INNATE IMMUNITY ON ANTIPHOSPHOLIPID SYNDROME: TLRs IMPLICATION ON INFLAMMATION

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BACKGROUND

Antiphospholipid Syndrome (APS) is an acquired autoimmune disorder clinically determined by a history of recurrent vascular thrombosis and/or specific obstetric complications (fetal loss).
APS can be divided into two types; primary APS (PAPS), where the disease occurs alone, or secondary APS, where it is found together with other autoimmune diseases, frequently systemic lupus erythematosus (SLE).
The APS is characterized by an adaptive immune response against self membrane phospholipids or associated plasma proteins resulting in the generation of anti-phospholipid specific antibodies (aPL), but it is unclear what factors lead to its generation. Currently these following circulating antibodies are measured for a APS diagnosis: an anticardiolipin antibody (aCL) and an anti-beta2-glycoprotein I (ß2GPI) antibody.
Interestingly, presence of antibodies with similar antigen specificity produce different clinical manifestations, but in the other hand, finding aPLs does not necessarily mean that the patient suffers of APS. 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.
Still, there is evidence that the presence of aPL is necessary for the manifestation of APS, but it is not sufficient and it is been clarified that additional factors are needed for the clinical manifestations of the syndrome.
A 'two hit hypothesis' has been suggested to explain those data, and according to it, the antibody (representing the first hit) induces a thrombophilic state, but clotting takes place only in the presence of a second hit, frequently related to mediators of innate inflammatory immune responses (e.g. a toll-like receptor ligand).
At present, toll-like receptors (TLRs) are the most characterized innate immune receptors and are of great interest due to the fact that these have a central role in triggering innate immunity and coordinating innate and adaptive immunity. 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. Deregulation of these TLR signaling pathways may have severe consequences, and causes many autoimmune diseases and chronic pathological inflammation.
In this study we try to dive through the relationship between the expression of TLRs and other genes of their signaling pathways with the different clinical manifestations of APS.

AIMS

To relate TLRs expression/function and IgG anti-ß2GPI domain I antibodies with the Antiphospholipid Syndrome (APS) through its different manifestations: Primary APS with vascular thrombosis, Primary APS with obstetric complications, and Secondary APS.

METHODS

PATIENTS.

This study is based on a cohort of 100 patients suffering of APS. Blood samples have been collected from 2009.

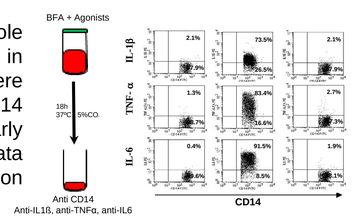
CLINICAL DATA.

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

TLR EXPRESSION AND FUNCTION.

Flow Cytometry. 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 by flow cytometry: Cells from whole blood were polyclonally stimulated for 18 hours with different human TLR1 to TLR9 agonists 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 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.



qPCR. 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). 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 5 patients with APS and 3 healthy controls.

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.

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

RESULTS

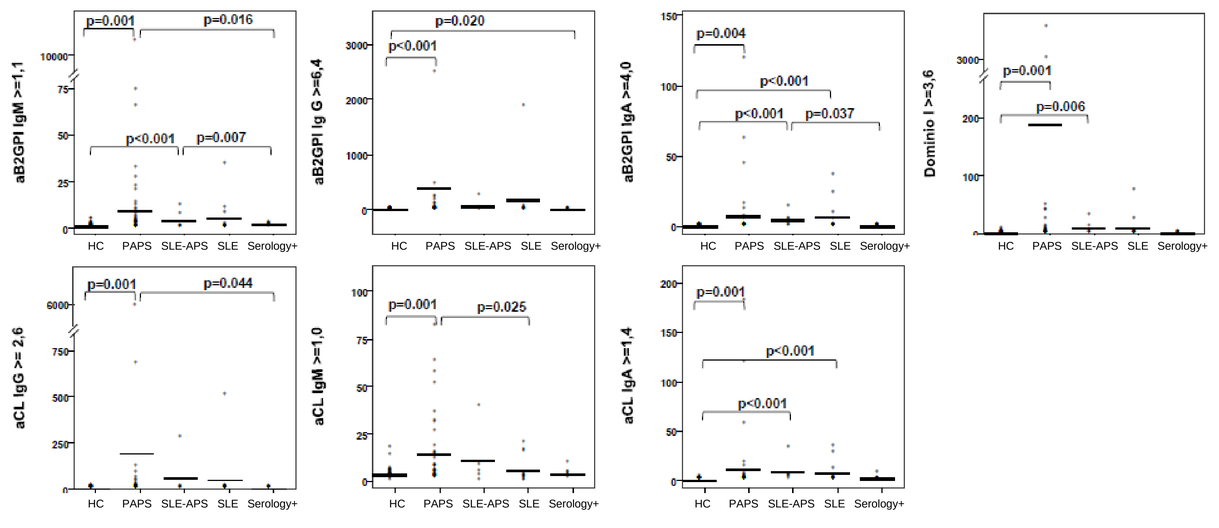


Fig 1a. There were statistically significant differences in all the antibody titers between Healthy Controls and PAPS, as expected. We can observe the same situation for anti-ß2GPI domain I antibodies.

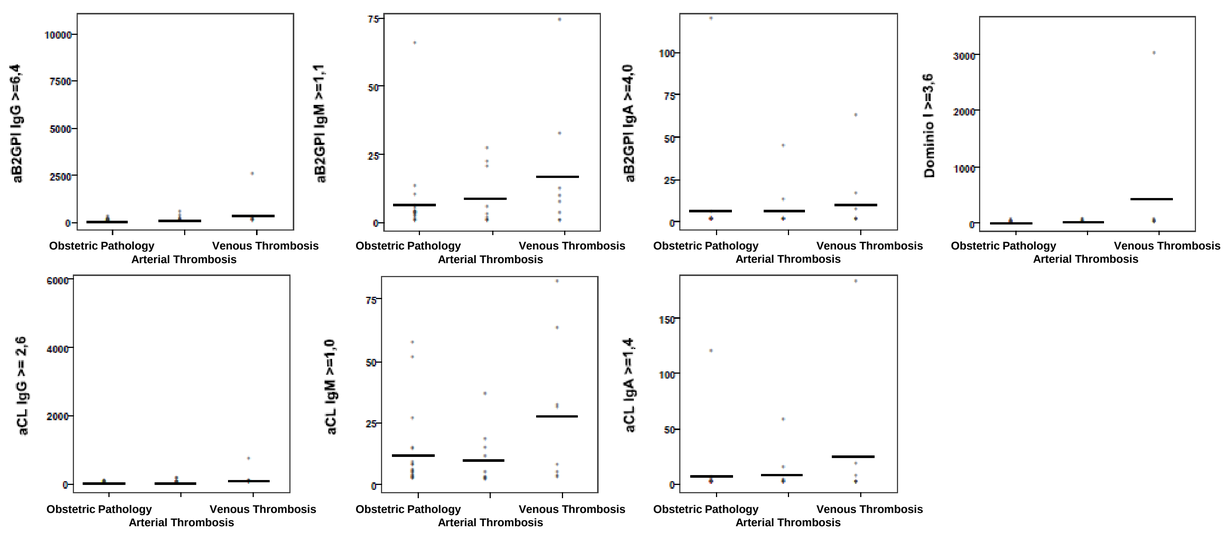


Fig 1b. The differences between clinical manifestations regarding to each autoantibody were not considered statistically significant. These results let us think of other factors, like inflammation, that may produce those different symptoms in APS.

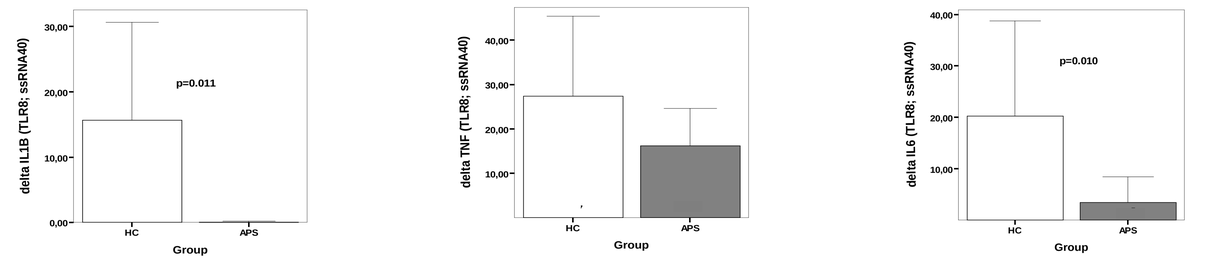


Fig 2. Agonist stimulated monocytes show a statistically significant TLR8 response reduction.

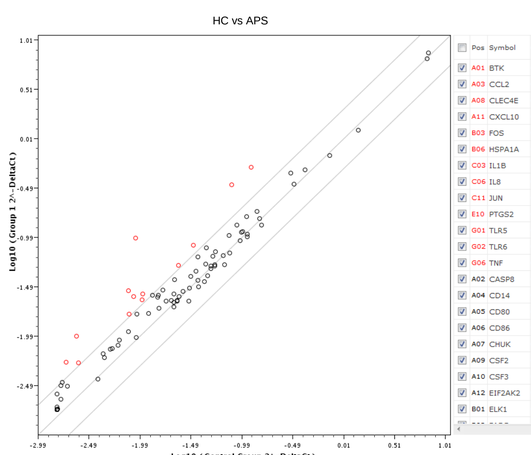


Fig 3a. Healthy Controls vs patients with PAPS. Red dots show the genes overexpressed in patients suffering of APS.

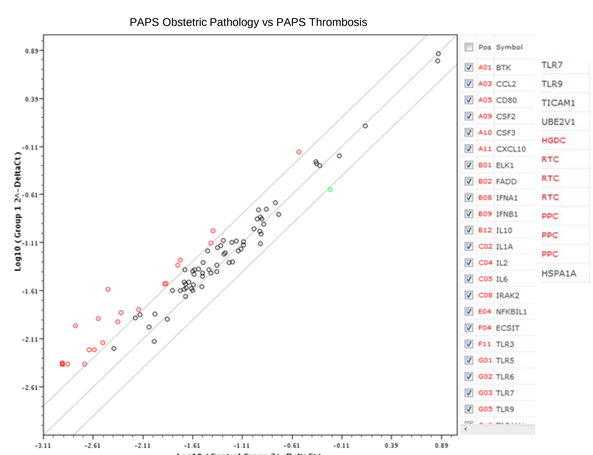


Fig 3b. Patients with PAPS developing obstetric pathology (taken as control) vs PAPS developing thrombosis. Red dots show the genes overexpressed in PAPS with thrombosis.

Examples of Scatter plots comparing the expression of genes involved in the TLR-mediated signal transduction and innate immunity. 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). p<0.05 are consistently up- or downregulated.

ACKNOWLEDGEMENTS

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CONCLUSIONS

- Deregulation of genes involved in TLRs pathways seems to differentiate among different forms of APS.
- TLR8 response among other receptors or molecules involved on inflammation is decreased in PAPS.
- IgA aPL, but not domain I, antibodies could be important in APS diagnosis and clinical differentiation.
- The association of the clinical, serological, genetic and cellular findings will be part of a prospective ongoing project.