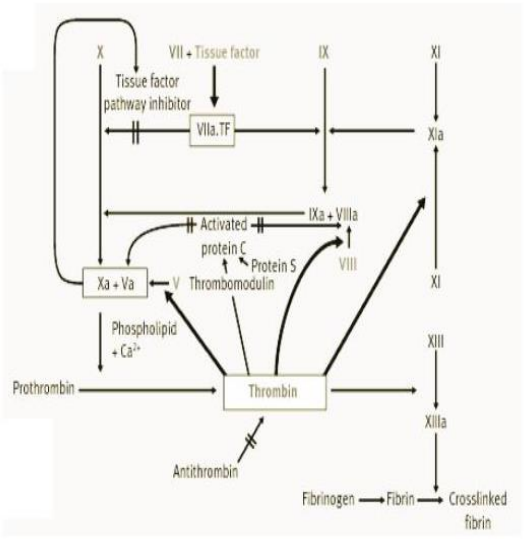

Figure 2: Cell-Based Model of Haemostasis (Allford, 2007).

In this model, the coagulation starts when tissue factor (TF) comes in contact with circulating factor VII activated (VIIa). In this way, the intrinsic and extrinsic pathway are integrated.

2.4 ANTIGENIC TARGETS OF ANTIPHOSPHOLIPID AUTOANTIBODIES (aPL)

The term “Antiphospholipid Syndrome” is used to connect the clinical manifestations to the presence of aPL (Amengual, 2003).

Initially it was thought that these autoantibodies were directed against anionic phospholipids, but in the last decade, different groups of investigators have been demonstrated that aPL are part of a family of autoantibodies against phospholipid-binding plasma proteins or phospholipid-protein complexes. Several antigenic targets have been identified among which high and low molecular weight kininogens, protein C, annexin V and protein S. However, the most common and best characterized target for aPL are β2-Glycoprotein I (β2GpI) and prothrombin (PT). All these antigenic targets are involved in coagulation system, giving an explication of high incidence of thrombotic events in patients with APS (Amengual, 2003).

2.4.1 β2-GLYCOPROTEIN I

The main antigenic target for aPL is β2GpI, also known as apolipoprotein H. In 90’s, it has been demonstrated that aCL associated with APS, were not directed against cardiolipin alone, in fact they require a cofactor that is a plasmatic protein: β2GpI (Amengual, 2003).

In human, this protein is synthesized by different cells: hepatocytes, endothelial, and trophoblast cells. β2GpI circulates in blood at high concentration: the mean serum level is about 200 µg/ml (Miyakis,2004; Mahler, 2012). β2GpI is a 50-KDa anionic phospholipid- binding glycoprotein that belongs to the CCP superfamily (complement control protein).

The CCP domain functions as a protein-protein interaction module in many different proteins. β2GpI is organized in five CCP domains: the first four domains have regular, conserved sequences, while the fifth domain is aberrant and has additional amino acids (multiple lysine). This amino acid strain creates a positively charged domain that is responsible for the binding to the anionic phospholipids. The crystal structure shows that the phospholipid-binding site

is located at the bottom side of domain V and predicts that the potential binding site for aβ2GpI is located in domain I (Groot, 2011; Miyakis,2004).

β2GpI can exist in plasma in two different conformations: closed/circular or open/hockey-stick like conformations. As shown in figure 3, binding of β2GpI to anionic surfaces results in a conformational change: the conversion from closed to open conformation leads to expose the antibody-binding site, that is not accessible to autoantibodies in the closed conformation (Groot, 2011; Harper,2011).

About its physiological role, not many other information are available, but it is supposed that β2GpI plays an important role in biology, since it shares high homology with different mammalian species (Miyakis, 2004). The homology with other proteins involved in innate immunity suggests that β2GpI could play a role in host defense against bacteria (Groot, 2011).

Multi-centric studies have found a strong association between aβ2GpI antibodies and history of thrombosis (Mahler, 2012). Moreover recent studies have shown that aβ2GpI antibodies associated with major risk of thrombosis, bind the domain I of the β2GpI, that is exposed in the open conformation (Harper, 2011), as already demonstrated by *Andreoli et. al*, who have demonstrated that only autoantibodies directed against domain I of β2GpI are associated with increased risk of thrombosis, while a significant lower risk of thrombosis has been found in case of aβ2GpI antibodies targeting other domains of β2GpI (Harper, 2011; Andreoli,2010).

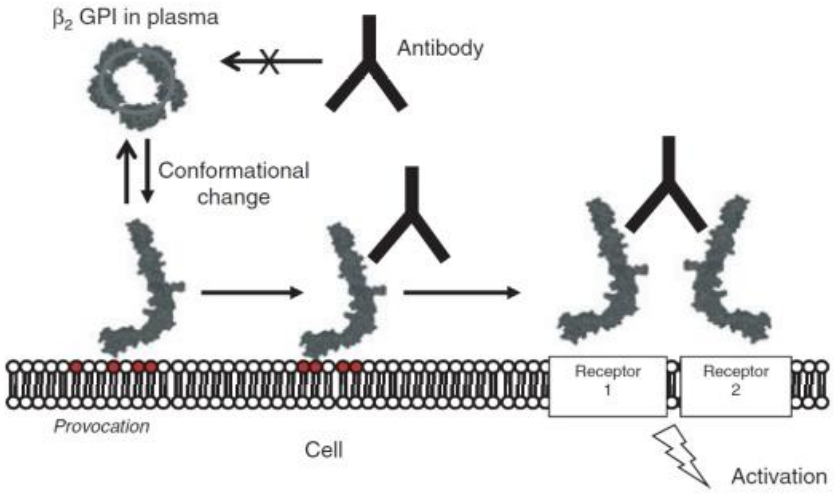

Figure 3: Model of cell activation by autoantibodies against β2GpI (Tripodi, 2011)

β2GpI circulates in plasma in a closed conformation. When β2GpI binds anionic phospholipids, changes its conformation from a closed to an open structure. In this way, the epitope for autoantibodies are exposed. Then β2GpI is able to interact with receptor on the surface of the cells.

2.4.2 PROTHROMBIN

Prothrombin is another major phospholipid-binding protein recognized by aPL. It is a vitamin K-dependent proenzyme synthesized in the liver as inactive zymogen that circulates in blood at concentration of 100 μg/ml (Amengual, 2003).

Mature human prothrombin is a protein of 579 amino acids with a molecular weight of 72-KDa. To exert its procoagulant activity converting fibrinogen to fibrin, prothrombin is physiologically activated by the prothrombinase complex (Xa-Va-calcium-phospholipids): the vitamin K-dependent carboxylation of the γ-carboxyglutamic domain located in fragment 1 of prothrombin allows to bind to the negatively charged phospholipids and consequently the activation of FII (Amengual, 2003; Sciascia and Khamashta, 2014).

2.5 APS AND THROMBOSIS

A strong association between aPL and thrombosis has been demonstrated, but nevertheless the pathogenic role of aPL in the development of thrombosis should be clarified (Gomez-Puerta, 2014).

2.5.1 THE "TWO-HIT" HYPOTHESIS

In APS, the key elements involved in pathogenic mechanism of thrombosis are:

- cellular component of vessels;
- humoral components that regulate haemostasis (coagulation factor, natural anticoagulants and fibrinolytic system);
- inflammation (inflammatory cells, soluble inflammatory mediators and infectious agents) (Willis, 2015).

The complex interaction of these elements, lead to proinflammatory and prothrombotic state, for this reason, it is referred to as the "two-hit" hypothesis. Theory postulates that even though the persistence of elevated levels of aPL is a necessary condition, the occurrence of APS is seemingly triggered by an additional "second hit", such as trauma or infection (Willis, 2015; Brandt, 2013).

2.5.1.1 CELLULAR COMPONENTS

The aPL are directed against anionic phospholipids, therefore several studies have investigated the interaction of β2GpI with cellular membranes and cells. Both in vitro and in vivo studies have shown that the complex β2GpI-aβ2GpI can bind and activate many different cells such as endothelial cells (ECs), monocytes and platelets (Tripodi, 2011).

Activation of endothelial cells by aPL is a major thrombogenic mechanism. The specific binding of aβ2GpI to EC up-regulates the cell-surface expression of cell adhesion molecules (CAMs): intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, promoting leukocyte adhesion (Willis, 2015; Brandt, 2013).

Furthermore, activated EC up-regulate the expression of TF (the key initiator of the coagulation pathway), microparticle formation, fibrinolysis inhibitor PAI-1 and inflammatory cytokines/chemokines such as IL-6, monocyte chemotactic protein-1 (MCP-1), fractalkine (Meroni, 2001). On the other side activation of endothelium leads to decrease expression of thrombomodulin (Meroni, 2001; Rikarni, 2015). These finding suggest that the complex β2GpI-aβ2GpI can induce an endothelial activation either directly or by cytokine autocrine loop (Meroni, 2001). Another mechanism involved in thrombus formation is vasoconstriction: endothelium regulates vessel tone through endothelin-1 peptide, the most potent endothelium-derived contracting factor (Meroni, 2001); supporting this idea, Atsumi et al, reported that plasma level of endothelin-1 peptide significantly correlated with history of thrombosis in APS patient (Atsumi, 1998). All these mechanisms lead to a procoagulant/proinflammatory phenotype that increases the risk of thrombotic occlusions (Brandt, 2013).

The monocytes are another player in the development of thrombosis in APS patients, indeed exposed to aPL, monocytes up-regulate expression of TF via p38 MAPK pathway, resulting in nuclear factor-kB (NF-kB) activation (Willis, 2015; Brandt, 2013). TF expression is associated with increased plasma levels of vascular endothelial growth factor (VEGF) and cell surface expression of both VEGF and the Flt-1 tyrosine kinase receptor. Stimulation of Flt-1 tyrosine kinase receptor by VEGF results in TF mRNA and protein expression. Furthermore, monocytes derived from APS patients or normal monocytes exposed to aPL, show an increased expression of protease-activated receptor 1 (PAR-1) and PAR-2. This is very significant because that PAR-1 and PAR-2 mediate several effects of thrombin such as up-regulation of proinflammatory cytokines (IL-6, IL-8, MCP-1) (Harper, 2011; Willis, 2015).

In vivo, platelets are central to arterial thrombus formation, indeed in APS patients, platelet activation is increased. Plentiful evidence from epidemiological and mechanistic studies indicates that aPL activate platelets,

resulting in increased expression of thromboxane B2 (TXB2), fibrinogen receptor glycoprotein IIb/IIIa (GPIIb/IIIa) and consequently platelet aggregation (Harper, 2011; Willis, 2015). *Urbanus et al.* demonstrated that plasma β2GpI does not bind to platelets, whereas β2GpI in complex with aβ2GpI does bind to platelets (Urbanus, 2008). The platelet receptors involved in the interaction of β2GpI/aβ2GpI complex are apolipoprotein E receptor 2 (ApoER2) and von Willebrand factor receptor glycoprotein Ibα (GPIbα) (Harper, 2011; Urbanus, 2008). Therefore β2GpI/aβ2GpI complex can bind to ApoER2 and/or GPIbα and this binding mediates the activation of platelets and the induction of thromboxane A2 synthesis (Urbanus, 2008).

Finally, activated platelets secrete platelet factor 4 (PF4), a member of the CXC chemokine family with multiple prothrombotic effects (inhibition of inactivation of thrombin by antithrombin, potentiation of platelet aggregation and accelerating cleavage of activated protein C) and also and antigenic target in APS (Harper, 2011; Giannakopoulos, 2013).

The activation of all these cell types by aPL coupled with the release of several proinflammatory mediators has linked to the development of thrombosis in APS animal models and in same case in human APS patients (Willis, 2015).

2.5.1.2 HUMORAL FACTORS

aPL act at various levels of the coagulation cascade leading to uncontrolled fibrin formation and impaired thrombus resolution (Willis, 2015).

Direct activation of prothrombin binding to the surface of ECs, has been demonstrated in APS patients and has been attributed to anti-prothrombin antibodies (aPT) with LA activity; this activation induces TF expression and thrombosis (Willis, 2015; Amengual, 2003). Furthermore, aPL bind directly to antithrombin III (ATIII) resulting in reduced inactivation of FIXa and FXa (Willis, 2015; Harper, 2011).

aPL interfere with the fibrinolytic system: the action of β2GpI/aβ2GpI complex on endothelium leads to decrease thrombomodulin expression (TM) and tp

increase of plasminogen activator inhibitor (PAI-1), this might be one of the causes of thrombophilic diathesis in APS (Meroni, 2001; Rikarni ,2015).

Modulation of activated protein C (APC), a phospholipid-dependent major antithrombotic pathway, has been found in APS patients who have increased resistance to APC resulting in greater thrombin generation overtime (Meroni, 2001; Harper, 2011; Willis, 2015).

Finally, it has been hypothesized an involvement of annexin 5 (A5): normally A5 binds to phosphatidylserine surfaces of ECs, forming a shield that inhibits the formation of procoagulant complex; in a model of the pathogenesis of the antiphospholipid syndrome, aβ2GpI that bind to the domain 1 of the β2GpI can disrupt the A5 antithrombotic shield present on the endothelial cells (Giannakopoulos, 2013; Tripodi, 2015).

Overall, the resistance of activated coagulation factors to inactivation and the reduced activity of natural anticoagulant and fibrinolytic agents, potentiate unchecked fibrin formation and the thrombogenic state (Willis, 2015; Tripodi, 2011).

2.5.1.3 INFLAMMATION

In addition to activation of the coagulation pathway, APS is characterized by proinflammatory changes. Indeed, inflammation acts as a key trigger event for the thrombotic manifestation of APS and it is very important for changes in antigen conformation and immune cell activity, critical elements in aPL ontogeny (Harper, 2011; Willis, 2015).

Several studies have demonstrated that APS patients are characterized by increased oxidative stress: paraoxonase activity (a glycoprotein that prevents oxidation of low-density lipoprotein-LDL- cholesterol) is significantly decreased in these patients, whereas 8-epi-prostaglandin $F_{2\propto}$, a biomarker of lipid peroxidation, is upregulated (Giannakopoulos, 2013).

In APS patients, oxidative stress has an effect on β2GpI, in fact APS patients frequently show high levels of oxidized β2GpI. Oxidative stress acts on β2GpI at different levels:

- increases β2GpI production through gene promoter up-regulation via nuclear factor kappa B (NFkB);
- increases the immunogenicity of β2GpI through post-translational modification;
- induces conformational changes exposing hidden epitopes of β2GpI, important for aPL production (Willis, 2015)

It has been proposed that disturbance of redox balance in patients with APS could constitute the "first hit" which allows the formation of β2GpI/aβ2GpI complex on ECs (Giannakopoulos, 2013).

Furthermore, oxidative stress can up-regulate annexin II (A2) expression, an endothelial receptor that mediates the binding of β2GpI to ECs (Ma, 2000), and, in a murine model of thrombosis, induces platelet aggregation, EC stimulation and von Willebrand factor expression (Nishimura, 2011).

Patients with APS have decreased levels of plasma nitrite, as compared with controls. This suggests an abnormal activity of endothelial nitric oxide synthase (e-NOS). Endothelium-derived nitric oxide is fundamental for normal function of endothelium and a reduced expression of e-NOS results in superoxide and peroxynitrite production (Giannakopoulos, 2013).

Activation of the complement cascade also contributes to the pathogenic effects of aPL; in particular, the anaphylatoxins C3a and C5a induce the inflammatory vascular phenotype of APS and are necessary players connecting EC, monocytes, and platelet activation by aPL and the thrombotic manifestation (Willis, 2015).

2.6 APS AND PREGNANCY

The risk for adverse pregnancy outcomes, such as recurrent miscarriage, fetal demise, placental insufficiency, preeclampsia and intrauterine growth restriction (IUGR) in women with APS is greatest from the 10^{th} week of gestation onward (Hanly,2003; Mulla, 2013).

There is also evidence that these women have an increased risk of giving birth to a premature infant because of pregnancy-associated hypertension and utero-placental insufficiency (Hanly, 2003).

Unlike the systemic APS that is a prothrombotic and proinlammatory disease, obstetric APS (OAPS) is primarily a proinflammatory syndrome (Mulla, 2013). Indeed, the first hypothesis that OAPS was due to an intraplacental thrombosis with consequently alteration of maternal-fetal blood exchanges, has not been confirmed by histological studies (Khamashta, 2016).

Different groups of investigators have been postulated two mechanisms for aPL-induced pregnancy morbidity: defective placentation and inflammation (Khamashta, 2016).

In the placenta, aβ2GpI can react with both sides, maternal and fetal (Simone, 2000). This ability induces direct placental damage with different mechanisms:

- inhibiting trophoblast differentiation and syncytialization;
- inducing trophoblast apoptosis;
- impairing trophoblast invasiveness;
- affecting trophoblast expression of adhesion molecules that regulate its adhesion to and invasion of the maternal tissue;
- inhibiting production of angiogenic factor by trophoblasts (Khamashta, 2016; Tong, 2014).

Moreover, has been proposed as an additional mechanism for preeclampsia due to the internalization of aPL by trophoblasts with the subsequent acceleration of cell death and release of debris that can activate maternal endothelial cells (Khamashta, 2016).

It has been proved that inflammation has an important role in OAPS. This idea is based on:

- the histological demonstration of complement deposition, neutrophil infiltration, and tumor necrosis factor α (TNFα) secretion in decidual tissue;

- the observation that complement deficiency in animal models or complement inhibition in vivo are protective against obstetrical complications;
- the evidence of a protective effect of heparin linked to its anti-complement activity;
- the observation in in vitro studies that aPL can induce trophoblasts to produce interleukin-1 β (IL1β) by activation of inflammasome (Mulla, 2013; Khamashta, 2016; Müller-Calleja, 2015).

2.6.1 aPL AND INFLAMMASOMES

Inflammasomes (NLR) are large soluble cytoplasmatic complexes that are capable of activating the cystein protease caspase-1 in response to a wide range of stimuli including microbial and self-molecules. The activation of inflammasome leading to the processing and activation of pro-IL1β and pro-IL18 through caspase-1 (Chen, 2009). Inflammasome includes several members: NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, NLRC4 and NAIP proteins (Barbè, 2014). The most well characterized are NLRP1 and NLRP3 (figure 4) and different studies on OAPS, have associated the production of IL1β with the activation of NLRP3 (Mulla, 2013; Khamashta, 2016; Müller-Calleja, 2015).

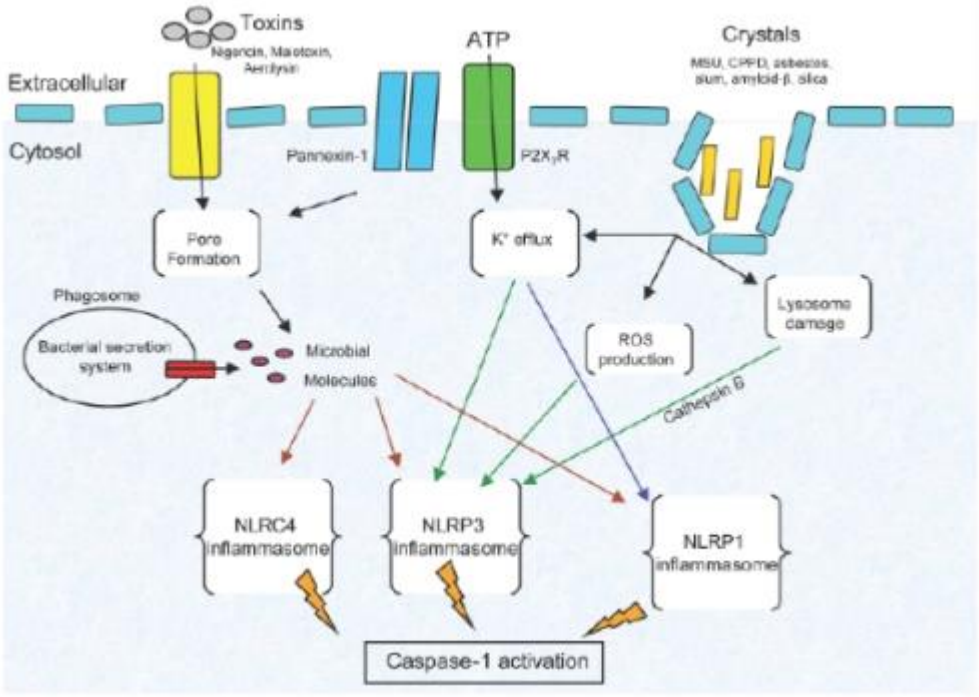

Figure 4: Mechanism of Inflammasome.

Three different Inflammasome (NLRC4, NLRP3 and NLRP1) activate caspase-1 in response to several stimuli such as microbial component or crystal; in this way, pro-IL1β and pro-IL18 are processed

2.7 RISK FACTORS OTHER THAN aPL IN APS PATIENTS

Recently, the role of vascular risk factors in the development of clinical events in patients with APS has been established (Khamashta, 2016). The presence of multiple risk factors such as hypertension, smoking, hypercholesterolemia, or estrogen use may increase the occurrence of thrombosis in patients with aPL (Erkan, 2002).

SLE is a risk factor for thrombosis per se: in patients with SLE there are a higher-than-expected incidence of vascular events, which are not completely explained by traditional vascular risk factors (Esdaile, 2001). The combination of SLE and aPL positivity has been shown to increase the risk of thrombosis. Indeed, in SLE patients with aPL positivity, the annual risk of first thrombosis is higher than in healthy aPL positive subjects without other cardiovascular risk (4% vs < 1%) (Khamashta, 2016).

Thrombotic risk assessment should be considered also in patients with primary APS, such as women with a history of pregnancy morbidity due to aPLs (OAPS). In fact, these patients have a higher thrombotic event rate than healthy women (3.3 vs 0-0.5⁄100patients-years) (Lefevre, 2011).

Moreover, there are many aPL carriers that never develop APS, only few cases will develop thrombosis or obstetrical manifestations and only a very small group will develop CAPS. In this scenario, it could be of great advantage to make a risk stratification of thrombotic/obstetric events in such patients.

To date, three score model have been proposed for risk stratification: the first two scores are focused on the aPL profile, while the third, the Global APS Score (GAPSS) included other variables such as autoimmune profile or cardiovascular risk factor. This model seems to be the better one (Khamashta, 2016).

To help clinicians in patient management, in addition to GAPSS, it would be useful to identify a new specific plasmatic biomarker, independent from the other classic risk factors for thrombosis.

2.7.1 PLASMATIC PLATELET-ACTIVATING FACTOR ACETYLHYDROLASE ACTIVITY (PAF-AH)

Platelet activating factor acetylhydrolase activity (PAF-AH) is a Ca^{2+}-independent A2 phospholipase, also known as lipoprotein-associated phospholipase A2 (Lp-PLA2). The plasmatic PAF-AH is constitutively active and circulates bound to LDL, HDL and other lipoproteins. PAF-AH hydrolyzes the ester bond at the sn-2 position of phospholipids, such as PAF and PAF mimetics, that are early mediators of inflammation (McIntyre, 2008). PAF activates a variety of cells of the innate immune system promoting migration, adhesion and inflammatory effects. Thus, PAF-AH while inactivating PAF, is considered an important factor to prevent an exaggerated inflammatory response and to protect cells from uncontrolled oxidative damage (Rosenson, 2012). Several studies have shown an association between high levels of PAF-AH activity and the severity of cardiovascular diseases and identified PAF-AH

as a marker of vascular inflammation involved in the atherosclerotic plaque instability (Davidson, 2008; Maiolino, 2012). To date, there are not study on PAF-AH activity and APS.

3. AIM OF THE STUDY

Numbers of recent papers underlined the important role of aPS/PT. In particular, the combination of aβ2GPI, aPS/PT and LA demonstrates the best diagnostic accuracy for APS and aPS/PT were recently recommended as a surrogate of LA when specific inhibitors and/or analytical variables may affect its interpretation (Bertolaccini, 2011). Despite these recommendations, very few clinical laboratories include aPS/PT in routine analyses so far. Moreover, no definite recommendations are available to guide the therapeutic approach in patients positive only for aPS/PT antibodies. To clarify their role in APS diagnosis and treatment, a better comprehension of its pathogenic mechanisms is needed. Thus, the principal aim of this thesis is to investigate the pathogenic mechanism underlying the thrombotic manifestations associated to the presence of anti-phosphatidylserine-prothrombin antibodies. To address this issue, since aβ2GpI antibodies represent the most studied and recognized player in APS, I decided to compare the biological effects sustained in vitro by aPS/PT to those sustained by aβ2GpI, by developing an experimental model able to investigate the thrombotic effect. Beside this principal study, to better assess the atherosclerotic risk in APS population and improve the risk management of these patients in the follow-up, I will investigate a new potential prognostic biomarker, such as the plasmatic activity of the PAF-AH (Platelet Activating Factor Acetylhydrolase), that is a specific marker of vascular inflammation involved in the atherosclerotic plaque instability.

4. MATERIAL AND METHODS

4.1 PATIENTS

For in vitro experiments, total IgG were purified from six selected patients, in particular three positive only for aβ2GpI IgG and three positive only for aPS/PT

IgG, all were LA positive. Patients positive for aβ2GpI IgG all recognized also the domain I. As controls, total IgG were purified from five blood donors (BD), who were tested negative for LA, aβ2GpI and aPS/PT antibodies.

The plasmatic PAF-AH activity was evaluated in a series of 167 consecutive unselected patients (124 females and 69 males; mean age: 51±16 years) screened for the presence of aPL at the Laboratory of Immunopathology of the University Hospital of Udine in a routinely context of thrombotic events, risk of thrombosis or obstetric complications. Patients were compared to 77 blood donors (BDs; 39 females and 38 males; mean age: 39±13 years) enrolled at the Transfusion Unit of the same Hospital.

All patients and controls gave their informed consent to these studies according to the Declaration of Helsinki and to the Italian legislation (Authorization of the Privacy Guarantor No. 9, 12 December 2013).

4.2 ANTIBODY DETERMINATION AND ANTIGENIC SPECIFICITY

4.2.1 LA

Plasma samples were tested for the presence of LA at the Laboratory of Haemostasis of the University Hospital of Udine, according to the recommended criteria from the ISTH Subcommittee on Lupus Anticoagulant-Phospholipid-dependent antibodies.

These criteria require a three-step procedure that is summarized in figure 5 (Tripodi,2011).

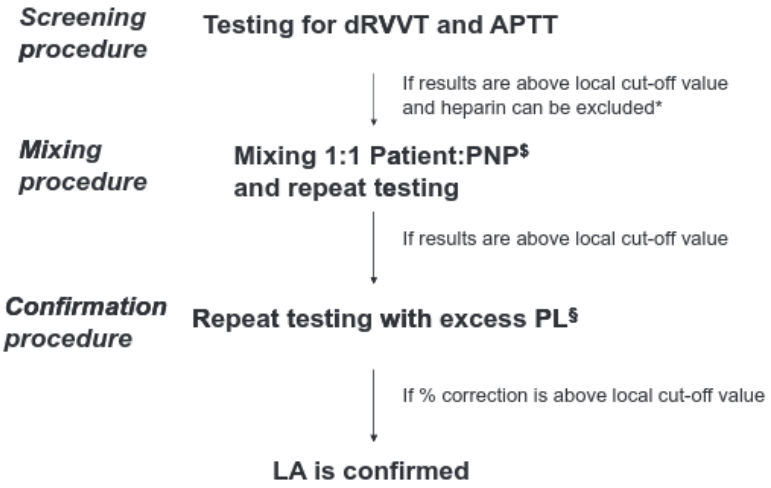

Figure 5: Flowchart for the Laboratory Detection of Lupus Anticoagulants (Tripodi, 2011).

** the presence of heparin is ruled out by a normal thrombin clotting time; § PNP, pooled normal plasma; PL, phospholipids.*

4.2.2 aCL and aβ2GpI

Anti-cardiolipin (aCL) IgG/IgM and anti-β2GpI (aβ2GpI) IgG/IgM antibodies (figure 6) were detected by commercial methods (CLIA, Zenit RA, Menarini Diagnostic; cutoff IgG 10, IgM 20). Anti-β2GpI IgG antibodies specifically directed against domain I were detected by CLIA using the Inova Diagnostic Kit (Bioflash; cutoff 20).

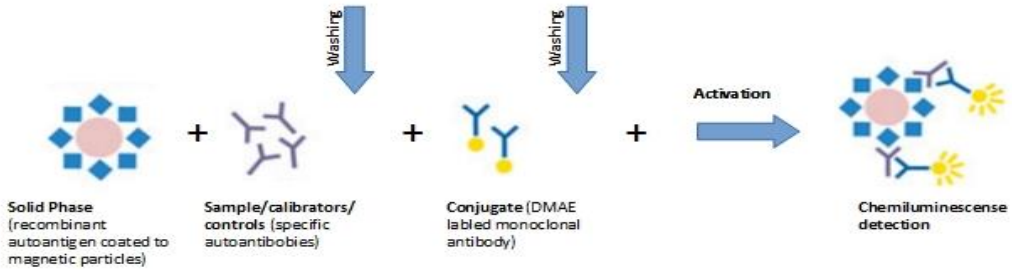

Figure 6: CLIA System for detection of aCL IgG/IgM and aβ2GpI IgG/IgM (Menarini Diagnostic)

The assay is based on a two-step indirect chemiluminescent method that generates quantitative results. This particular technique uses autoantigen-coated magnetic particles as slid phase and an antibody labeled with dimethyl acridinium ester (DMAE) as detection marker.

4.2.3 aPS/PT

The aPS/PT IgG and IgM antibodies, in serum samples, were analyzed by ELISA (figure 7) using the Quanta Lite aPS/PT IgG/IgM ELISA kit (Inova Diagnostic Inc, San Diego, CA; cutoff IgG 40 AU/ml, IgM 30 AU/ml).

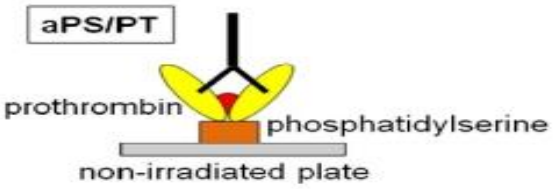

Figure 7: ELISA System for the detection of aPS/PT (Sciascia and Khamashta, 2014). Antibodies are able to bind prothrombin (PT) when it is exposed to immobilized anionic phospholipids.

4.3 MEASUREMENT OF PAF-AH ACTIVITY

The plasmatic PAF-AH activity was assessed by a colorimetric assay (PAF-AH Assay Kit- Cayman Chemical Company, Ann Arbor, Michigan, USA). Briefly (figure 8) serum samples were incubated with the substrate 2-thio PAF, that is

hydrolyzed by PAF-AH at the sn2-position releasing free thiols detected by DTNB Ellman's reagent (5,5'-dithio-bis-2-nitrobenzoic acid).

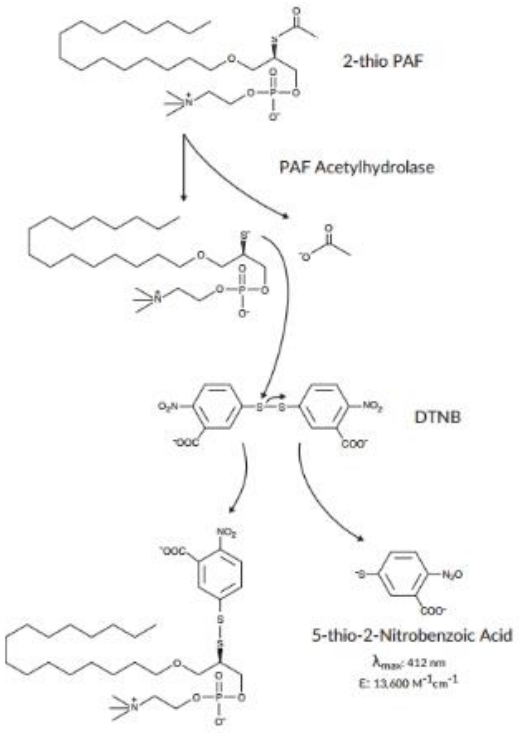

Figure 8: PAF-AH Assay Scheme (Cayman Chemical Company)

4.4 ISOLATION OF IgG

Total IgG from patients and controls were purified from serum samples with two different methods:

- affinity chromatography (figure 9);
- immunopurification with magnetic beads (figure 10).

For affinity chromatography, it was used Rec.Protein A-Sepharose® 4B Conjugate (Life Technologies) according to the manufacturer's instruction. Briefly, Rec.Protein A-Sepharose® is a bead-formed agarose-based gel filtration matrix where Protein A from Staphylococcus aureus was immobilized. Protein A binds to Fc region of immunoglobulins (Igs) through interaction with heavy chain. To elute IgG from Protein A-Sepharose®, 0.1 M glycine buffer pH 3.0 was

used; to preserve the activity of purified IgG, the pH of fractions was neutralized by addition of 1: 10 vol/vol of 1 M Tris-HCl pH 9.0.

For the immunopurification of IgG with magnetic beads, PureProteome^{TM} Protein A Magnetic Beads (Millipore) was used, according to the manufacturer's instruction. As for affinity chromatography, Fc region of IgG binds recombinant Protein A from Staphylococcus aureus covalently coupled with polymer-coated inorganic beads. A glycine buffer (0.2 M, pH 2.5) was used also to elute the bound IgG; after the elution, the solution with IgG was neutralized with Tris-HCl 1 M pH 8.5.

The purity of immunopurified IgG was verified by immunofixation diagnostic assay performed on the fully automated gel electrophoresis instrument InterlabG26 (Interlab).

The immunopurified IgG was quantified by spectrophotometry and checked by Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE).

High titer of aβ2GpI or aPS/PT were measured in the IgG fraction purified from patient sera, while the IgG fraction obtained from BD sera remained negative.

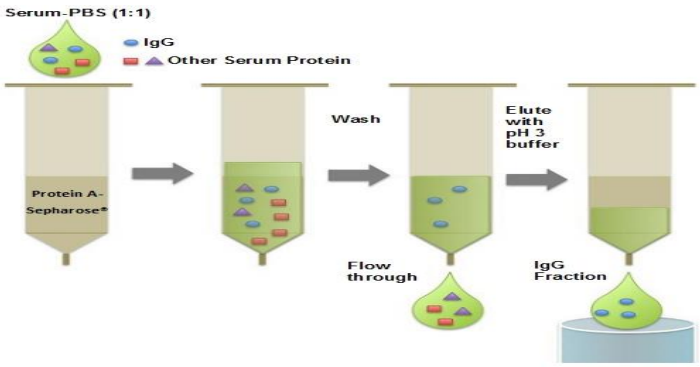

Figure 9: Isolation of IgG Fraction from Serum with Affinity Chromatography

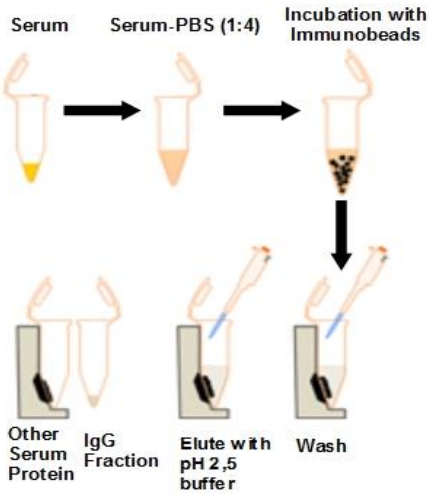

Figure 10: Isolation of IgG Fraction from Serum with Immunobeads

4.5 CELL CULTURE

4.5.1 ISOLATION OF MONOCYTES

Monocytes were isolated from fresh peripheral blood mononuclear cells (PBMCs). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood of five blood donors by gradient centrifugation (Ficoll-Paque Plus). The cells were collect and washed with PBS. Monocytes were isolated from PBMCs by negative selection using the Human Monocyte Enrichment Kit (Stemcell Technologies) according to the manufacturer's protocol. Isolated monocytes were cultured overnight in RPMI-1640 (Sigma-Aldrich) supplemented with 0.02 M HEPES (Sigma-Aldrich), 100 µM penicillin-streptomycin (Sigma-Aldrich), and 10 vol% heat-inactivated Fetal Bovine Serum (FBS,Gibco) in humidified atmosphere (5 vol % CO_2, 37°C).

4.5.2 HUVEC

Human Umbilical vein endothelial cells (HUVECs) (Gibco), were maintained under 5 vol% CO_2 at 37°C in M199 (Sigma-Aldrich) supplemented with 100 µM penicillin-streptomycin (Sigma-Aldrich), and 10 vol% heat-inactivated Fetal Bovine Serum (FBS, Gibco).

4.6 PROCOAGULANT CELL TREATMENT

To test prothrombotic effect of the fraction of IgG aPL positive, cells were treated as shown in table 3 for 4, 16 and 24 hours (Oku, 2013; Raschi, 2014). Monocytes and HUVECs were treated for 4 h for mRNA analysis and for 8, 16 and 24 h for cytokine and chemokine expression.

TABLE 3. PROCOAGULANT TREATMENT FOR MONOCYTES AND HUVECs

	UN***	LPS	BD	aβ2GpI	aPS/PT
Ca^{2+} (2.5mM) *		✓	✓	✓	✓
PT **		✓	✓	✓	✓
LPS (1ng/ml) ***		✓	✓	✓	✓
IgG BD (500µg/ml)			✓		
IgG aβ2GpI (500µg/ml)				✓	
IgG aPS/PT (500µg/ml)					✓

*This concentration of Ca^{2+} was sufficient to facilitate the binding of PT to phosphatidylserine

**PT (prothrombin) were added to monocytes at a concentration of 10µg/ml and to HUVECs at a concentration of 15µg/ml.

***LPS (lipopolysaccharide) were added to pre-activate cells and to mimic the “second-hit”

****UN unstimulated cells

4.7 RNA ISOLATION AND REAL-TIME PCR

Total RNA was extracted from the cells using ReliaPrep™ RNA Cell Miniprep System according to the manufacturer's protocol and stored at -80°C until use. RNA quantification was determined with NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, Wilmington, Del). The purity of the RNA samples was evaluated with the optical density 260:280 and 260:230 ratio and with denaturing agarose gel electrophoresis and ethidium bromide staining.

Complementary DNA (cDNA) was generated using the iScript™ Select cDNA Synthesis Kit (Bio-Rad Laboratories) according to the random primer protocol provided by the manufacturer.

In order to evaluate mRNA relative expression of TF, IL1β, NLRP1 and NLRP3, real-time PCR was performed using SsoAdvance universal SYBR green supermix (Bio-Rad Laboratories) and a LightCycler 480 (Roche Diagnostics Ltd) according to the manufacturer's instructions. The primers used are shown in table 4. The result of mRNA expression was analyzed by measuring threshold cycle and the value was normalized with GAPDH using ΔΔct method.

TABLE 4: PRIMER SEQUENCES* USED FOR TF, IL1β, NLRP1 AND NLRP3 GENE EXPRESSION ANALYSES

Genes Analysed	Forward (sequence 5'-3')	Reverse (sequence 5'-3)
TF	TGTTCAAATAAGCACTAAGTCAGGAGAT	TCGTCGGTGAGGTCACACTCT
IL1β	TGCCCGTCTTCCTGGGAGGG	GGCTGGGGATTGGCCCTGAA
NLRP1	GACCTGGCCTCTGTGCTTAG	AGTCCCCAAAGGCTTCGTAT
NLRP3	CTGTGTGTGGGACTGGAAGCAC	GCAGCTCTGCTGTTTCAGCAC
GAPDH	AGTATGACAACAGCCTCAAG	TCTAGACGGCAGGTCAGGTCCAC

*Primers were projected to target two consecutive exons of gene in order to prevent the amplification of any contaminating genomic DNA

4.8 MEASUREMENT OF NITRIC OXIDE (NO) PRODUCTION

After stimulation of HUVECs for 16 as described previously, supernatants were aspirated, centrifuged at 15500 g (5 min, 4°C), and stored at -80°C until quantification of NO production.

The measurement of NO levels was performed using a colorimetric assay (NITRATE/NITRITE COLORIMETRIC Assay Kit- Cayman Chemical Company, Ann Arbor, Michigan, USA) according to the manufacturer's instruction. Briefly, NO is scavenged rapidly ($t_{1/2}$), the final products (NOx) in vivo are nitrite (NO_2^-) and nitrate (NO_3^-), thus the best index of total NO production is the sum of nitrite and nitrate. This assay is a two-step process (figure 11). The first step provides a conversion of nitrate to nitrite through the nitrate reductase. Griess Reagent is added during second step, converting nitrite in a deep purple azo-compound. Concentrations were calculated by comparing absorption of samples and a standard curve.

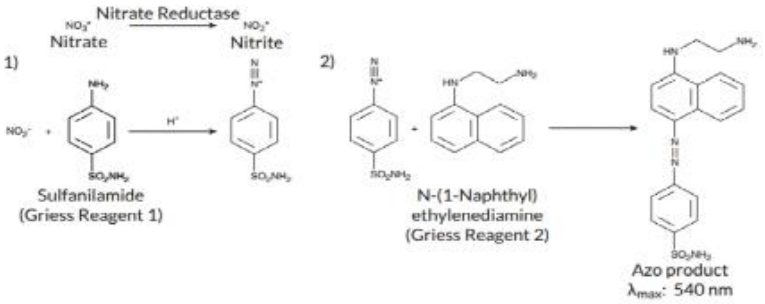

Figure 11: Griess Reagent Chemistry (Cayman Chemical Company)

4.9 QUANTIFICATION OF CYTOKINES AND CHEMOKINES

After stimulation of HUVECs for 16 and 24h as described previously, supernatants were aspirated, centrifuged at 15500 g (5 min, 4°C), and stored at -80°C until quantification of cytokines and chemokines. For the quantification was used Bio-Plex Pro™ Human 2-Plex Panel (ICAM-1 and VCAM-1) and Bio-Plex Pro™ Human Chemokine 40-Plex Panel (table 4) with Bio-Plex 200 system (Bio-Rad Laboratories). This simultaneous dosage is based on Luminex® Technologies (figure 12). Briefly paramagnetic beads are internally dyed with red and infrared fluorophores of differing intensities, each dyed bead is given a unique number allowing the differentiation of one bead from another. In this way, multiple analyte-specific beads can then be combined in a single well of a 96-well microplate-format assay to detect and quantify different targets simultaneously.

Data were analyzed using Bio-Plex Data Pro™ software.

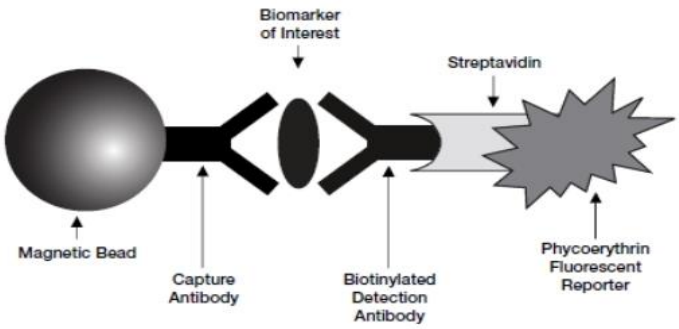

Figure 12: Bio-Plex sandwich immunoassay

Table 4 Bio-Plex Pro™ Human Chemokine 40-Plex Panel

CCL21	CXCL1	IL16	CCL3
CXCL13	CXCL2	CXCL10	CCL15
CCL27	CCL1	CXCL11	CCL20
CXCL5	IFNϒ	CCL2	CCL19
CCL11	IL1β	CCL8	CCL23
CCL24	IL2	CCL7	CXCL16
CCL26	IL4	CCL13	CXCL12
CX3CL1	IL6	CCL22	CCL17
CXCL6	IL8	MIF	CCL25
GM-CSF	IL10	CXCL9	TNFα

4.10 STATISTICAL ANALYSIS

Quantitative variables were expressed as mean ± standard deviation (SD) and checked for normality distribution by the Shapiro Wilk test. To compare biomarker serum levels between patient and control series, either Mann-Whitney or unpaired t-test was used when appropriate. Correlation analysis were performed using the Pearson's or the Spearman's rank correlation coefficient, when appropriate. Statistical analyses were performed with GraphPad Prism software. P values less than 0.05 were considered as significant.

5. RESULTS

5.1 IgG PURIFICATION

The IgG fractions were obtained with both methods, affinity chromatography and immunopurification with magnetic beads. The purification was checked with two different techniques: SDS-page and immunofixation.

As shown in figure 13, to identify the fraction of interest, the different eluates were verified by SDS-page. The absence of contamination by other immunoglobulins, was verified by immunofixation technique (figure 14).

A highly-purified IgG fractions, were obtained both by affinity chromatography and immunopurification. The only difference between these two methods, was the quantity of input material versus the final yield: immunopurification starts from a small amount of serum while in affinity chromatography, the quantity of input material is proportional to the volume of Sepharose®. In order to obtain sufficient IgG for procoagulant treatment, the affinity chromatography was finally chosen.

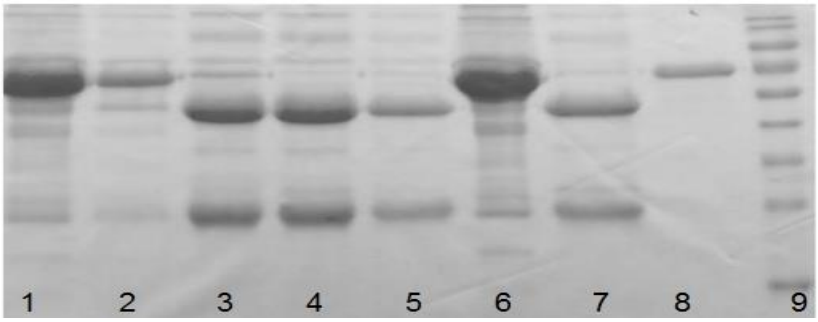

Figure 13: Purification of IgG from Human Serum with affinity chromatography (lanes 1-5) and immunoprecipitation (lanes 6-7)

Lines 1 and 6 show the input material, lane 2 shows serum depleted from IgG, lanes 3-5 and 7 show the bound IgG fraction, lane 8 shows bovine serum albumin (BSA) and lane 9 shows molecular weight markers

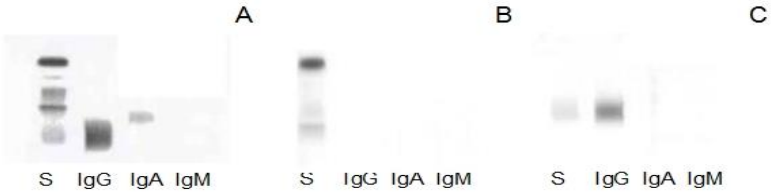

Figure 14: Immunofixation of the input material (A), serum depleted of IgG (B) and IgG fraction.

As shown in panel B, in serum depleted, IgG are absent and (panel C) the fraction of IgG is highly pure. S= serum protein

5.2 SETTING OF PROCOAGULANT TREATMENT

To mimic the "two-hit" theory, cells were first stimulated with LPS at concentration of 1 ng/ml (Raschi, 2014).

Then, PT was always added to the medium, since β2GpI is produced by monocytes and HUVECs under LPS-stimulation, while PT is produced only by liver cells, thus PT has to be added in each experimental condition. To facilitate the binding of PT to phosphatidylserine, the calcium concentration was adjusted to 2.5 mM (Oku, 2013).

Lastly, to be sure that the effects seen were due to aβ2GpI IgG and/or aPS/PT IgG, and not to general IgG immunoglobulins, monocytes and HUVECs were treated with IgG fraction extracted from BD.

5.3 EFFECT OF IgG FROM BLOOD DONORS' SERUM ON MONOCYTES AND HUVECs

The possible effect of IgG fraction extracted from BD, was evaluated in term of mRNA expression after four hours of treatment. In particular, in monocytes we evaluated TF and IL1β mRNA expression, in HUVECs TF mRNA expression.

In monocytes, compared to the unstimulated cells, no difference in TF up-regulation was found between treatment with LPS alone (6.4±0.2 fold) or LPS

plus the IgG fraction from BD (5.2±1.4 fold) (figure 15, panel A). A similar scenario was found for IL1β expression (figure 15, panel B), no difference was observed among treatment with LPS alone or combined with IgG fraction from BD (respectively 8±2-fold vs 8.4±1 fold, p=0.7).

In contrast, the treatment with BD IgG plus LPS on HUVECs determined a downregulation of TF mRNA expression compared to the LPS alone stimulation (figure 16; 18.4±5.8 fold for LPS alone, 9.6±2.4 fold for LPS plus IgG BD, $p<0.05$).

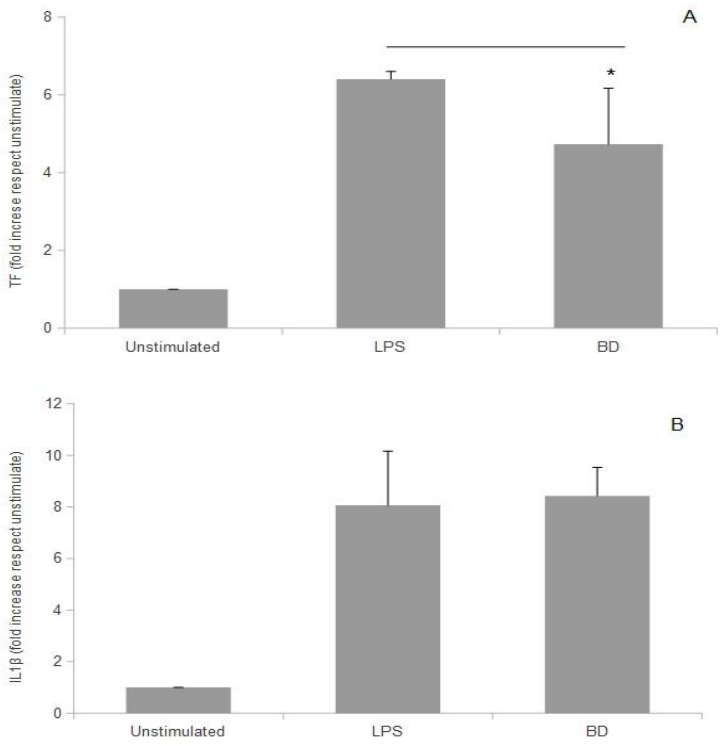

*Figure 15: Monocytes obtained from five BD were stimulated as described below for four hours. LPS (1ng/ml) was added alone or with IgG BD fractions (0.5mg/ml). The bars represent the mean ± S.E. of three independent experiments. The expression levels of mRNA were detected by PCR real-time with ΔΔct method. * p< 0.05. (A) Relative TF mRNA expression levels in monocytes. (B) Relative IL1β mRNA expression in monocytes.*

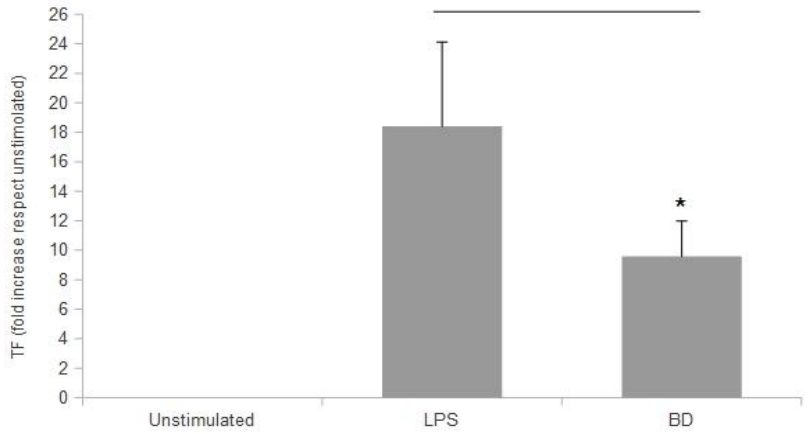

Figure 16: HUVECs were stimulated as described below for four hours.

*LPS (1ng/ml) was added alone or with IgG BD fractions (0.5mg/ml). The bars represent the mean ± S.E. of three independent experiments. The expression levels of mRNA were detected by PCR real-time with ΔΔct method. * p< 0.05. Relative TF mRNA expression levels in HUVECs.*

5.4 EFFECT OF IgG FROM APS PATIENTS' SERUM ON MONOCYTES

To analyses and compare aPS/PT and aβ2GpI effect in vitro in monocytes and HUVECs, we stimulated cells either with the aPS/PT or the aβ2GpI IgG extract and with a mix 1:1 of the two extract. As shown in figure 17, in monocytes, the addition of IgG from APS patients significantly up-regulated the mRNA expression of TF compared to LPS alone. No difference was found between aPS/PT, aβ2GpI and the mix of the two antibodies.

A different result was found when evaluating a proinflammatory effect in term of IL1β mRNA expression. In this case, while IgG aβ2GpI did not affect LPS-induced IL1β expression, aPS/PT significantly reduced the effect of LPS on monocytes (figure 18). Treatment with IgG positive for both aβ2GpI and aPS/PT on monocytes, reflected the effects obtained separately with IgG positive for aβ2GpI or aPS/PT, since the negative effect of aPS/PT was partially reverted by aβ2GpI.

According to these results concerning IL1β expression regulation, treatment with LPS in monocytes was able to switch-on the specific expression of NLRP3

(figure 19) and the IgG aβ2GpI further increased LPS-induced NLRP3 expression, while aPS/PT did not, moreover, when mixed to aβ2GpI, they abolished the effect of aβ2GpI.

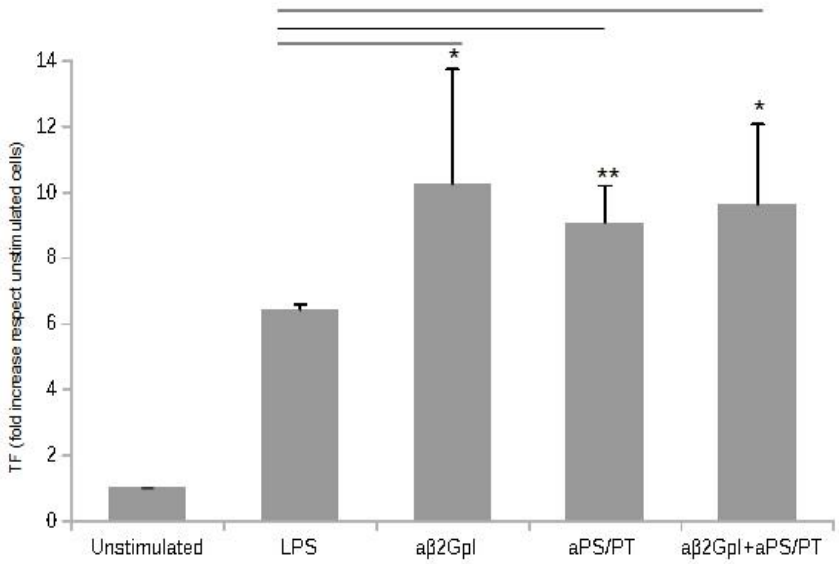

Figure 17: Relative TF mRNA expression levels in monocytes

*Monocytes obtained from five BD were stimulated as described below for four hours. LPS (1ng/ml) was added alone or with IgG fractions (0.5mg/ml) extracted from APS patients positive for aβ2GpI, aPS/PT or both.The bars represent the mean ± S.E. of three independent experiments. The expression levels of mRNA were detected by PCR real-time with ΔΔct method. * p< 0.05 **p<0.001*

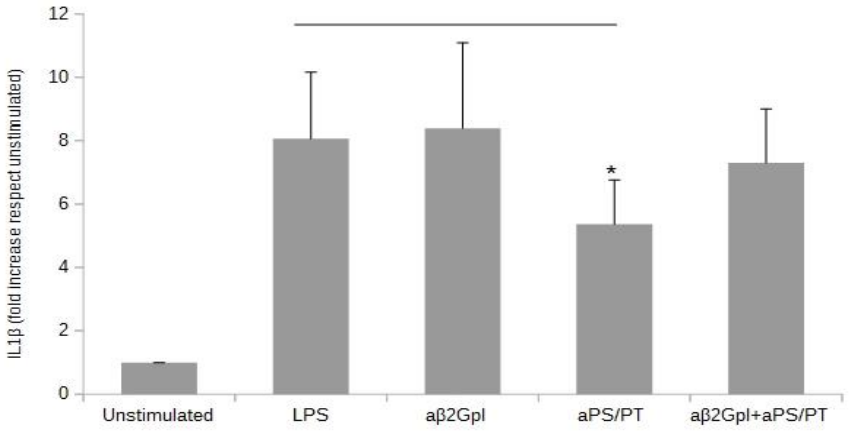

Figure 18: Relative IL1β mRNA expression in monocytes.

*Monocytes obtained from five BD were stimulated as described below for four hours. LPS (1ng/ml) was added alone or with IgG fractions (0.5mg/ml) extracted from APS patients positive for aβ2GpI, aPS/PT or both. The bars represent the mean ± S.E. of three independent experiments. The expression levels of mRNA were detected by PCR real-time with ΔΔct method. * p< 0.05*

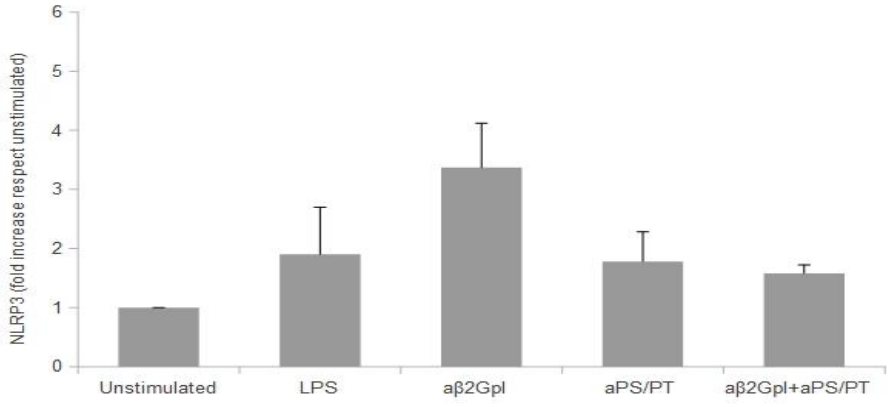

Figure 19: Relative NLRP3 mRNA expression in monocytes.

Monocytes obtained from five BD were stimulated as described below for four hours. LPS (1ng/ml) was added alone or with IgG fractions (0.5mg/ml) extracted from APS patients positive for aβ2GpI, aPS/PT or both. The bars represent the mean ± S.E. of three independent experiments. The expression levels of mRNA were detected by PCR real-time with ΔΔct method.

5.5 TF EXPRESSION IN HUVECs TREATED WITH IgG OBTAINED FROM APS PATIENTS' SERUM

HUVECs were treated with LPS alone or in combination with IgG fraction extract from APS patients positive for aβ2GpI or aPS/PT or both. The thrombotic effect was evaluated in term of TF mRNA expression.

The addition of IgG from APS patients caused a significantly increase ($p<0.001$) of TF expression compared to LPS alone, in line with their pathogenic role in APS (figure 20): 18.4±5.8 fold for LPS alone, 89.5±6.8 fold for LPS plus IgG aβ2GpI, 86.8±2.4 fold for LPS plus IgG aPS/PT, 58.3±3.9 fold for LPS plus IgG positive for both. Of note, the mix disclosed the lowest effect, as compared to the single antibodies.

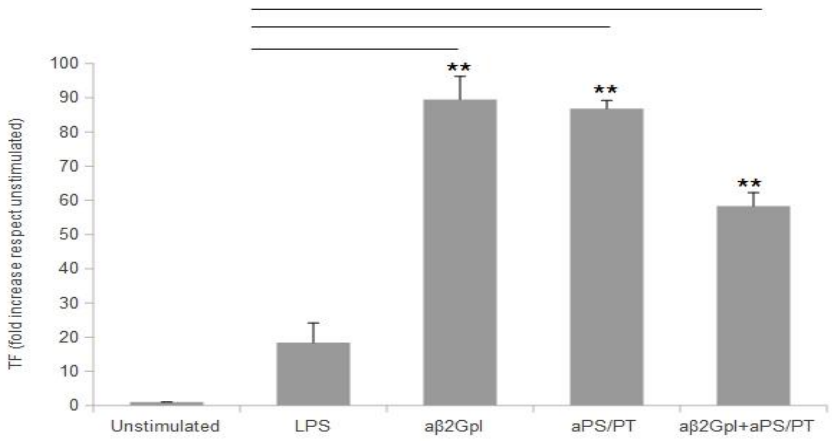

Figure 20: Relative TF mRNA expression in HUVECs.

*Cells were stimulated as described below for four hours. LPS (1ng/ml) was added alone or with IgG fractions (0.5mg/ml) extracted from APS patients positive for aβ2GpI, aPS/PT or both. The bars represent the mean ± S.E. of three independent experiments. The expression levels of mRNA were detected by PCR real-time with ΔΔct method. *p< 0.05, ** p<0.001*

5.6 THE EFFECT OF IgG OBTAINED FROM APS PATIENTS' SERUM ON NITRIC OXIDE (NO) PRODUCTION IN HUVECs

NOx levels were found to be increased in cells treated respect unstimulated cells. In particular, as shown in figure 21, after 16 hours of treatment, NOx levels were significantly increased in endothelial cells treated with LPS plus IgG aβ2GpI or IgG aPS/PT. No difference was found between aPS/PT, aβ2GpI and the mix of the two antibodies.

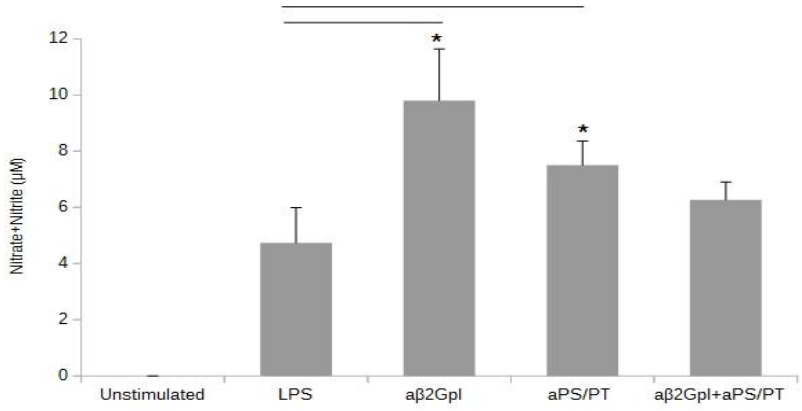

Figure 21: Production of NOx in HUVECs.

Cells were stimulated as described below for 16 hours. LPS (1ng/ml) was added alone or with IgG fractions (0.5mg/ml) extracted from APS patients positive for aβ2GpI, aPS/PT or both. Nox levels were measured in supernatant of treated-cells. The bars represent the mean ± S.E. of three independent experiments.
**p< 0.05*

5.7 SOLUBLE FACTOR RELEASED BY HUVECs AFTER PROCOAGULANT TREATMENT: PRELIMINARY DATA

In order to identify soluble factor released specifically by HUVECs under procoagulant treatment, supernatants were analyzed by Luminex® technology, investigating 42 cytokines, chemokines and growth factors. Preliminary data are shown in table 6 and 7, where only molecules significantly upregulated were reported. HUVECs stimulated both by aβ2GpI IgG and aPS/PT IgG, compared to LPS alone, did not show different pro-inflammatory cytokine or

chemokine effects, while they released higher amount of the specifically endothelial cells activation markers, such as VCAM-1 and ICAM-1.

TABLE 5: SOLUBLE FACTORS RELEASED FROM HUVECs AFTER 16h PROCOAGULANT TREATMENT

16h	CXCL5 (pg/ml)	CX3CL1 (pg/ml)	IL8 (pg/ml)	CCL2 (pg/ml)	VCAM1 (pg/ml)	ICAM1 (pg/ml)
UNSTIMULATED	504	9	148	27	0	37
LPS	1885	83	11151	806	5	149
aβ2GpI	1501	60	7841	600	152	301
aPS/PT	1885	97	10925	1094	143	290

TABLE 6: SOLUBLE FACTORS RELEASED FROM HUVECs AFTER 24h PROCOAGULANT TREATMENT

24h	CXCL5 (pg/ml)	CX3CL1 (pg/ml)	IL8 (pg/ml)	CCL2 (pg/ml)	VCAM1 (pg/ml)	ICAM1 (pg/ml)
UNSTIMULATED	504	9	148	27	0	37
LPS	1851	69	9749	664	7	231
aβ2GpI	1668	69	9461	867	119	442
aPS/PT	1849	96	13316	880	83	478

5.8 PAF-AH

5.8.1 PAF-AH PLASMATIC ACTIVITY IN PATIENTS AND CONTROLS: CORRELATION WITH LIPOD METABOLIC MARKERS

PAF-AH plasmatic activity in BDs disclosed a mean value of 15.6±4 nmol/min/ml (range 5.9 – 28.4). As expected (Maiolino, 2012), a significant correlation was found between PAF-AH activity and total cholesterol (r=0.25; p=0.032) with direct strong correlation with Low Density Lipoprotein (LDL) (r=0.46, p<0.0001) and significant inverse correlation with High Density Lipoprotein (HDL) (r= -0.45, p<0.0001). Instead, no correlation was found with age. 116 /167 patients undergoing aPL investigation, showed at least one positive aPL among LAC, aCL, aβ2GpI or aPS/PT antibodies, while 51/167 resulted all negative. PAF-AH activity was clearly more elevated in the overall patients (19.8±5.5 nmol/min/ml) than in BDs (p<0.0001), but no difference was found between aPL positive and aPL negative patients (19.9±5.8 versus 19.6±4.7 nmol/min/ml; figure 22). The analysis on total cholesterol shown that levels of cholesterol did not differ significantly between BDs and the patients. In particular, no difference was observed between BDs and aPL positive patients (188±38 mg/dl versus 198±42 mg/dl; p=0.10) and between aPL positive and aPL negative patients (206±52 mg/dl; p=0.47). However, LDL serum levels were higher in aPL negative patients than in BDs (127±42 mg/dl vs 104±35 mg/dl; p=0.0073) as well as in aPL positive patients (109±35 mg/dl; p=0.032 vs aPL negative; p=ns vs BDs). The significant correlation between PAF-AH activity and cholesterol, LDL and HDL serum levels persisted in aPL positive patients (r=0.21, p=0.041; r=0.23, p=0.024 and r= -0.31, p=0.0027 respectively), while in aPL negative patients this correlation was evident only for LDL (r=0.29, p=0.14; r=0.25, p=0.0027 and r= -0.25, p=0.21 respectively).

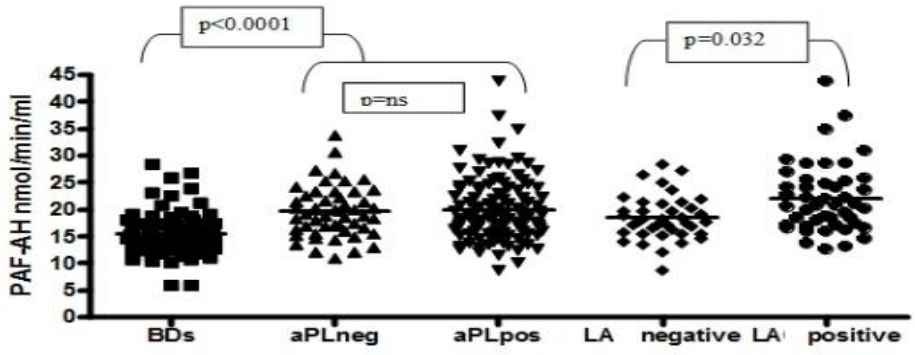

Figure 22: PAF-AH Plasmatic Activity in Patients and Controls. PAF-AH plasmatic activity was clearly more elevated in the all patients (19.8±5.5 nmol/min/ml) than in BDs (p<0.0001), but no difference occurred between aPL positive and aPL negative patients (19.9±5.8 versus 19.6±4.7 nmol/min/ml; p=ns). LA positive patients disclosed higher PAF-AH than LA negative (22.1±6.4 versus 19.5±4.1 nmol/min/ml; p=0.0032)

5.8.2 PAF-AH PLASMATIC ACTIVITY IN PATIENTS DISCLOSING DISTINCT PATTERN OF aPL POSITIVITY

Dividing aPL positive patients based on LA assay, PAF-AH activity was higher in LA positive patients than LA negative patients, as shown in figure 22 (22.1±6.4 versus 19.5±4.1 nmol/min/ml; p=0.0032). Of note, total cholesterol levels did not differ between LA positive and LA negative patients (202±39 mg/dl versus 201±34 mg/dl; p=ns), as well as LDL (113±39 mg/dl versus 108±26 mg/dl; p=ns) and HDL serum levels (60±21 mg/dl versus 63±21 mg/dl; p=ns). Moreover, LA positive patients disclosed higher PAF-AH than aPL negative patients (p=0.03), with again no difference as regard to HDL (62±24 mg/dl in aPL-negative; p=ns) and LDL (127±42 mg/dl in aPL-negative; p=ns). As illustrated in figure 23, patients presenting aβ2GpI IgG positive antibodies disclosed higher PAF-AH activity than patients presenting only aβ2GpI IgM positive antibodies (23.1±7.2 nmol/min/ml versus 20.1±5.3 nmol/min/ml; p=0.035), but they did not differ with regard to LDL and HDL serum levels. Patients who were negative for aβ2GpI IgG or IgM antibodies, but who showed either isolated LA or aCL or aPS/PT positive antibodies demonstrated

significantly lower PAF-AH activities, that appeared comparable to those measured in BDs (figure 17; 16.9±3.8 nmol/min/ml; p=ns versus BDs; p=0.003 versus aβ2GpI IgM positive). Total cholesterol, LDL and HDL serum levels in the latter subgroup of patients did not differ from those measured in patients with aβ2GpI IgM positive or IgG positive antibodies. Overall, aPS/PT IgG positive patients disclosed PAF-AH activity close to that of aPS/PT IgM positive patients (17.3±3 nmol/min/ml versus 16.1±3.9 nmol/min/ml; p=ns). Finally, patients disclosing aβ2GpI IgG positive antibodies together with aPS/PT IgG positive antibodies tended to show higher PAF-AH activity than patients disclosing only aβ2GpI IgG positive antibodies (23.4±7 nmol/min/ml versus 21±4.7; p=ns).

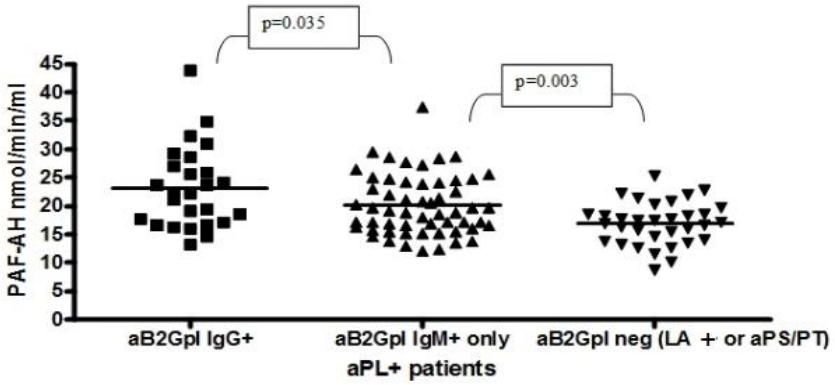

Figure 23: PAF-AH plasmatic activity in patients with distinct aPL specificity. Patients presenting aβ2GpI IgG + antibodies disclosed higher PAF-AH plasmatic activity than patients presenting only aβ2GpI IgM+ antibodies (23.1±7.2 nmol/min/ml versus 20.1±5.3 nmol/min/ml; p=0.035). Patients negative for aβ2GpI IgG or IgM antibodies, showing either isolated LA or aCL or aPS/PT positive antibodies demonstrated significantly lower PAF-AH activity (16.9±3.8 nmol/min/ml; p=0.003 versus a aβ2GpI IgM+)

6. DISCUSSION

At present, the laboratory diagnosis of APS is frequently complicated: among APS patients, there is a subgroup defined “seronegative” that shows clinical criteria but results negative for all “criteria” aPL (LA, aCL and aβ2GpI isotype IgG and/or IgM). To improve APS laboratory diagnosis, it has been proposed several autoantibodies that are directed against other plasma proteins from the coagulation cascade (i.e. PS-PT complex), or interfere with the anticoagulant activity of A5 (Khamashta, 2016).

aPS/PT antibodies have been proposed as potential new biomarkers for thrombosis and/or pregnancy morbidity in the setting of APS. The introduction of the aPS/PT IgM and IgG antibodies among the routinely investigated aPL antibodies, leads to an improvement in APS laboratory diagnostic performance, as shown in a recent observational study performed in our laboratory (Fabris, 2014). Moreover, given their elevated correlation with LA activity, aPS/PT could help when immunological deficits or anticoagulant therapy avoid a correct LA interpretation. However, their pathogenic mechanism is still substantially undefined.

To date, aPS/PT antibodies are not included among “criteria” aPL. To better correlate the presence of aPS/PT and APS clinical manifestation, an in vitro study was carried out to compare the prothrombotic effect of these autoantibodies versus the criteria antibodies, such as the aβ2GpI. Several studies have already demonstrated the prothrombotic effect of aβ2GpI IgG on monocytes and endothelial cells (Rikarni, 2015). Indeed, aβ2GpI induce TF expression on these cells. On the other side, there are few information about the effect of aPS/PT (Oku, 2013) and no direct comparison between aβ2GpI and aPS/PT.

In this study, for the first time, a pro-coagulant treatment for the contemporary analysis of the effect of aβ2GpI IgG and aPS/PT IgG on

monocytes and ECs was employed. Compared to the stimulation by the IgG fraction obtained by BD, either the IgG fractions by aβ2GpI positive and aPS/PT positive patients determined a significant activation of monocytes and HUVECs, showing a procoagulant phenotype. While the effect was similar in term of TF mRNA expression and release of specific endothelial activation factors (NO, ICAM, VCAM), a different effect was noticed in terms of IL-1b and NLRP3 expression, since aPS/PT antibodies seem to have an opposite effect compared to aβ2GpI. Further studies are needed to clarify these results.

Endothelial cells are actively involved into the inflammatory process with specific adaptive response that includes formation of reactive oxygen species (ROS) with upregulation of nitric oxide (NO) production (Assis, 2002; Laurindo, 1994). Furthermore, endothelial dysfunction plays an important role in atherosclerotic disease (Pasaoglu, 2014). The upregulation of NO production in response to both aβ2GpI and aPS/PT antibodies indicate that they both induce an adaptatively response on endothelial cells.

Several studies demonstrated that PAF-AH is a cardiovascular risk marker independent respect the traditional risk factors for CV. Its increased expression was correlated with the vulnerability of atherosclerotic plaques. Therefore, in order to assess the CV risk, PAF-AH dosage has been proposed to ensure a better stratification of at risk populations, (Corson, 2008). To date, PAF-AH has never been investigated in the context of APS patients, or, even less, in patients at risk to develop an overt APS (i.e. asymptomatic carriers of aPL antibodies).

This study was conducted on patients routinely screened for APS, demonstrating a significant association between the presence of aPL antibodies (LA and aβ2GpI IgG in particular) and PAF-AH activity upregulation in plasma.

Atherosclerosis is definitively recognized as a chronic inflammatory response due to the accumulation of lipoproteins in the walls of arteries (Libby, 2016). PAF-AH is manly associated with LDL and it is predominantly express in the

necrotic centre of atherosclerotic plaques and in the macrophage-rich areas releasing pro-inflammatory mediators, such as lysophospholipids and oxidized fatty acids (Rosenson, 2012).

In addition to the presence of LA, several different targets of aPL could be determined by many analytical methods, with frequent discordant results, that could make the laboratory diagnosis of APS extremely complicated. The main role of the aβ2GpI antibodies, especially those specifically targeting domain I (Giannakopoulos, 2013), is widely accepted and present results seem to further confirm their importance with regard to CV risk stratification, since PAF-AH appeared particularly elevated in aβ2GpI positive patients and more so in those displaying LA activity and carrying the IgG isotype. This particular association may be explained by the fact that IgG aβ2GpI antibodies are able to recognize the stable complex between oxLDL and β2GpI, thus facilitating macrophage-derived foam cell formation in patients with APS (Zhang, 2014). The immune-pathological mechanisms sustained by oxLDL/β2GpI complexes are not yet fully understood, but TLR4 was recently shown to be involved (Zhang, 2014). TLR4 could be the key player linking PAF-AH up-regulation to aβ2GpI IgG antibodies in APS, as evidenced by a mouse model of preterm delivery which demonstrated that PAF effects and signalling depend upon TLR4 stimulation (Agrawal, 2014).

Lp-PLA2 activity proved to be markedly reduced in vivo when the enzyme is bound to HDL (Rosenson, 2012), and this is in line with our observation that aβ2GpI IgG+ patients disclosed higher PAF-AH and lesser HDL than BDs. This is not true for other subgroups of patients, such as aPL-negative patients or those presenting only isolated LAC or aCL or aPS/PT antibodies. Compared to these patients, PAF-AH plasmatic activity up-regulation in aβ2GpI IgG+ cases appeared to be at least partially disconnected from the lipoprotein levels and specifically linked to the presence of such aPL antibodies.

Therefore, PAF-AH up-regulation arose as a specific thrombotic risk marker in patients carrying aβ2GpI antibodies and is not generally associated with other

aPL antibodies possibly implicated in APS manifestations, but further studies are needed to confirm this observation.

Unfortunately, in two large randomized clinical trials, an inhibitor of PAF-AH (darapladib) (O'Donoghue, 2014; Wallentin, 2016) failed to reduce the risk of major coronary events as compared to placebo. In addition, it was associated with significantly higher rates of drug discontinuation and adverse effects. These results suggested that PAF-AH may be a biomarker of vascular inflammation, rather than a causal pathway of CV diseases (Wallentin, 2016). Therefore, high PAF-AH activity could reflect a response to pro-inflammatory stress characteristic both of atherosclerosis and APS (Marsthe, 2014).

The leading cause of death in primary and secondary APS patients are cardiovascular events due to accelerated atherosclerosis, which often progresses more rapidly, compared with the general population (Silva, 2014). Some key pro-inflammatory proteins correlate with APS clinical manifestations (Becarevic, 2016) and common radiological markers of subclinical atherosclerosis and CV risk were often reported in such patients (Ambrosino, 2014). However, to date, besides the presence of aPL itself, no serological biomarkers specifically associated with aPL-related pathogenic mechanisms have been identified as useful to improve the classification of CV risk in aPL+ patients with and without overt APS clinical manifestations.

In this scenario, present findings on PAF-AH assume a relevant place, possibly representing a reliable and affordable biomarker useful to identify patients at higher risk in which to take a more cautious therapeutic attitude in the follow-up.

Moreover, studying PAF-AH metabolic pathway may help to better explain the pathogenesis of APS and to improve management and interpretation of aPL-related issues, from the analytical result, to the final therapeutic decision.

Even if we and others recently demonstrated an important role of aPS/PT antibodies in the laboratory diagnosis of APS (Fabris, 2014; Amengual, 2016), the therapeutic management of patients characterized by the presence of

isolated aPS/PT remains an open issue. Patients with isolated aPS/PT antibodies disclosed lower (BDs-like) PAF-AH as compared to patients with positive aβ2GpI antibodies. Nevertheless, aPS/PT antibodies, may exert their distinct pathogenic role through pathways in which PAF-AH is not involved, as shown previously as regard to IL1b and NLRP3 expression on monocytes treated with such antibodies compared to aβ2GpI.

7. CONCLUSIONS

The introduction of aPS/PT antibodies in the diagnostic process of APS is highly recommended, since they disclosed diagnostic laboratory performances at least equal to the aCL and aβ2GpI antibodies and a high correlation with LA activity, such that they can be a viable alternative.

Data obtained by our in vitro study, even if preliminary, confirmed that aPS/PT exert similar pro-thrombotic effects on monocytes and HUVECs as compared to the aβ2GpI antibodies. The different effect of aβ2GpI and aPS/PT on expression of IL1β and NLRP3, and the different impact on PAF-AH production, may suggest that these classes of antibodies, while disclosing similar pro-thrombotic effects, probably activate different metabolic pathways. Further studies are needed to better clarify these issues.

Anyway, the prognostic information conveyed by plasmatic PAF-AH activity in patients with positive aPL antibodies appeared to be independent to that of common lipid metabolism markers (i.e. LDL), as previously reported by other authors in the context of patients with major coronary events (Maiolino, 2012) and, based on present results, PAF-AH plasmatic activity may represent a new prognostic biomarker also in the context of aPL antibodies, to identify patients at major risk and favouring more tailored therapeutic interventions. Further prospective studies on selected patients are ongoing.

8. PUBLISHED ABSTRACTS

Dubsky de Wittenau G, Radovic S, Pascolo R, Cesca F**, Cifù A**, Curcio F, Lonigro R.De-novo deletion or uniparental disomy as SMA determining cause: a case report. *European society of human genetics. Milan (Italy), 2014*

CifùA, Zago S, Poz A, Giacomello R, Tonutti E, Curcio F, Fabris M. Anti-phosphatidylserine/ prothrombin Autoantibodies Significantly Improve the Laboratory Diagnostic Process Of Anti-Phospholipids Antibody Syndrome. *Secondo congresso dell'area di patologia e diagnostica di laboratorio. Palermo (Italy), 2014*

Fabris M, Giacomello R, **Cifù A**, Tanzi N, Curcio F, Tonutti E. Phosphatidylserine-dependent antiprothrombin antibodies in clinical practice. *SIPMET meeting the young scientists -Alba (Italy), 2015*

CifùA, Poz A, Tonutti E, Giacomello R, Pistis C, Curcio F, Fabris M. Studio pilota: PAF (platelet activating factor) -acetilidrolasi quale nuovo possibile marker di rischio trombotico nei pazienti affetti da sindrome da anticorpi anti-fosfolipidi. *Primo congresso nazionale della società italiana di patologia clinica e medicina di laboratorio - Roma (Italy), 2015*

Tonelli V, Pistis C, **Cifù A**, Tonutti E, Vescini F, Curcio F, Grimaldi F, Fabris M.Elevati livelli di B-Lymphocyte Stimulator (BLyS) nei pazienti con

tumore neuroendocrino identificano una malattia più severa e non responsiva alla terapia. *Primo congresso nazionale della società italiana di patologia clinica e medicina di laboratorio - Roma (Italy), 2015*

Cifù A, Fabris M, Giacomello R, Pistis C, Tonutti E, Curcio F. The plasmatic Platelet-Activating Factor acetylhydrolase activity: a possible risk biomarker in patients with anti-phospholipid syndrome.
10th international congress of autoimmunity - Leipzig (Germany),2016

Fabris M, Tanzi N, Giacomello R, **Cifù A**, Poz A, Curcio F, Tonutti E. Anti-phospholipid antibodies testing by ELISA versus CLIA: a comparative Italian study. *10th international congress of autoimmunity - Leipzig (Germany),2016*

Cifù A, Sanvilli N, Tanzi N, Pistis C, Fabris M, Fontana DE, Giacomello R, Curcio F, Gigli G, Janes F. Asymmetrical Dimethylarginine (ADMA) In Asympomatic Cerebral Small Vessel Disease. *The American Journal of Pathology- Supplement, Volume 186 (2016) S6*

Domenis R, Zanutel R, **Cifù A**, Pistis C, Di Benedetto P, Causero A, Pozzi M, Bassini F, Fabris M, Niazi KR, Curcio F. Characterization of the Proinflammatory Profile of Synovial Fluid-Derived Exosomes of Patients

with Gonarthrosis. *The American Journal of Pathology- Supplement, Volume 186 (2016) S23*

Fabris M, **Cifù A**, Pistis C, Giacomello R, Siega-Ducaton M, Tonutti E, Curcio F. Elevated Plasma Platelet-Activating Factor Acetylhydrolase Activity is Significantly Associated with High Risk Anti-Phospholipid Antibody Antigenic Specificities. *The American Journal of Pathology- Supplement, Volume 186 (2016) S24*

Cifù A, Pistis C, Domenis R, Curcio F, Fabris M. Comparison between Anti-β2Glycoprotein I Antibody and Anti-Phosphatidylserine/Prothrombin Antibody Biological Effects on Peripheral Blood Monocytes. *The American Journal of Pathology- Supplement, Volume 186 (2016) S27*

Cifù A, Pistis C, Fabris M, Domenis R, Zanello A, Gandolfo S, De Vita S, Curcio F. Characterization of Parotid Gland Epithelial Cell Primary Cultures from Patients Affected by Sjogren's Syndrome. *The American Journal of Pathology- Supplement, Volume 186 (2016) S31*

Pistis C, Domenis R, Moretti M, Vicario A, **Cifù A**, Di Benedetto P, Causero A, Pozzi M, Bassini F, Fabris M, Niazi KR, Curcio F. The immunomodulatory properties of adipose mesenchymal stem cells are strengthened by treatment with osteoarthritic synovial fluid. *PISA 2016 Breakthroughs in*

biology: from underlying pathogenesis to translational medicine- Houston (Texas, USA),2016

9. PUBLICATIONS

Fabris M,**Cifù A**, Pistis C, Siega-Ducaton M, Fontana DE, Giacomello R, Tonutti E, Curcio F.Exploring the Plasmatic Platelet-Activating Factor Acetylhydrolase Activity in Patients with Anti-phospholipid Antibodies. *Autoimmunity Highlights* – ***ahead of print***

Domenis R, Zanutel R, Caponnetto F, Toffoletto B, **Cifù A**, Pistis C, Di Benedetto P, Causero A, Pozzi M, Bassini F, Fabris M, Niazi KR, Soon-Shiong P, Curcio F. Characterization of the proinflammatory profile of synovial fluid-derived exosomes of patients with osteoarthritis. *Mediators of Inflammation* - ***under review***

10. REFERENCES

Agrawal V, Jaiswal MK, Ilievski V, Beaman KD, Jilling T, Hirsch E. Platelet-Activating Factor: a Role in Preterm Delivery and an Essential Interaction with Toll-Like Receptor Signaling in Mice. Biology of Reproduction 2014;91(5):119.

Aguiar CL, Erkan D. Catastrophic antiphospholipid syndrome: how to diagnose a rare but highly fatal disease. Therapeutic Advances in Musculoskeletal Disease 2013;5(6):305–14.

Allford SL, Machin S. Haemostasis. Surgery (Oxford) 2007;25(6):241–4.

Ambrosino P, Lupoli R, Minno AD, Iervolino S, Peluso R, Minno MNDD. Markers of cardiovascular risk in patients with antiphospholipid syndrome: A meta-analysis of literature studies. Annals of Medicine 2014;46(8):693–702.

Amengual O, Forastiero R, Sugiura-Ogasawara M, et al. Evaluation of phosphatidylserine-dependent antiprothrombin antibody testing for the diagnosis of antiphospholipid syndrome: results of an international multicentre study. Lupus 2017;26(3):266–76.

Amengual O, Atsumi T, Koike T. Specificities, properties, and clinical significance of antiprothrombin antibodies. Arthritis & Rheumatism 2003;48(4):886–95.

Andreoli L, Nalli C, Motta M, et al. Anti- 2-glycoprotein I IgG antibodies from 1-year-old healthy children born to mothers with systemic autoimmune

diseases preferentially target domain 4/5: might it be the reason for their 'innocent' profile? Annals of the Rheumatic Diseases 2010;70(2):380–3.

Arachchillage DJ, Greaves M. The chequered history of the antiphospholipid syndrome. British Journal of Haematology 2014;165(5):609–17.

Assis MCD, Plotkowski MC, Fierro IM, Barja-Fidalgo C, Freitas MSD. Expression of inducible nitric oxide synthase in human umbilical vein endothelial cells during primary culture. Nitric Oxide 2002;7(4):254–61.

Atsumi T, Khamashta MA, Haworth RS, et al. Arterial disease and thrombosis in the antiphospholipid syndrome: A pathogenic role for endothelin 1. Arthritis & Rheumatism 1998;41(5):800–7.

Barbé F, Douglas T, Saleh M. Advances in Nod-like receptors (NLR) biology. Cytokine & Growth Factor Reviews 2014;25(6):681–97.

Bertolaccini M, Amengual O, Atsumi T, et al. 'Non-criteria' aPL tests: report of a task force and preconference workshop at the 13th International Congress on Antiphospholipid Antibodies, Galveston, TX, USA, April 2010. Lupus 2011;20(2):191–205.

Bećarević M, Ignjatović S. Proinflammatory proteins in female and male patients with primary antiphospholipid syndrome: preliminary data. Clinical Rheumatology 2016;35(10):2477–83.

Brandt KJ, Kruithof EK, Moerloose PD. Receptors involved in cell activation by antiphospholipid antibodies. Thrombosis Research 2013;132(4):408–13.

Chen G, Pedra JH. The Inflammasome in Host Defense. Sensors 2009;10(1):97–111.

Corson MA, Jones PH, Davidson MH. Review of the Evidence for the Clinical Utility of Lipoprotein-Associated Phospholipase A2 as a Cardiovascular Risk Marker. The American Journal of Cardiology 2008;101(12).

Davidson MH, Corson MA, Alberts MJ, et al. Consensus Panel Recommendation for Incorporating Lipoprotein-Associated Phospholipase A2 Testing into Cardiovascular Disease Risk Assessment Guidelines. The American Journal of Cardiology 2008;101(12).

Erkan D. A cross-sectional study of clinical thrombotic risk factors and preventive treatments in antiphospholipid syndrome. Rheumatology 2002;41(8):924–9.

Esdaile JM, Abrahamowicz M, Grodzicky T, et al. Traditional Framingham risk factors fail to fully account for accelerated atherosclerosis in systemic lupus erythematosus. Arthritis & Rheumatism 2001;44(10):2331–7.

Fabris M, Giacomello R, Poz A, et al. The introduction of anti-phosphatidylserine/prothrombin autoantibodies in the laboratory diagnostic process of anti-phospholipid antibody syndrome: 6 months of observation. Autoimmunity Highlights 2014;5(2):63–7.

Giannakopoulos B, Krilis SA. The Pathogenesis of the Antiphospholipid Syndrome. New England Journal of Medicine 2013;368(11):1033–44.

Groot PGD, Meijers JCM. β2-Glycoprotein I: evolution, structure and function. Journal of Thrombosis and Haemostasis 2011;9(7):1275–84.

Gómez-Puerta JA, Cervera R. Diagnosis and classification of the antiphospholipid syndrome. Journal of Autoimmunity 2014;48-49:20–5.

Hanly JG. Antiphospholipid Syndrome: an overview. Canadian Medical Association Journal 2003; 168(13): 1675-1682

Harper BE, Willis R, Pierangeli SS. Pathophysiological mechanisms in antiphospholipid syndrome. International Journal of Clinical Rheumatology 2011;6(2):157–71.

Hoffman M. A cell-based model of coagulation and the role of factor VIIa. Blood Reviews 2003;17.

Khamashta M, Taraborelli M, Sciascia S, Tincani A. Antiphospholipid syndrome. Best Practice & Research Clinical Rheumatology 2016;30(1):133–48.

Laurindo FR, De APM, Barbeiro HV, et al. Vascular free radical release. Ex vivo and in vivo evidence for a flow- dependent endothelial mechanism. Circulation Research 1994;74(4):700–9.

Lefèvre G, Lambert M, Bacri J-L, et al. Thrombotic events during long-term follow-up of obstetric antiphospholipid syndrome patients. Lupus 2011;20(8):861–5.

Libby P, Bornfeldt KE, Tall AR. Atherosclerosis. Circulation Research 2016;118(4):531–4.

Ma K. High Affinity Binding of beta 2-Glycoprotein I to Human Endothelial Cells Is Mediated by Annexin II. Journal of Biological Chemistry 2000;275(20):15541–8.

Mahler M, Norman GL, Meroni PL, Khamashta M. Autoantibodies to domain 1 of beta 2 glycoprotein 1: A promising candidate biomarker for risk

management in antiphospholipid syndrome. Autoimmunity Reviews 2012;12(2):313–7.

Maiolino G, Pedon L, Cesari M, et al. Lipoprotein-Associated Phospholipase A2 Activity Predicts Cardiovascular Events in High Risk Coronary Artery Disease Patients. PLoS ONE 2012;7(10).

Maiolino G. Lipoprotein-associated phospholipase A2 prognostic role in atherosclerotic complications. World Journal of Cardiology 2015;7(10):609.

Mann KG, Bovill EG, Krishnaswamy S. Surface-Dependent Reactions in the Propagation Phase of Blood Coagulation. Annals of the New York Academy of Sciences 1991;614(1):63–75.

Marathe GK, Pandit C, Lakshmikanth CL, Chaithra VH, Jacob SP, D'souza CJM. To hydrolyze or not to hydrolyze: the dilemma of platelet-activating factor acetylhydrolase. The Journal of Lipid Research 2014;55(9):1847–54.

Mcintyre TM, Prescott SM, Stafforini DM. The emerging roles of PAF acetylhydrolase. The Journal of Lipid Research 2008;50(Supplement).

Mcmichael M. New Models of Hemostasis.Topics in Companion Animal Medicine 2012;27(2):40–5.

Meroni PL, Riboldi P. Pathogenic mechanisms mediating antiphospholipid syndrome. Current Opinion in Rheumatology 2001;13(5):377–82.

Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid

syndrome (APS). Journal of Thrombosis and Haemostasis 2006;4(2):295–306.

Miyakis S, Giannakopoulos B, Krilis SA. Beta 2 glycoprotein I-function in health and disease. Thrombosis Research 2004;114(5-6):335–46.

Mulla MJ, Salmon JE, Chamley LW, et al. A Role for Uric Acid and the Nalp3 Inflammasome in Antiphospholipid Antibody-Induced IL-1β Production by Human First Trimester Trophoblast. PLoS ONE 2013;8(6).

Müller-Calleja N, Köhler A, Siebald B, et al. Cofactor-independent antiphospholipid antibodies activate the NLRP3-inflammasome via endosomal NADPH-oxidase: implications for the antiphospholipid syndrome. Thrombosis and Haemostasis 2015;113(5):1071–83.

Nishimura S, Manabe I, Nagasaki M, et al. In vivo imaging visualizes discoid platelet aggregations without endothelium disruption and implicates contribution of inflammatory cytokine and integrin signaling. Blood 2011;119(8).

Norris LA. Blood coagulation. Best Practice & Research Clinical Obstetrics & Gynaecology 2003;17(3):369–83.

Oku K, Amengual O, Zigon P, Horita T, Yasuda S, Atsumi T. Essential role of the p38 mitogen-activated protein kinase pathway in tissue factor gene expression mediated by the phosphatidylserine-dependent antiprothrombin antibody. Rheumatology 2013;52(10):1775–84.

O'Donoghue ML, Braunwald E, White HD, et al. Effect of Darapladib on Major Coronary Events After an Acute Coronary Syndrome. Jama 2014;312(10):1006.

Panteleev MA, Dashkevich NM, Ataullakhanov FI. Hemostasis and thrombosis beyond biochemistry: roles of geometry, flow and diffusion. Thrombosis Research 2015;136(4):699–711.

Pasaoglu OT, Turkozkan N, Ark M, Polat B, Agilli M, Yaman H. The Effect of Taurine on the Relationship Between NO, ADMA and Homocysteine in Endotoxin-Mediated Inflammation in HUVEC Cultures. Inflammation 2014;37(5):1439–43.

Raschi E, Chighizola CB, Grossi C, et al. β2-glycoprotein I, lipopolysaccharide and endothelial TLR4: Three players in the two-hit theory for anti-phospholipid-mediated thrombosis. Journal of Autoimmunity 2014; 55:42–50.

Rikarni, Dharma R, Tambunan KL, Isbagyo H, Dewi BE, Acang N, Setiabudy R, Aman AK. Prothrombotic effect of Anti-beta-2 Glycoprotein-1 Antibodies on the expression of Tissue Factor, Thrombomodulin, and Plasminogen Activator Inhibitor-1 in Endothelial Cells. Acta Med Indones. 2015;47(1):31-7

Rosenson RS, Hurt-Camejo E. Phospholipase A2 enzymes and the risk of atherosclerosis. European Heart Journal 2012;33(23):2899–909.

Sciascia S, Bertolaccini M. Antibodies to phosphatidylserine/prothrombin complex and the antiphospholipid syndrome. Lupus 2014;23(12):1309–12.

Sciascia S, Khamashta MA, Bertolaccini ML. New Tests to Detect Antiphospholipid Antibodies: Antiprothrombin (aPT) and Anti-Phosphatidylserine/Prothrombin (aPS/PT) Antibodies. Current Rheumatology Reports 2014;16(5).

Silva FFD, Levy RA, Carvalho JFD. Cardiovascular Risk Factors in the Antiphospholipid Syndrome. Journal of Immunology Research 2014; 2014:1–6.

Simone ND, Meroni PL, Papa ND, et al. Antiphospholipid antibodies affect trophoblast gonadotropin secretion and invasiveness by binding directly and through adhered β2-glycoprotein I. Arthritis & Rheumatism 2000;43(1):140–50.

Tripodi A, Groot PGD, Pengo V. Antiphospholipid syndrome: laboratory detection, mechanisms of action and treatment. Journal of Internal Medicine 2011;270(2):110–22.

Urbanus RT, Pennings MTT, Derksen RHWM, Groot PGD. Platelet activation by dimeric2-glycoproteinI requires signaling via both glycoproteinIb and apolipoproteinE receptor2. Journal of Thrombosis and Haemostasis 2008;6(8):1405–12.

Wallentin L, Held C, Armstrong PW, et al. Lipoprotein-Associated Phospholipase A 2 Activity Is a Marker of Risk But Not a Useful Target for

Treatment in Patients With Stable Coronary Heart Disease. Journal of the American Heart Association 2016;5(6).

Watson H, Davidson S, Keeling D. Guidelines on the diagnosis and management of heparin-induced thrombocytopenia: second edition. British Journal of Haematology 2012;

Willis R, Gonzalez EB, Brasier AR. The Journey of Antiphospholipid Antibodies From Cellular Activation to Antiphospholipid Syndrome. Current Rheumatology Reports 2015;17(3).

Wilson WA, Gharavi AE, Koike T, et al. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: Report of an International workshop. Arthritis & Rheumatism 1999;42(7):1309–11.

Zhang X, Xie Y, Zhou H, et al. Involvement of TLR4 in Oxidized LDL/β2GPI/Anti-β2GPI-Induced Transformation of Macrophages to Foam Cells. Journal of Atherosclerosis and Thrombosis 2014;21(11):1140–51