INNATE IMMUNITY ON ANTIPHOSPHOLIPID SYNDROME: TLRs IMPLICATION ON IMFLAMMATION

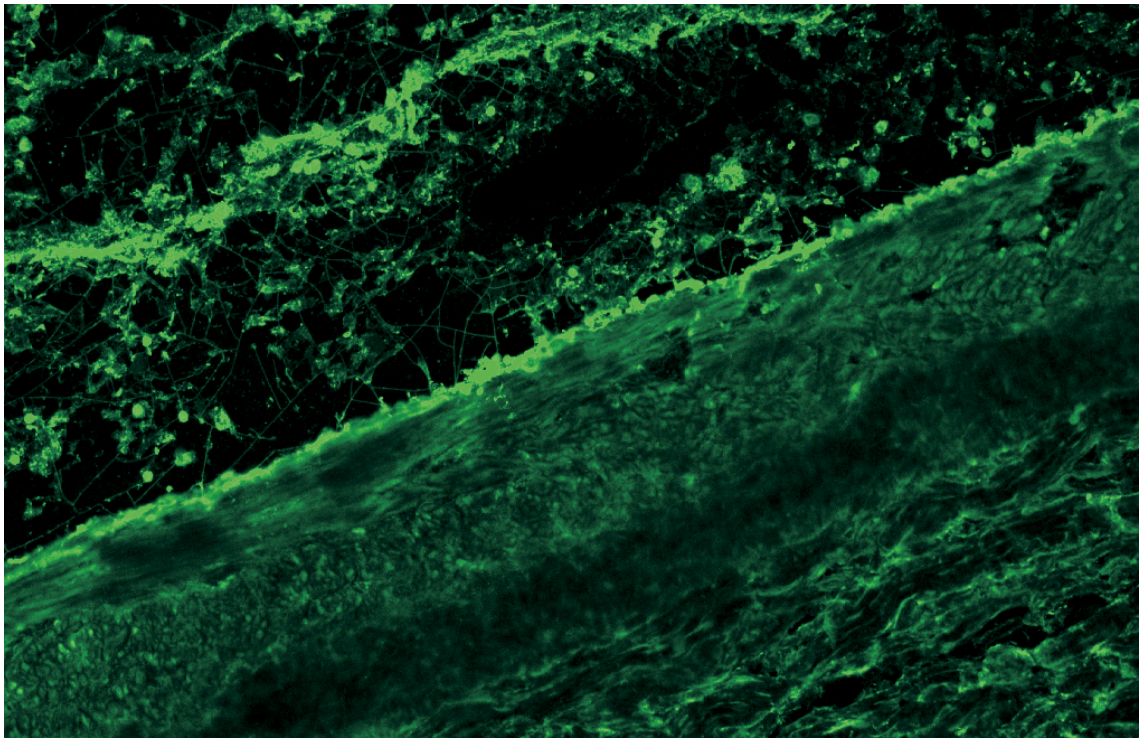

Fig. Endothelial cell staining of an umbilical vein by antibodies against β2GPI. (Atlas of tissue autoantibodies. RG Hughes et al. 2008)

Autor: Ana Franco González de Canales

Director: Marcos López Hoyos. Unidad de Inmunología. Hospital Marqués de Valdecilla. IFIMAV

Máster de Biología Molecular y BioMedicina. Junio 2013

Universidad de Cantabria

BACKGROUND

In 1983, the rheumatologist Dr. Graham R. V. Huges, who worked at the Louise Coote Lupus Unit at St Thomas' (London Hospital), introduced the term 'Antiphospholipid Syndrome' (APS) to characterize a group of patients in whom positivity for anti-cardiolipin antibodies was associated with an increased risk of thrombosis (sometimes called Hughes syndrome). Later on, the syndrome has been better defined as an acquired autoimmune disorder clinically determined by a history of recurrent venous or arterial thrombosis and/or specific obstetric complications (fetal loss). It is a heterogeneous systemic disease affecting many disciplines of medicine including haematology, immunology, rheumatology, obstetrics and neurology (Brock E Harper et al, 2011).

APS can be divided into two types; primary APS where the disease occurs alone or secondary APS where it is found alongside other autoimmune diseases, frequently systemic lupus erythematosus (SLE). However, the clinical and laboratory features of primary and secondary APS do not differ. Both primary and a secondary APS can be a devasting disease. A small subgroup of patients may be diagnosed as having catastrophic APS, an acute and devasting syndrome with significant morbidity and mortality characterized by simultaneous clots in multiple small vascular beds in at least three organ systems over a period of days or weeks leading to multiorgan failure. The long-term prognosis of the APS is determined mainly by recurrent thrombosis, which may occur in up to 29% of patients, sometimes despite antithrombotic therapy.

The APS is characterized by an adaptive immune response against self membrane anionic phospholipids or associated plasma proteins resulting in the generation of anti-phospholipid specific antibodies (aPL), but it remains unclear what factors (genetic and/or environmental) lead to the generation of those aPL. aPL are found in approximately 20–30% of patients with systemic lupus erythematosus, and are the major autoantibodies in patients with primary APS. Although the presence of aPL support the induction of thrombus formation, little is known concerning the direct and indirect impact of aPL and the participation of various cell types. Why antibodies with similar antigen specificity produce different clinical manifestations is not clear. We still do not know why, or how, a relatively homogeneous population of autoantibodies mediates different pathogenic mechanisms, presumably leading to the divergent clinical

manifestations. This scenario begs the question of what additional factors are necessary to induce thrombotic events (*Yehuda Shoenfeld et al. 2008*).

aPL are both pathogenic drivers of, and diagnostic markers for APS (*Pier Luigi Meroni et al, 2011*). But finding aPL does not always mean that the patient suffers of APS, and also thrombosis and obstetric complications have a high incidence not related to APS, but diagnostic laboratories should determine whether a patient suffers from APS or not. To make the diagnosis of APS, aPL should be persistently present in the blood, although more than occasionally persons without clinical signs that are compatible with the APS test positive in one or more of the assays. It is then unclear why only certain individuals with aPL develop clinical events.
Indeed, recent studies suggest that IgG anti-ß2GPI domain I antibodies are highly associated with thrombosis while antibodies against other domains of ß2GPI were not. Nonetheless, there is not clear evidence about their role as either pathogenic or diagnostic.

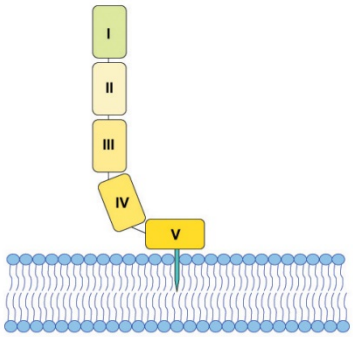

Figure1. ß2GPI antigen structure schema*. Domain I in green.*

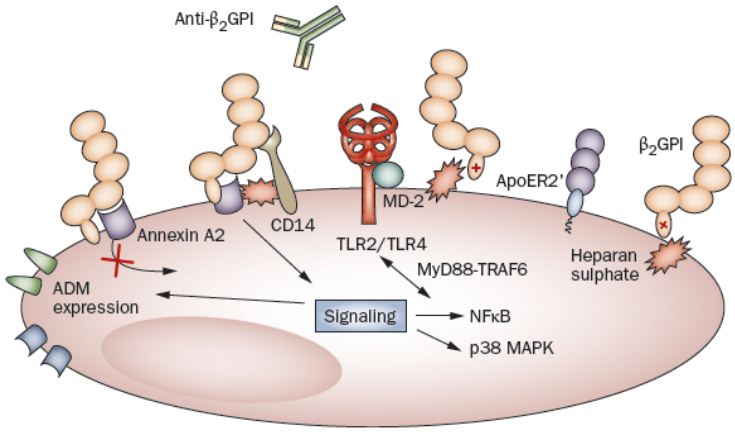

Figure2. Anti-ß2GPI autoantibodies activates endothelial cell through different mechanisms *(Pier Luigi Meroni et al. 2011).*

The revised classification criteria for the antiphospholipid syndrome (2006) emphasize the presence of medium/high titer specific autoantibodies as an essential component of the diagnosis. Currently three assays are available according to the official Sydney Criteria: lupus anticoagulant (LAC), an anticardiolipin antibody (aCL); and an anti-beta2-glycoprotein I (beta2GPI) antibody.

Patients can be diagnosed of APS if at least one of the clinical criteria (vascular thrombosis and/or complication of pregnancy) and one of the laboratory criteria explained above are met (*Bill Giannakopoulos et al. 2013*).

SECOND HIT HYPOTHESIS

There is evidence that the presence of aPL is necessary for the manifestation of APS, but they are not sufficient and need to be accompanied by additional factors for the clinical manifestations of the syndrome. A 'two hit hypothesis' has been suggested to explain the clinical observation that thrombotic events occur only occasionally, in spite of the persistent presence of aPL. According to this principle, the antibody (representing the first hit) induces a thrombophilic state, but clotting takes place only in the presence of another thrombophilic condition (the second hit), like a concomitant trigger of innate immunity that must be present for thrombosis to occur (e.g. a toll-like receptor ligand). Although the presence of aPL is a necessary pre-condition, APS-associated clotting is seemingly triggered by an additional 'second hit', frequently related to mediators of innate inflammatory immune responses. This hypothesis fits well with the potential involvement of pattern recognition receptors (such as TLRs) in sensing microbes and triggering an inflammatory response. It has been suggested that infectious processes might constitute the second hit, as they frequently precede full-blown APS and might be the initiator of the catastrophic subtype.

Then, we can say that innate immune activation plays a dual role in the pathophysiology of APS. First for initiating the production of aPL, but also for precipitating a thrombotic event. Pathogen-derived TLR ligands may trigger the initial production of aPL, while endogenous TLR ligands may be important in local endothelial changes that enable circulating aPL to activate the coagulation cascade. Therefore, innate immunity contributes critically to the pathogenesis of APS in two distinct phases: an 'initiation (or immunologic) phase' and an 'effector (or pathologic) phase'. During the 'initiation phase', the role of innate immunity is to amplify the adaptive immune response (e.g. to phospholipid-binding proteins such as β2GPI), resulting in the long-lived production of aPL and other SLE autoantibodies. Subsequently, during the 'effector phase', the role of innate immunity is to enhance the prothrombotic effects of aPL via priming of the vascular endothelium (e.g. cellular activation and/or disruption) at the site of eventual thrombosis. During both phases, innate immunity may be triggered by events such as injury, infection, inflammation, infarction, or ischemia.

Growing evidence suggests a role of innate immune cells, in particular polymorphonuclear neutrophils (PMN) and Toll-like receptors (TLR), to induce inflammatory responses. These responses could contribute to the innate immune activation and the manifestation of APS, and aPL apparently have a direct impact on complement activation. One can speculate that the combination of the effect of infection plus the perturbation of TLR function mediated by the autoantibodies overcome the threshold for triggering thrombosis. Alternatively, infections or inflammation might increase the expression of the aPL target antigen or the expression of antigenic epitopes that are hidden in resting conditions. Since PMN are important participants of innate immune responses and constitutively express various TLRs, there might be also a direct role for TLR mediated activation of PMN in the course of APS phenotype. PMN are well known to be involved in trophoblastic injury by complement activation and via the interaction of C5a with the C5a receptor inducing the PMN activation leading to the generation of reactive oxygen species (ROS) and releasing of granular components. Also, PMN express tissue factor (TF) upon C5a-induced activation which is an important contributor to neutrophil-mediated fetal injury and loss. Thrombus is induced dependent on the activation of C5 and C6 as well as on b2-GPI-reactive aPL. The interaction of C5a with the C5a receptor induces the activation of polymorphonuclear leukocytes (PMN). Altogether, these findings suggest innate immunity activation to be required for aPL to exert APS pathogenesis, and also provides new insight into the reason why some individuals with aPL develop APS, while others do not.

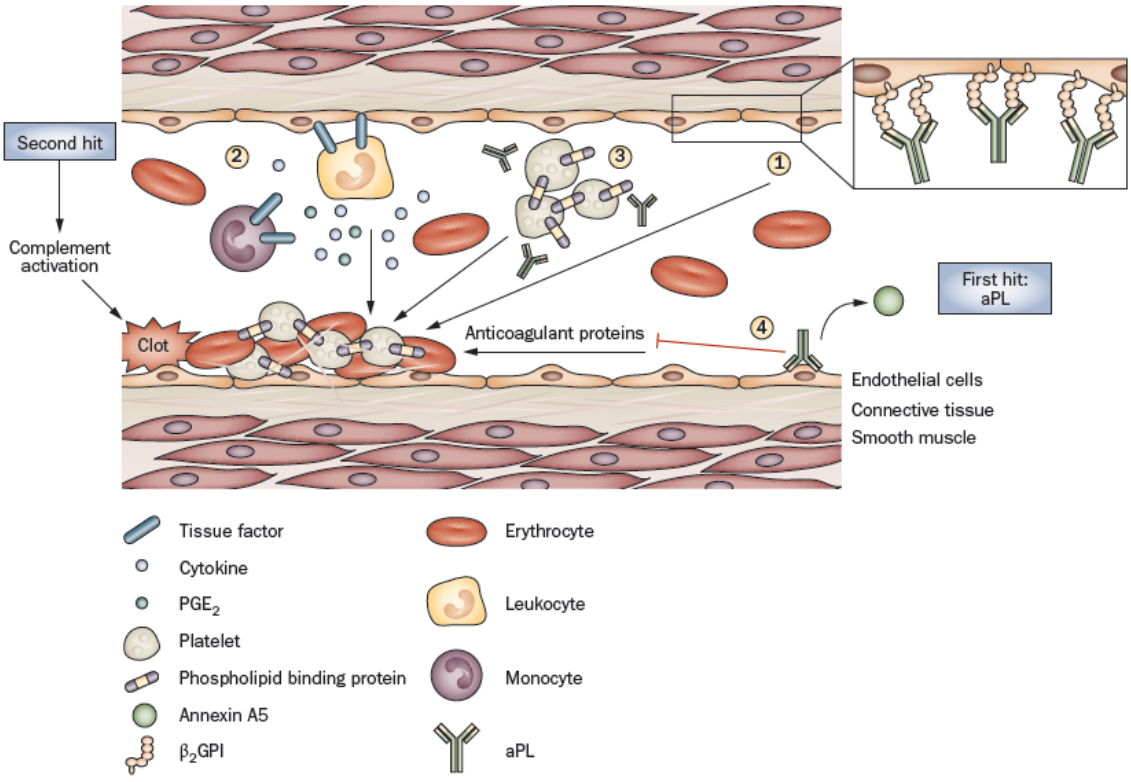

Figure3. Pathogenic clotting mechanisms mediated by aPL*. Clot formation is favored by aPL through several routes. (1) aPL-endothelial cells interaction, primarily β2GPI binds on the cell surface. It induces a procoagulant and proinflammatory endothelial phenotype. (2) On endothelial cells and blood monocytes, aPL upregulate the expression of tissue factor, and promote the adhesion of endothelial leukocyte, secretion of cytokine and synthesis of PGE2. (3) aPL bind to platelets because they recognize phospholipid-binding proteins, potentiating platelet aggregation induced by another agonist. (4) aPL inhibit anticoagulant activity, affect fibrinolysis and displace annexin A5 binding to anionic structures, interfering with some components of the coagulation cascade. All these mechanisms contribute to a procoagulant state. But that is not sufficient for clotting, which seems to require another procoagulant condition, a 'second hit', maybe complement activation (Pier Luigi Meroni et al. 2011).*

Overview of innate immunity

Innate immunity is programmed to detect highly conserved molecular motifs called pathogen associated microbial patterns (PAMPs) via specialized receptors, being the first line of defense against microbes, controlling or eliminating host infection through different mechanisms to hold it until the adaptive immune system is able to recognize it as a pathogen, or otherwise directly to clear it.

These defense mechanisms are of course regulated by receptor systems that are usually able to discriminate between normal self molecules on uninfected cells and infectious nonself ligands, thus controlling triggering of pro-inflammatory protein secretion. These proteins are chemokines and cytokines, as well as other biologically active molecules that act together at the site of infection, and initiate the process of inflammation.
The activation of the complement, which is a group of plasma proteins, may also trigger inflammation. When activated, it´s recognized and then it is able to bound specific complement receptors *(Taro Kawai et al 2010).*

Amongst several families of pattern-recognition receptors (PPRs), Toll-like receptors (TLRs) are the most characterized so far, they are the most extensively studied sensors of damage that participate in the initiation of inflammation. At least thirteen different TLRs have been identified in mammals, each one with a certain degree of specificity for a range of ligands.

These innate immune receptors, TLRs, are of great interest due to the fact that they have a central role both triggering innate immunity and coordinating innate and adaptive immunity. TLRs are crucial at all stages of the inflammatory response and in tissue repair and regeneration.

Immune-regulation capacity level looks to be directly related to TLR expression. Thus, these receptors are to be related too with some other autoimmune diseases because they are responsible for the recognition of exogenous conserved motifs on pathogens, but also, potentially, some endogenous molecules. Therefore, deregulation of these TLR signaling pathways may have severe consequences, and cause many autoimmune diseases and chronic pathological inflammation.

In human, ten TLRs and their respective ligands have been identified, which are members of the pattern recognition receptor (PRR) family. They are homologues of the Drosophila melanogaster Toll protein, preserved throughout evolution and are classified like Type I transmembrane receptors. They are expressed on the cell surface or intracellularly in many cell types, especially in cells of the innate immune system, where they function as sensors of infection or damage. TLRs recognize their ligands by binding to PAMPs. As we can imagine, PAMPs include a broad spectrum of microbial components like microbial peptides, lipopolisacharids (LPS), lipoteichoic acids, bacterial DNA, and viral single or double-stranded RNA (see Table1), but also endogenous molecules released by injured tissue. Identification of these ligands by TLRs activates intracellular signaling pathways that result in the activation of several key transcription factors, especially NF-κB, activator protein 1 (AP-1), and members of the interferon regulatory factor (IRF) family, leading to the subsequent expression of numerous genes involved in a defensive response. The products of these genes lead to the secretion of pro-inflammatory cytokines initiating inflammation, coordinate the effector functions of innate immunity, instruct and modulate adaptive immunity and initiate tissue repair and regeneration. Much intracellular signal transduction after activation occurs through MyD88, a common adaptor protein for TLRs, but some TLRs also trigger MyD88-independent pathways. TLR signaling leads to innate immune activation, which can in turn, result in an inflammatory response. Upon activation, tissue-resident macrophages release pro-inflammatory cytokines (tumor necrosis factor-alpha [TNF-α], interleukin-1 beta [IL-1β], and interleukin-6 [IL-6]) that coordinate both local and systemic inflammatory responses.14 TNF-α and IL-1β activate the local endothelium, inducing vasodilation and increased vascular permeability. The activated endothelium expresses increased levels of tissue factor, leading to local activation of the coagulation cascade. Together, IL-1β and IL-6 activate hepatocytes to produce a number of acute phase proteins, including complement, that further amplify the innate immune response (*J Rauch et al. 2010*).

TLR	Localization	Pathogen-derived agonists	Endogenous agonists	Synthetic agonists
TLR1 – TLR2	Extracellular	Bacteria: peptidoglycan, lipoproteins, LTA Fungi: zymosan	---	Pam_3Cys
TLR2 – TLR6	Extracellular	Bacteria: lipoproteins	Veriscan	MALP2
TLR3	Intracellular	Viruses: dsRNA	mRNA	PolyI:C
TLR4	Extracellular	Bacteria: LPS Viruses: RSV fusion protein Fungi: mannan Protozoa: glycoinositolphospholipids	Saturated fatty acids, β-defensins, oxLDL*, amyloid-β*	Lipid A derivatives
TLR5	Extracellular	Bacteria: flagellin	---	---
TLR7 – TLR8	Intracellular	Viruses: ssRNA	Self RNA	Imiquimod, R-848
TLR9	Intracellular	Bacteria: CpG DNA Viruses: CpG DNA Protozoa: CpG DNA, haemozoin	Self DNA	CpG-ODNs
TLR11	Extracellular	Uropathogenic bacteria Protozoa: profilin-like molecule	---	---

CpG-ODNs, CpG-containing oligodeoxynucleotides; dsRNA, double-stranded RNA; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MALP2, mycoplasma macrophage-activating lipopeptide 2; oxLDL, oxidized low-density lipoprotein; polyI:C, polyinosinic–polycytidylic acid; RSV, respiratory syncytial virus; ssRNA, single-stranded RNA. *Amyloid-β and oxLDL bind to CD36 and a TLR4–TLR6 heterodimer.

Table1. Toll like receptors. *Exogenous, endogenous and synthetic TLR agonists examples.*

We can imagine the importance of TLRs, the significance of these receptors in susceptibility to infection and their involvement in the pathogenesis of a large number of non-infective inflammatory disorders such as cancer, allergy, autoimmunity, inflammatory bowel disease, or atherosclerosis, when reading the literature about different mutations and experimental models with alter TLR function.

As already introduced above, these receptors are to be related with some autoimmune diseases, like APS which we are interested on, because they are responsible for the recognition of exogenous conserved motifs on pathogens, but also, potentially, some endogenous molecules driving to a sterile inflammation sustained by innate immune cells. This contributes to a loss of tolerance. Also, many autoantigens are generated by tissue injury and are able to stimulate innate immunity through TLRs. This supports the idea that many of them are autoantigens, because they act as autoadjuvants which directly activate innate immunity to induce an immune response directed to self tissues or molecules.

Thus, we can think of TLRs like important targets and of course in the future for new vaccines and therapies that may prevent or treat human diseases especially, in our case, autoimmune disorders.

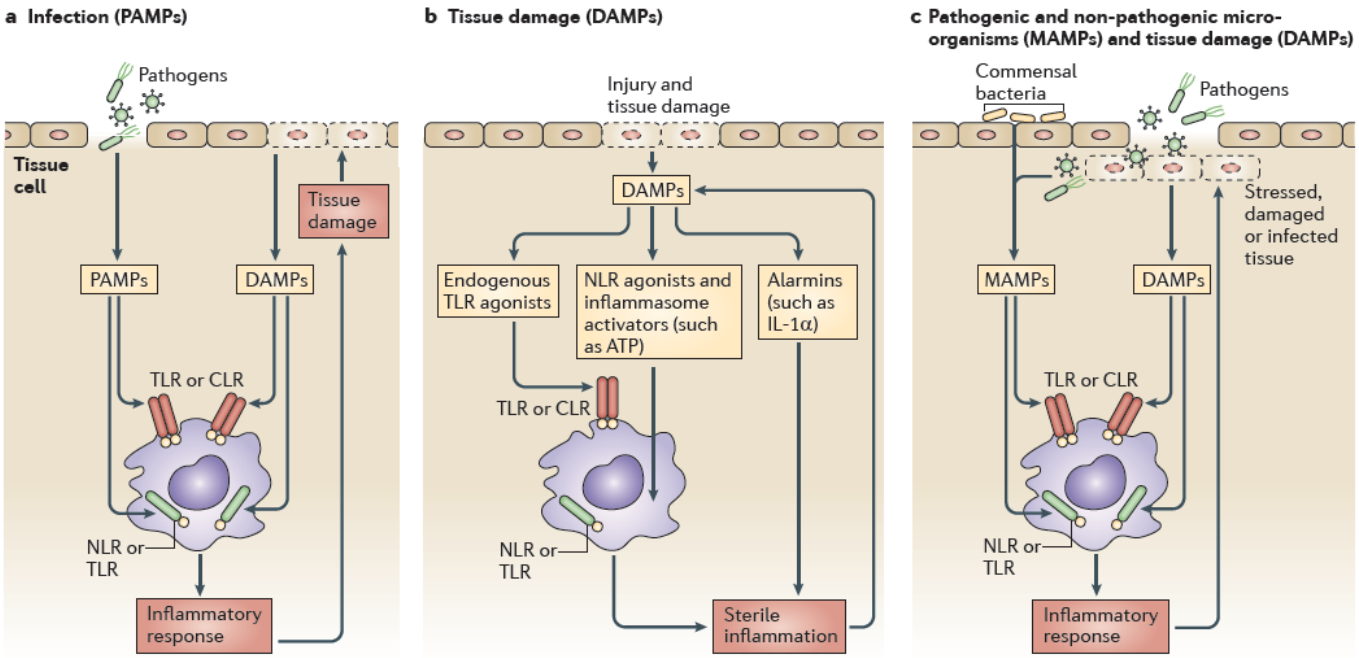

Figure4. - Autoimmune responses activation mediated by PAMPs, DAMPs and MAMPs*. For an inflammatory response optimal activation in innate immune cells, such as dendritic cells (DCs) and macrophages, two signals appear to be required, derived from exogenous or endogenous molecules. In this figure, three models for the autoimmune responses activation are represented.* ***A*** *Pathogen-associated molecular patterns (PAMPs), (Toll-like receptor (TLR), NOD-like receptor (NLR) and C-type lectin receptor (CLR) ligands), trigger pro-inflammatory cytokines production in response to infection. This cytokine production initiates an inflammatory response which produces tissue damage and leads to the damage-associated molecular patterns (DAMPs) production, which may contribute with PAMPs inducing inflammatory responses by innate immune cells, and therefore activating autoimmune T cell responses.* ***B*** *DAMPs released from damaged cells include TLR agonists and inflammasome activators, that induce the production of pro-inflammatory cytokines from tissues. Interleukin-1α (IL-1α) released from necrotic cells is a key alarmin that drives sterile inflammation.* ***C*** *Microorganism-associated molecular patterns (MAMPs) include TLR, NLR and CLR agonists and inflammasome activators. DAMPs released from host cells combined with MAMPs mediate pro-inflammatory cytokines production in response to pathogens, which could promote autoreactive T cells activation. (Kingston H. G. Mill, 2011).*

Toll-like receptors activation on immune and vascular cells could potentiate atherogenesis, because signalling cascade of these receptors can lead to pro-inflammatory cytokine release, lipid uptake, and foam cell formation and activation of the adaptive immune system, which are now considered to be major factors in the development of autoimmunity. (*Jennifer E. Cole et al, 2010).*

In this reasoning line, TLR mediated signals have been also related to the activation cascade of aPL induced thrombus formation. There is evidence for instance that TLR4 and the signalling cascade via MyD88 contribute to the APS phenotype. Furthermore, the participation of other TLRs like TLR7 on plasmacytoid dendritic cells and TLR8 on monocytes should not be excluded. PMN might act as stimulators of APS phenotype since they constitutively express various TLRs (*Gerd Gladigau et al, 2012*).

Multiple cells of the innate immune system express TLRs, including macrophages, dendritic cells (DCs), neutrophils, mucosal epithelial cells, and endothelial cells. These receptors not only alert the immune system to infection but it also stimulates the adaptive immune responses and has something to do with its nature improving its effectiveness. The innate immune response provides a link between innate and adaptive immunity (*J Rauch et al. 2010*)

Inflammatory T cells are induced by molecules derived from pathogens or commensal microorganisms, as well as by endogenous stress-induced self molecules. These respective microorganism-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs) induce inflammatory T cells either indirectly, through the induction of pro-inflammatory cytokine production by innate immune cells, or directly, by binding to PRRs on T cells (*Kingston H. and G. Mills 2011).*

Preliminary studies suggest some genes implicated on innate immune response, like TLR8 and CD14, to play a relevant role on APS pathogenicity. APS associated genetic profile suggest innate immune response like a clear influence on APS, which in fact is associated with an elevated pro-inflammatory cytokine production like IL1 or TNFα, that are induced, among other mechanisms, through TLR activation.

Nowadays, TLR8 expression and function in patients suffering of APS has not been studied, and could open new lines not only for the disorder etiology identification, but also to deeply understand the relationship between TLRs and APS.

AIMS

A huge ignorance exists about the pathogenicity implicated on APS development, but as an autoimmune disorder, presumably immune system control mechanisms may be altered in the context of a genetic predisposition also currently ignored. So the most important propose of this study is to elucidate as much as possible about the molecular mechanisms of this disorder, the APS, in order to improve the patients prognosis in a near future. The following are our first steps on this intention.

- The main aim of this study is to evaluate the function of the TLRs and the expression levels of the genes involved in the TLR pathway on blood mononuclear cell populations from healthy controls and patients suffering of different subtypes of antiphospholipid syndrome, and then to be able to relate those results with APS different clinical manifestations: Primary APS with vascular thrombosis, Primary APS with obstetric complications, Secondary APS, and Seropositive patients with no clinical manifestation.

  In case to confirm that this relation exists, we could really be sure that the innate immune responses have something to do with APS manifestation, and its molecular mechanisms would be closer to be known and better explained, which is really important for treatment development.

- A beta trial in collaboration with the manufacturer, Inova, is to be done with a novel immunoassay system based on chemiluminescence that measures the amount of certain autoantibodies or molecules in the patients' sera.

  Specifically for this study, the antibodies of interest were IgA, IgG and IgM anticardiolipin (aCL), IgA, IgG and IgM anti-beta2-glycoprotein I (ß2GPI) and IgG anti-ß2GPI domain I.

  Domain I of the ß2GPI antibodies are to be proved to be a good goal for APS diagnosis, and to be a thrombosis effector in patients suffering of APS.

MATERIALS AND METHODS

PATIENTS.

This study is based on a cohort of one hundred patients suffering of APS. Thirty seven primary APS and five secondary APS patients fulfilling the revised Sapporo criteria [13], six seropositive patients with no clinical data, eleven SLE patients fulfilling the revised American College of Rheumatology classification criteria [14], and forty one healthy patients similar in age and gender were included in this study (Table1). Blood samples have been collected from 2009. No patients were under immunosuppressant therapy.

Symptom / Pathology	Patients Number	Gender (Women %)	Arterial Thrombosis (%)	Venous Thrombosis (%)	Obstetric Pathology (%)
Health Control	41	68,3	---	---	---
PAPS	37	83,8	29,73	24,32	51,35
APS+SLE	5	100	40	40	20
SLE	11	100	0	0	0
SEROLOGY +	6	83,3	0	0	0

Table2. Patients vs Pathology or Symptom. One hundred patients and healthy controls were included in this study. The woman percentage is also indicated for the high women prevalence of the disease.

CLINICAL DATA.

Clinical data associated to this study cohort were already collected by the research group.

TLR EXPRESSION AND FUNCTION.

FLOW CYTOMETRY

Phenotypic analysis and functional assays.. TLR expression was assessed by flow cytometry on subpopulations of PBMCs. Briefly, cells were stained with fluorochrome-conjugated anti-CD19, anti-CD3 and anti-CD14 to identify B cells, T cells and monocytes respectively. To determine cell surface or intracellular expression of TLR, cells were surface or intracellularly stained with fluorochrome-conjugated antihuman TLR (Acris) or isotype control.

TLR function assessment in circulating monocytes: Cells from whole blood were polyclonally stimulated for 18 hours with different human TLR1 to TLR9 agonists (Table3) in the presence or absence of Brefeldine A in polypropylene tubes. Unstimulated cells were considered as controls. After culture, cells were stained with FITC-conjugated anti human-CD14 to identify the monocytes population. Later, cells were lysed, permeabilized and intracellularly stained with monoclonal antibodies (BD Biosicences) against three different cytokines (IL1b, TNFa, IL6). Data were acquired and analyzed in a FACScanto II Flow Cytometer (BD Biosciences). Expression and function of TLRs were studied from only 10 patients with PAPS during this work.

TOLL LIKE RECEPTOR	TLR AGONIST
TLR1	PamCSK4
TLR2	HKLM
TLR3	Poli (I:C)
TLR4	E.coli K12 LPS
TLR5	Flagellin
TLR6	FLS1
TLR7	Imiquimod
TLR8	ssRNA
TLR9	TypeB CpG Oligonucleotide

Table3. TLR agonists. The agonists used for each TLR in this study are summarized on this table.

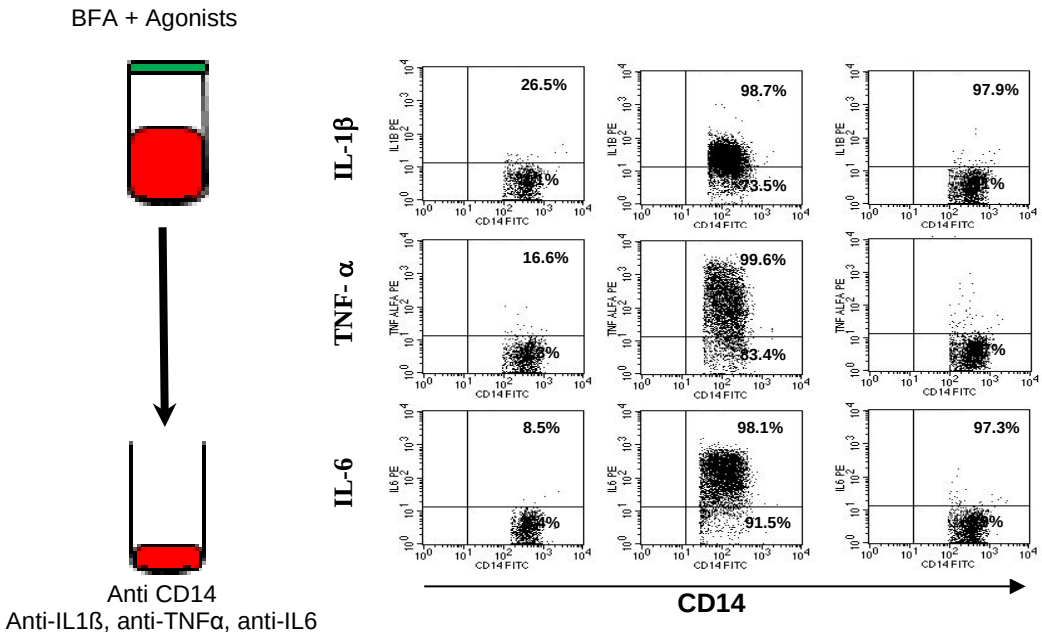

Figure5. TLR agonists' experiments and example of cytokine production vs monocyte plots.

RNA ISOLATION AND cDNA SYNTHESIS.

Total RNA was extracted using TRIzol (Invitrogen) from PBMCs after Ficoll separation from human peripheral whole blood. The RNA concentration was quantified with a Nano-Drop spectrophotometer and RNA quality was checked by electrophoresis on 1% agarose gel (Figure6). Good ribosomal RNA band integrity is important for optimal PCR Array results. Therefore, for q-PCR arrays, the RNA was cleaned after ethanol precipitation and cDNA was generated from 100ng of the total RNA using the SABiosciences's RT2 First Strand Kit, according to the manufacturer's protocol (Qiagen).

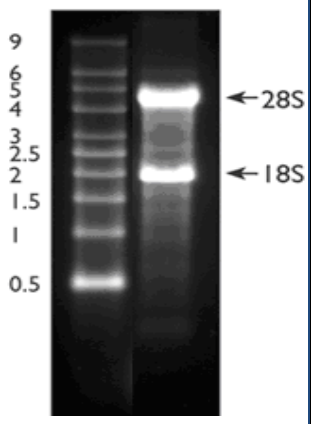

Figure6. Agarose gel *to check RNA quality*

qPCR ARRAY

Quantification of the expression of 84 genes involved in TLR pathway and 5 housekeeping genes was performed using Human Toll-Like Receptor (TLR) Signaling Pathway RT² Profiler PCR Array (SA Biosciences. PAHS-018Z) (Figure7). cDNA was synthetized from 100ng total RNA each plate. Results were analyzed using PCR Array Data Analysis Web Portal (Qiagen). The p values were calculated based on a Student's t-test of the replicate ($2^{-\Delta\Delta Ct}$) values for each gene in the control group and infected groups. A p-value less than 0.05 was considered statistically significant. SA Biosciences Online Software was used to obtain plots from the gene expression comparisons among groups. These studies were performed in 8 patients with APS and 2 healthy controls. Sample number 7 was included twice because the patient suffered both from obstetric complications and vascular thrombosis. Different patient groups depending on syndrome subtypes or clinical manifestations were compared following the next scheme.

1. - Healthy Controls (5, 6) vs APS (1, 2, 3, 4, 7, 8, 9, 10)
2. - PAPS obs. pathology (2, 7, 9) vs PAPS thrombosis (1, 7, 8, 10)
3. - Healthy Controls (5, 6) vs SLE + APS (3, 4)
4. - Healthy Controls (5, 6) vs PAPS obstetric pathology (2, 7, 9)
5. - Healthy Controls (5, 6) vs PAPS arterial thrombosis (7, 8, 10)
6. - LES + SAF (3, 4) vs PAPS (1, 2, 7, 8, 9, 10)

The entire list of genes present in the array is in table 4. Amplification, data acquisition, and the melting curve were carried out by means of the CFX-Manager software (BioRad). The PCR cycling program was set as follows: stage 1: 95ºC for 10 min (required to activate the DNA polymerase), stage 2 repeated for 40 cycles: 95ºC for 15 sec followed by 55ºC for 35 sec and then by 72ºC for 30 sec (SYBR Green fluorescence is detected and recorded from every well during the annealing step of each cycle). The threshold cycle (Ct) and melting curve of each gene were automatically established and recorded by the software.

Toll-Like Receptors: CD180 (LY64), SIGIRR, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10.

Pathogen-Specific Responses:

Bacterial: CCL2 (MCP-1), CD14, CD180 (LY64), FOS, HRAS, IL10, IL12A, IL1B, IL6, IL8, IRAK1, HMGB1, HSPA1A (HSP70 1A), JUN, LTA (TNFB), LY86 (MD-1), LY96, NFKBIA (IKBA/MAD3), PTGS2 (COX2), RELA, RIPK2, TLR2, TLR4, TLR6, TNFRSF1A, TICAM1 (TRIF).

Viral: EIF2AK2 (PRKR), IFNB1, IFNG, IL12A, IL6, IRF3, PRKRA, RELA, TBK1, TLR3, TLR7, TLR8, TNF, TICAM1 (TRIF).

Fungal/Parasitic: CLEC4E, HRAS, HSPA1A (HSP70 1A), IL8, TLR2, TIRAP.

TLR Signaling:

Negative Regulation: SARM1, SIGIRR, TOLLIP.

TICAM1 (TRIF)-Dependent (MYD88-Independent): IRF3, MAP3K7 (TAK1), MAP3K7IP1 (TAB1), NR2C2, PELI1, TBK1, TICAM2, TLR3, TLR4, TRAF6, TICAM1 (TRIF).

MYD88-Dependent: IRAK1, IRAK2, MAP3K7 (TAK1), MAP3K7IP1 (TAB1), MYD88, NR2C2, TIRAP, TLR1, TLR10, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TRAF6.

Downstream Pathways and Target Genes:

NFκB Pathway: BTK, CASP8, CHUK (IKKa), ECSIT (SITPEC), FADD, IKBKB, IL10, IL1B, IRAK1, IRAK2, IRF3, LY96, MAP3K1 (MEKK), MAP3K7, MAP4K4, NFKB1, NFKB2, NFKBIA (IKBA/MAD3), NFKBIL1, NFRKB, PPARA, REL, RELA, TNF, TNFRSF1A, UBE2N, UBE2V1.

JNK/p38 Pathway: ELK1, FOS, IL1B, JUN, MAP2K3 (MEK3), MAP2K4 (JNKK1), MAP3K1 (MEKK), MAP3K7, MAPK8 (JNK1), MAPK8IP3, TNF.

JAK/STAT Pathway: CCL2 (MCP-1), CSF2 (GM-CSF), IFNG, IL12A, IL2, IL6.

Interferon Regulatory Factor (IRF) Pathway: CXCL10 (INP10), IFNA1, IFNB1, IFNG, IRF1, IRF3, TBK1.

Cytokine-Mediated Signaling Pathway: CCL2 (MCP-1), CSF3 (GCSF), IL1A, IL1B, IL6, IRAK1, IRAK2, RELA, SIGIRR, TNF, TNFRSF1A.

Regulation of Adaptive Immunity: CD80, CD86, HSPD1, IFNG, IL10, IL12A, IL1B, IL2, MAP3K7, TRAF6.

Adaptors & TLR Interacting Proteins: BTK, CD14, HMGB1, HRAS, HSPA1A (HSP70 1A), HSPD1, LY86 (MD-1), LY96 (MD-2), MAPK8IP3, MYD88, PELI1, RIPK2, SARM1, TICAM1 (TRIF), TICAM2 (TRAM), TIRAP, TOLLIP.

Effectors: CASP8 (FLICE), EIF2AK2 (PRKR), FADD, IRAK1, IRAK2, MAP3K7 (TAK1), MAP3K7IP1 (TAB1), NR2C2, PPARA, PRKRA, ECSIT (SITPEC), TRAF6, UBE2N, UBE2V1.

Table4. Functional Gene Grouping. 84 genes involved in TLR pathway belong to the Human Toll-Like Receptor (TLR) Signaling Pathway RT² Profiler PCR Array used for this study.

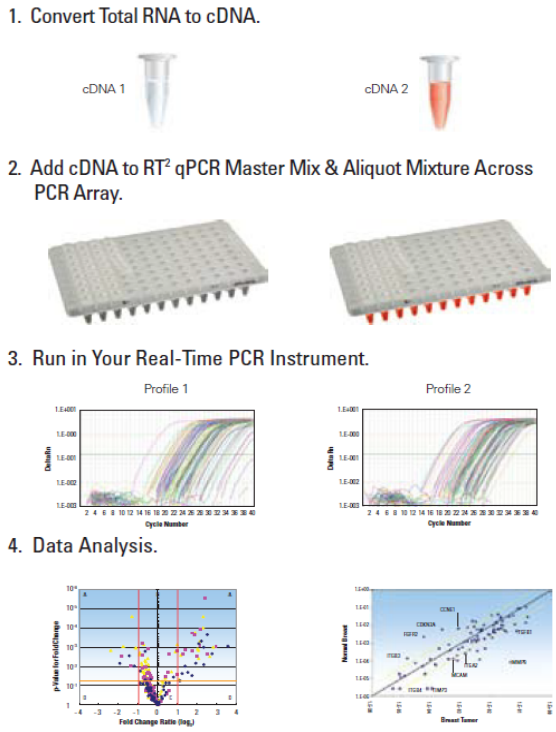

Figure7. PCR Array process. *Steps included on PCR Array process from RNA retro-transcription until data analysis.*

aPL MEASUREMENT.

BIOFLASH v1.0 (Inova). This is a novel immunoassay system, an analyzer based on chemiluminescence that measures the amount of certain autoantibodies or molecules in the patients sera. Specifically for this study, the antibodies of interest were IgA, IgG and IgM anticardiolipin (aCL), IgA, IgG and IgM anti-beta2-glycoprotein I (ß2GPI) and IgG anti-ß2GPI domain I. The measurement of these antibodies is part of a beta trial in collaboration with the manufacturer, Inova.

STATISTICAL ANALYSES

SPSS was used for the statistical analyses of TLRs and BioFlash data, carrying out comparisons among groups at a significance level of $P<0.05$.

PCR Array Statistical analysis

Results were analyzed using the PCR Array Data Analysis Web Portal (SABiosciences). The delta Ct (DCt) method was used for PCR array data analysis. The normalized (DCt) for each gene of interest (GOI) was calculated by subtracting the average Ct of the five housekeeping genes from the Ct of each GOI. Next, the double delta Ct (DDCt) for each GOI was calculated by deducting the average DCt of GOI in the sham group from the DCt of each GOI. The fold-change of each GOI compared with the sham group was calculated as 2_DDCt. Ct data were uploaded into the data analysis template on the manufacturer's website (http://www.sabiosciences.com/pcr/arrayanalysis.php) and the P values were calculated based on a Student's t-test of the replicate (2_DDCt) values for each gene in the control and infected groups. Fold-regulation represents fold-change results in a biologically meaningful way. A fold-change value greater than 2 indicates positive- or an upregulation, and the fold-regulation is equal to the fold-change. Fold-change values less than 2 indicate negative or down-regulation, and the fold-regulation is the negative inverse of the fold-change.

RESULTS

aPL measurement by chemiluminescence

Sera from a court of one hundred patients and controls were studied for this trial in order to determine the relationship between the titers of the most relevant antibodies regarding to APS and the different subtypes of this disorder. Specifically for this study, IgM, IgG and IgA of the anticardiolipin antibody (aCL), IgM, IgG and IgA of the anti-beta2-glycoprotein I (aß2GPI) and Domain I of the ß2GPI titers were analyzed obtaining, after statistical processing and normalization at a significance level of P<0.05, the following results.

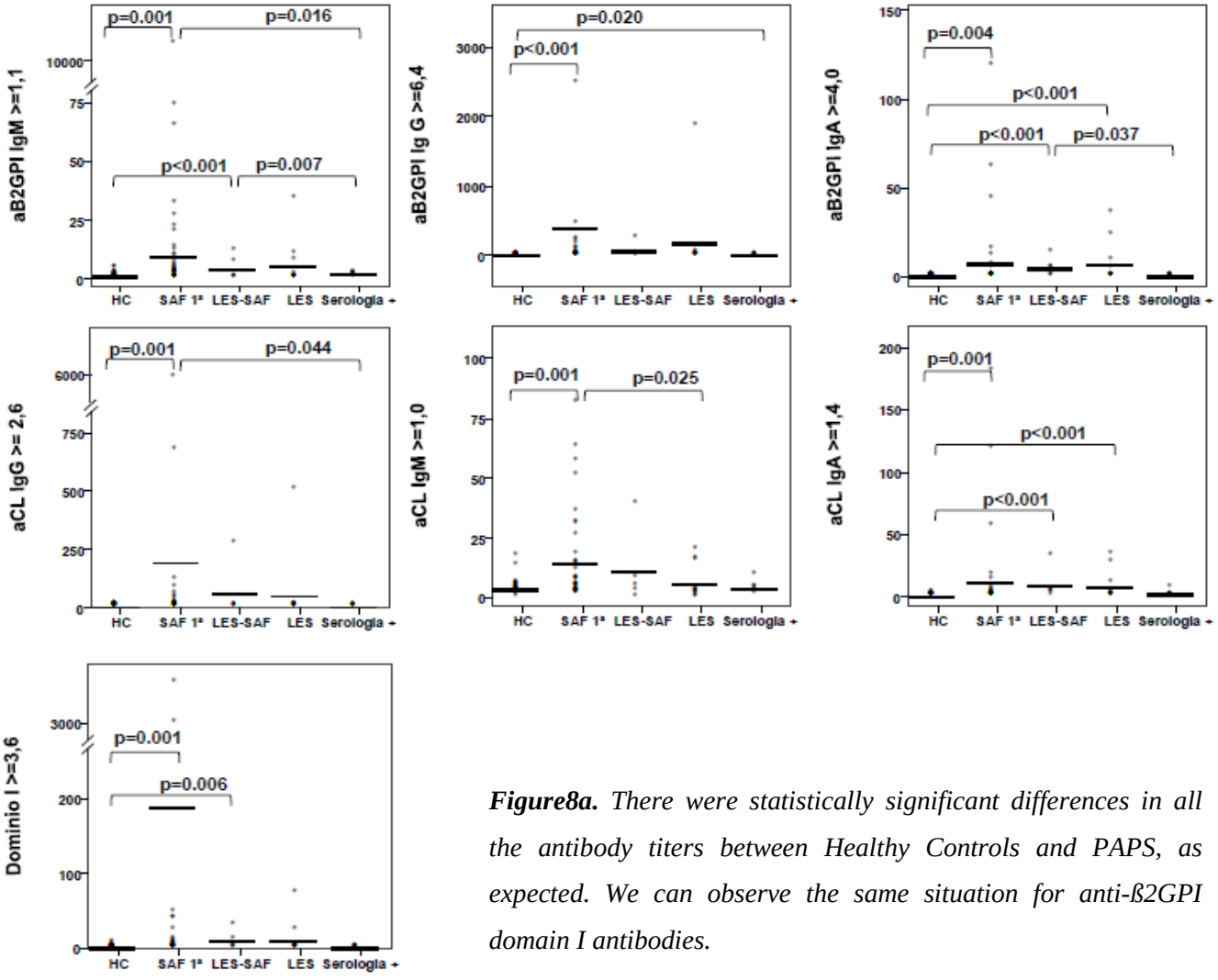

Figure8a. *There were statistically significant differences in all the antibody titers between Healthy Controls and PAPS, as expected. We can observe the same situation for anti-ß2GPI domain I antibodies.*

Statistically significant differences are found in all the antibody titers including IgA aPL, between healthy controls and the different subtypes of APS: Primary and Secondary APS, SLE and Positive serology, but mainly comparing with primary APS.

The same court of hundred patients and healthy controls sera were analyzed in order to determine the relationship between the titers of the same antibodies and the different clinical manifestations developed by patients suffering from this disorder. They were analyzed obtaining, after statistical processing and normalization at a significance level of $P<0.05$, these following results.

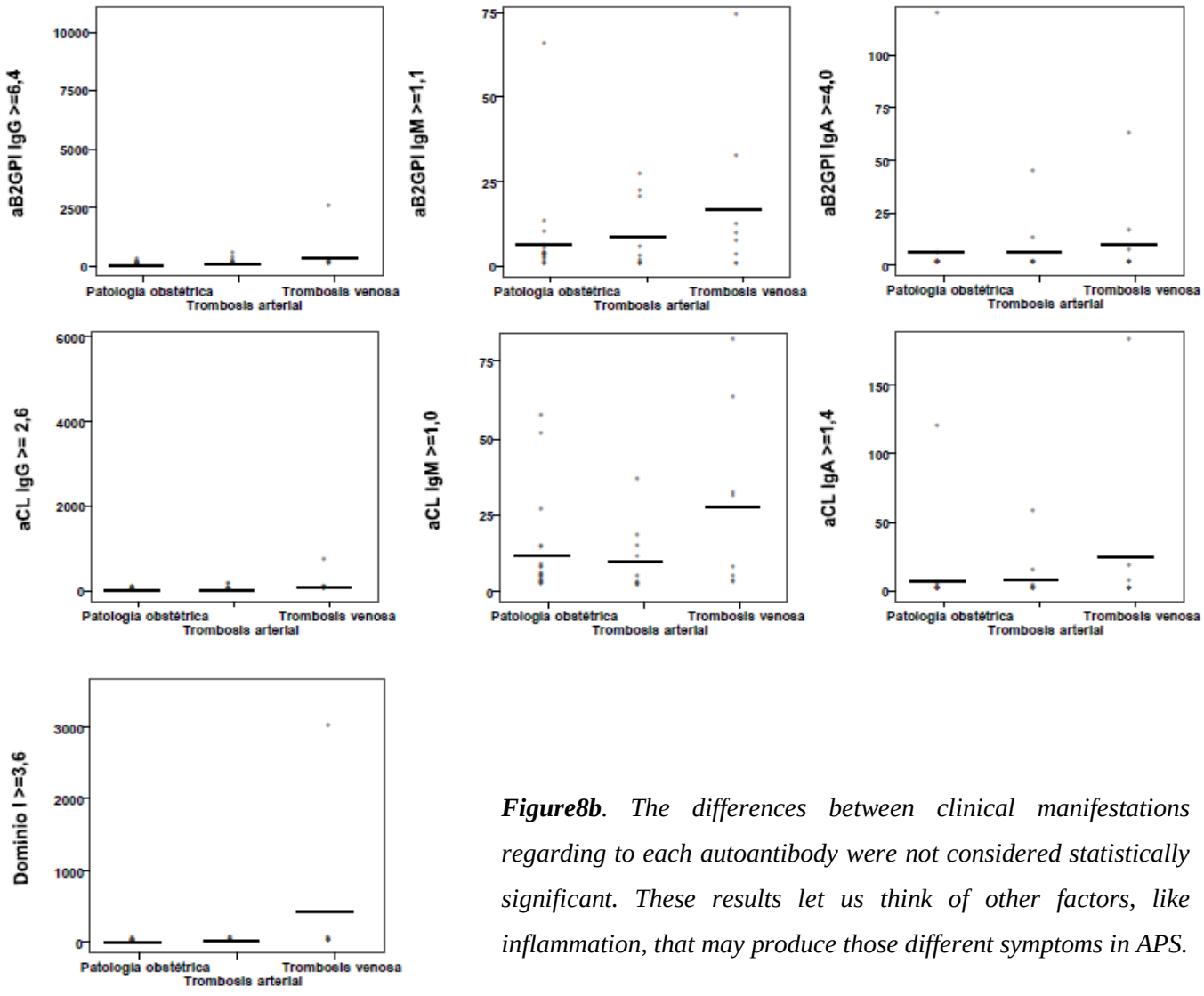

***Figure8b**. The differences between clinical manifestations regarding to each autoantibody were not considered statistically significant. These results let us think of other factors, like inflammation, that may produce those different symptoms in APS.*

In this case, the differences between the distinct clinical manifestations development regarding to each autoantibody, including IgA aPL, were not considered statistically significant. Symptoms of patients suffering of APS cannot be predicted or determined by any of these autoantibodies measurement so far.

TLR Function

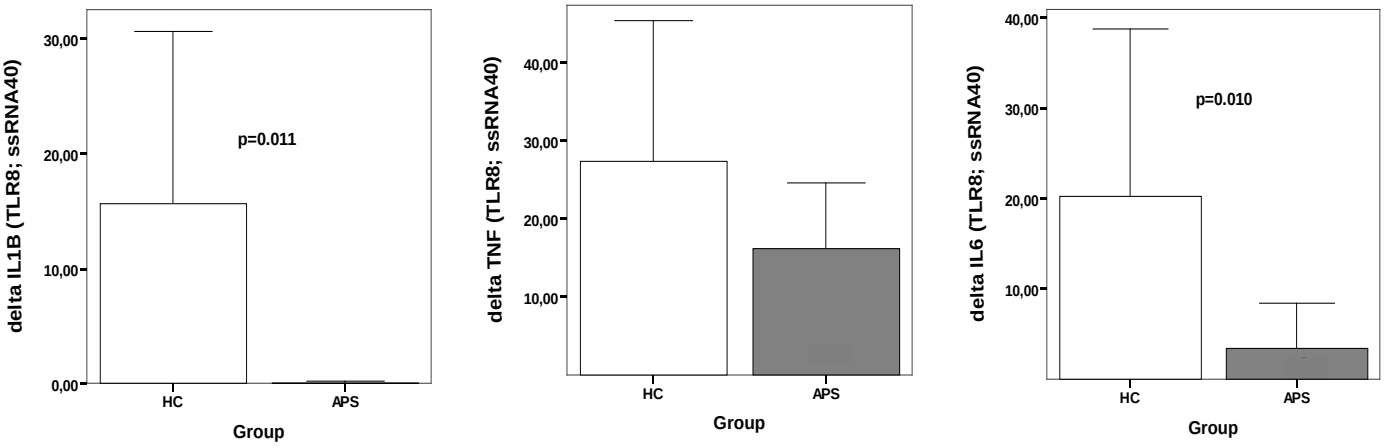

Figure9. *Agonist stimulated monocytes show a statistically significant TLR8 response reduction comparing patients suffering of APS with healthy controls.*

After stimulation for 18 hours with different human TLR1 to TLR9 agonists, cells from whole blood were surface stained with FITC-conjugated anti human-CD14 to identify the monocytes population, and intracellularly stained with monoclonal antibodies against three different cytokines (IL1b, TNFa, IL6). Once finished this process, data were acquired and analyzed in a FACScanto II Flow Cytometer to see inflammation relationship with APS.

Comparing agonist function of TLRs beyond healthy controls and patients suffering of APS, the clearest result is represented above and there it is found that TLR8 response looks to be significantly reduced in APS patients comparing with healthy controls (Fig. 2).

TLR Expression

Ten samples were analyzed to compare the expression of several genes implicated on the TLR mediated signal transduction between different APS subtypes and/or clinical manifestations. The boundary lines indicate a twofold difference. Red spots indicate up-regulated genes. Green spots indicate down-regulated genes. Black dots inside the boundary lines indicate no change in regulation (<2-fold in either direction).

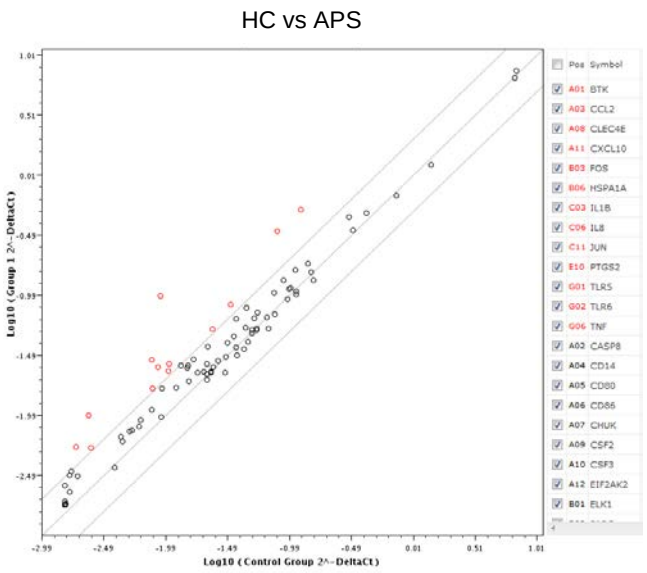

Figure10a. *Healthy Controls vs patients with APS.*

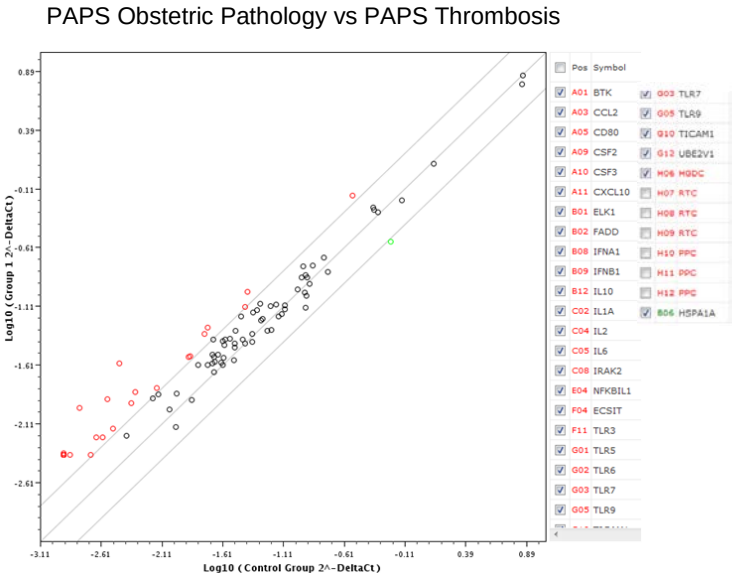

Figure10b. *Patients with PAPS developing obstetric pathology (taken as control) vs PAPS developing thrombosis.*

Fig 3a. Healthy Controls vs patients with APS. Red dots show the genes overexpressed in patients suffering of APS. The genes that have been found to be upregulated in patients suffering of APS were IL1B, BTK (NFκB Pathway), CCL2 (JAK/STAT Pathway), CLEC4E (Fungal/Parasitic Specific Responses), CXCL10 (Interferon Regulatory Factor (IRF) Pathway), FOS, PTGS2, HSPA1A (Bacterial Specific Responses), JUN (JNK/p38 Pathway), TLR5, TLR6 (Toll-Like Receptors) and TNF (Viral Specific Responses).

Fig 3b. Patients with PAPS developing obstetric pathology (taken as control) vs PAPS developing thrombosis. Red and green dots show the genes deregulated in PAPS with thrombosis. The genes that have been found to be up-regulated were UBE2V1, NFKBIL1, BTK (NFκB Pathway), CSF2, CCL2 (JAK/STAT Pathway), IL2, IL10,CD80 (Regulation of Adaptive Immunity), IL1A, IL6, IRAK2, CSF3 (Cytokine-Mediated Signaling Pathway), IFNA1, IFNB1, CXCL10 (Interferon Regulatory Factor (IRF) Pathway), ELK1 (JNK/p38 Pathway), ECSIT, FADD (Effectors), TLR3, TLR5, TLR6, TLR7, TLR9 (Toll-Like Receptors), TICAM1 (Bacterial Specific Responses), HGDC. The gen that has been found to be down-regulated was HSPA1A (Adaptors & TLR Interacting Proteins).

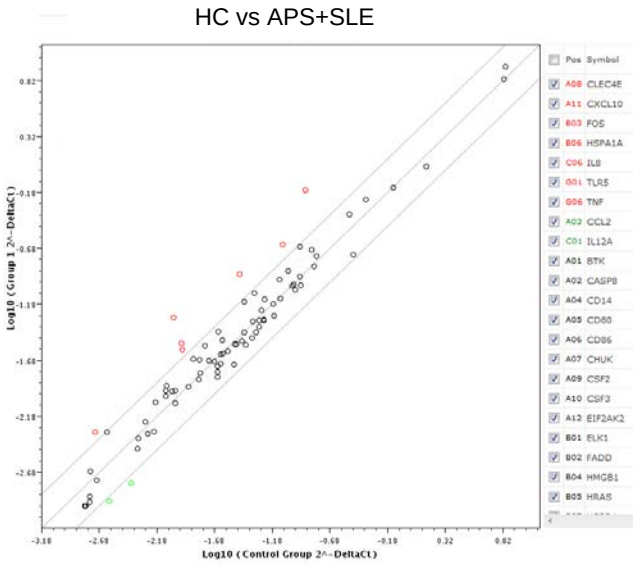

***Figure10c**. Healthy Controls vs patients with SLE+APS.*

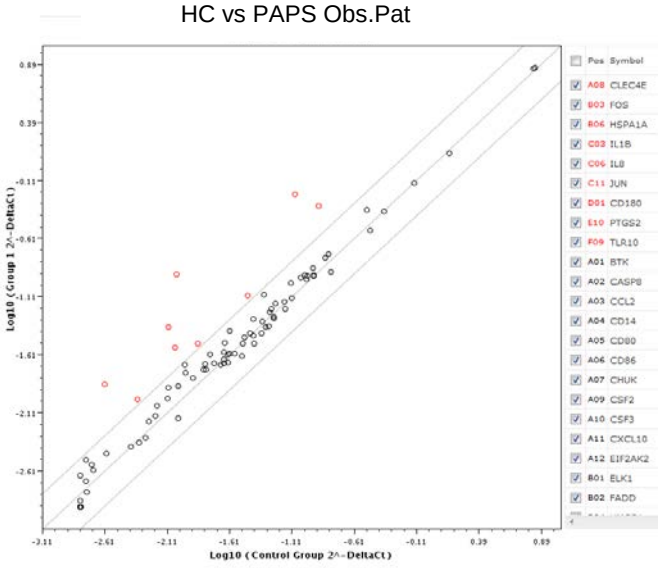

***Figure10d**. Healthy Controls vs patients with PAPS developing Obstetric Pathology.*

Fig 3c. Healthy Controls vs patients with SLE+APS. Red and green dots show the genes deregulated in patients suffering of secondary APS. The genes that have been found to be up-regulated in patients suffering of secondary APS were: IL8, CLEC4E (Fungal/Parasitic Specific Responses), CXCL10 (Interferon Regulatory Factor (IRF) Pathway), HSPA1A, FOS (Bacterial Specific Responses), TLR5 (Toll-Like Receptors) and TNF (Viral Specific Responses). The genes that have been found to be down-regulated were: CCL2 and IL12A (JAK/STAT Pathway).

Fig 3d. Healthy Controls vs patients with PAPS developing Obstetric Pathology. Red and green dots show the genes deregulated in patients suffering of PAPS with obstetric pathology. The genes that have been found to be up-regulated in patients with APS developing obstetric complications were: IL8, CLEC4E (Fungal/Parasitic Specific Responses), PTGS2, FOS, JUN, HSPA1A (Bacterial Specific Responses), CD180 and TLR10 (Toll-Like Receptors). No genes were found to be down-regulated in this case.

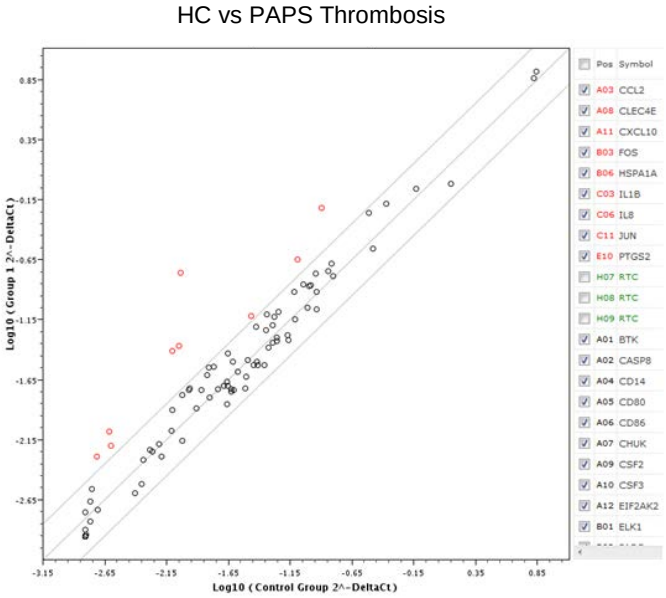

Figure 10e. Healthy Controls vs patients with PAPS developing Vascular Thrombosis.

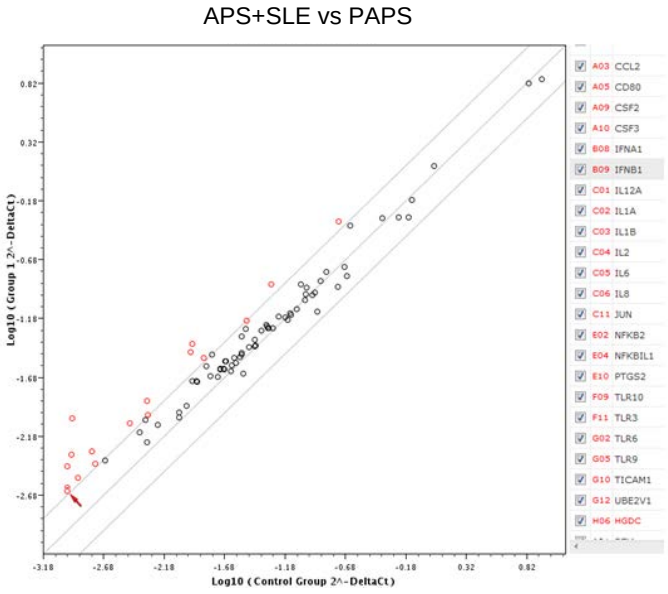

Figure 10f. Patients with SLE+APS vs patients suffering of PAPS.

Fig 3e. Healthy Controls vs patients with PAPS developing Vascular Thrombosis. Red and green dots show the genes deregulated in patients suffering of PAPS with vascular thrombosis. The genes that have been found to be up-regulated in patients with PAPS developing vascular thrombosis were: CCL2, IL8, CLEC4E (Fungal/Parasitic Specific Responses), CXCL10 (Interferon Regulatory Factor (IRF) Pathway), JUN, FOS, HSPA1A, PTGS2 (Bacterial Specific Responses) and IL1B (Cytokine-Mediated Signaling Pathway).

Fig 3f. Patients with SLE+APS vs patients suffering of PAPS. Red and green dots show the genes deregulated in patients suffering of secondary APS. The genes that have been found to be up-regulated in patients with secondary APS compared to patients suffering of PAPS were: IL8, JUN, PTGS2, TICAM1, CCL2 (Bacterial Specific Responses), IL12A, CD80 (**Regulation of Adaptive Immunity)**, IL2, IL6, CSF2 (JAK/STAT Pathway), CSF3 (Cytokine-Mediated Signaling Pathway), IFNB1, IFNA1 (Interferon Regulatory Factor (IRF) Pathway), IL1B, IL1A (Cytokine-Mediated Signaling Pathway), NFKB2, UBE2V1, NFKBIL1 (NFκB Pathway), TLR10, TLR3, TLR6 and TLR9 (**Toll-Like Receptors)**.

DISCUSSION

Although more patients sera should be added to these results for more consistent statistics, some interesting ideas arise from our experiments that could contribute in the future to a better understanding of this autoimmune disorder, the APS, maybe opening treatment possibilities that could offer patients a better life quality.

In the last years, aPLs antibodies have been studied in order to determine their implication over the different clinical manifestations of the APS and also for a better diagnosis and prognosis of the disorder. In some current articles, Domain I is said that seem be related to the thrombosis development in patients suffering of APS. We have assessed the aPL antibodies titers used currently for APS diagnosis, and also the anti Domain I ß2GPI antibodies titer for a beta trial and also to prove these relation Domain I – Thrombosis, but we have not found a clear and statistically significant relationship. So there is still something else that we do not know yet that should set the differences.

IgA antibodies measurement could be also good for APS diagnosis. Although nowadays it is IgM and IgG the ones determined to make a patient diagnosis, what we can conclude after aPL titers measurement and it statistical analyses is that IgA ß2GPI and IgA aCL antibodies could play a valuable role in the diagnosis of the APS disease and its clinical manifestations differentiation, at least at the same level as IgM and IgG.

TLR8 response among other receptors or molecules involved on inflammation is decreased in PAPS and seems to be required to produce different APS phenotypes. This is surprising together with previous findings proving a TLR8 up-regulation in the same group of patients comparing with healthy controls. This contradiction could be explained by a receptor saturation, so they may be over-expressed, while their function could be reduced because a saturation by high ligand concentration.

Several genes affecting TLR mediated signal transduction have been sought to be clearly deregulated comparing not only different subtypes of APS and healthy controls, but also distinct clinical manifestations. This is very important but it is only the beginning and deeper studies in this field are needed to elucidate which of these genes are the most transcendent ones, what their implication and their influence are over the APS development, and how they could be regulated so that the disorder could be more properly treated.

Some of the genes found to be up-regulated are common among the different subtypes of APS, which let us think of it like a tool for APS diagnosis and treatment, and some others are specific

of one of the subtypes or even directly related to a specific clinical manifestation, what could facilitate a deeper knowledge of the disease molecular mechanisms.

Also, analysis based not only on different subtypes and clinical manifestations, but also on aPL antibodies is still to be done, and more samples should be added for a more consistent statistical analysis.

The association of all these results, the clinical, serological, genetic and cellular findings will be part of a prospective ongoing project so that the can be better explained and related to each other.

Characterization of the molecular basis of the pathogenic mechanisms involved, including the second hits and the role of complement activation are needed to find a better treatment. So immunological research is needed!

CONCLUSIONS

We still do not know why, or how, a relatively homogeneous population of autoantibodies mediates different pathogenic mechanisms, presumably leading to the divergent clinical manifestations, but nowadays we can say that innate immune response and specifically TLRs activation leading to inflammation have something to do on it. After this four months study, we can through, even being still a lot to do in this field, some interesting data and ideas:

- There is a clear deregulation of several genes involved in TLRs pathways which seems to differentiate not only among different forms of APS, but also between distinct clinical manifestations.
  Some of the genes found to be up-regulated are common among the different subtypes of APS, which let us think of it like a tool for APS diagnosis and treatment, and some others are specific of one of the subtypes or even directly related to a specific clinical manifestation, what could facilitate a deeper knowledge of the disease molecular mechanisms.

- TLR8 response among other receptors or molecules involved on inflammation is decreased in PAPS and seems to be required to produce different APS phenotypes.

- Due to the high incidence of thrombosis and/or pregnancy morbidity in the general population, there is a strong need for highly specific assays to detect aPL in order to prevent over-diagnosis and unnecessary prolonged treatment. That is why a beta trial has been done in that direction.

- IgA ß2GPI and IgA aCL antibodies could play an important role in the diagnosis of the APS disease and its clinical manifestations differentiation. Curiously, antibodies against the Domain I of the beta2glycoprotein I do not seem to contribute much in this field, and therefore in this disorder diagnosis.

The association of the clinical, serological, genetic and cellular findings will be part of a prospective ongoing project.

ACKNOWLEDGEMENTS

First of all, I thank Marcos López Hoyos, the head of the laboratory where I have learnt and performed all the experiments necessary for this study, for giving me this amazing opportunity, for his patience and his useful advices not only for this laboratory, but also in my possible future jobs.

I would like to greatefully and sincerely thank Lorena Álvarez-Rodríguez, José Ramos Vivas, Victor Martínez Taboada, David San Segundo, Silvia Torices, Carolina Santa Cruz, David Ramos Melendro, and Ignacio Beares for their excellent help in learning the methods used during my work for these last four months as well as theorical aspects of APS, in particular, and autoimmune diseases, in general. And for their input, valuable discussions and accessibility.

I also thank the Universities of Cantabria and País Vasco for this invaluable period, and of course the Marqués de Valdecilla Hospital and the Instituto de Formación e Investigación Marqués de Valdecilla (IFIMAV).

I am also grateful to all the patients for their participation.

REFERENCES

1. Gerd Gladigau, Philipp Haselmayer, Inge Scharrer, Markus Munder, Nadine Prinz, Karl Lackner, Hansjörg Schild, Pamela Stein and Markus P. Radsak. A Role for Toll-Like Receptor Mediated Signals in Neutrophils in the Pathogenesis of the Anti-Phospholipid Syndrome. *PLoS ONE.* e42176. Vol 7, Issue7 (2012)

2. J Rauch, M Dieudé, R Subang and JS Levine. The dual role of innate immunity in the antiphospholipid syndrome. *Lupus* 19(4): 347–353 doi:10.1177/0961203310361492 (2010)

3. Bill Giannakopoulos, M.B., B.S., Ph.D., and Steven A. Krilis, M.B., B.S., Ph.D. The Pathogenesis of the Antiphospholipid Syndrome. *The new england journal of medicine.* 368;11 (2013)

4. *Pier Luigi Meroni, M. Orietta Borghi, Elena Raschi and Francesco Tedesco.* Pathogenesis of antiphospholipid syndrome: understanding the antibodies. *www.nature.com/nrrheum* Volume7 (2011)

5. Kingston H. G. Mills TLR-dependent T cell activation in autoimmunity. Nature *Reviews Immunology*. Volume 11 (2011)

6. Jennifer E. Cole, Ektoras Georgiou, and ClaudiaMonaco. The Expression and Functions of Toll-Like Receptors in Atherosclerosis. *Hindawi Publishing Corporation. Mediators of Inflammation.* Volume 2010, Article ID 393946, 18p (2010)

7. Brock E Harper, Rohan Wills, and Silvia S Pierangeli, Pathophysiological mechanisms in antiphospholipid syndrome. *Int J Clin Rheumtol.* 1; 6(2): 157–171 (2011)

8. Yehuda Shoenfeld, Pier Luigi Meroni and Ricard Cervera. Antiphospholipid syndrome dilemmas still to be solved: 2008 status. *Ann Rheum Dis* Vol 67 No 4 (2008)

9. Taro Kawai and Shizuo Akira. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature immunology* volume 11 number 5 (2010)

10. Silvia S Pierangeli, Mariano E Vega-Ostertag, Elena Raschi, Xiaowei Liu, Zurina Romay-Penabad, Valeria De Micheli, Monica Galli, Marco Moia, Angela Tincani, Maria Orietta Borghi, Tracy Nguyen-Oghalai and Pier Luigi Meroni. Toll-like receptor and antiphospholipid mediated thrombosis: in vivo studies. *Ann Rheum Dis 66:1327–1333 (2010)*

11. M.T.MonteroVega and A.de AndrésMartín. The significance of toll-like receptors in human diseases. *Allergol Immunopathol(Madr) 37(5):252–263 (2009)*

12. I Bernales, A Fullaondo, MJ Marín-Vidalled, E Ucar, V Martínez-Taboada, M López-Hoyos and A. Zubiaga. Innate immune response gene expression profiles characterize primary antiphospholipid syndrome. *Genes and Immunity 9: 38-46 (2008)*

13. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost 4: 295–306 (2006)*

14. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus**.** *Arthritis Rheum* 40: 1725 (1997)

15. Pierangeli SS, et al. Toll-like receptor and antiphospholipid mediated thrombosis: in vivo studies*. Ann Rheum Dis;* 66: 1327-33 (2007)

16. Meroni PL, et al. Endotelial cell activation by antiphospholipid antibodies. *Clin immunol* 112: 169-74 (2004)

17. Salmon JE et al. The antiphospholipid syndrome as a disorder initiated by inflammation: implications for the therapy of pregnant patients. *Nature Clin Prat Rheumatol* 3: 140-7 (2007)

18. Giannakopoulos B et al. Current concepts on the pathogenesis of the antiphospholipid syndrome. *Blood*; 109: 422-430 (2007)

19. Shoenfeld Y et al. Infectious origin of the antiphospholipid syndrome. *Ann Rheum Dis*; 65: 2-6 (2006)

20. Sutmuller RP et al. Toll-like receptors on regulatory T cells: expanding immune regulation. *Trends Immunol*; 27: 387-93 (2006)

21. Takeda K et al. Toll-like receptors. *Annu Rev Immunol*; 21: 335-376 (2003)