**Primary antiphospholipid syndrome: a low-grade auto-inflammatory disease?**

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**Objective.** To test the inflammation and immune activation hypothesis in primary thrombotic APS (PAPS) and to identify clinical and laboratory factors related to inflammation and immune activation.

**Methods.** PAPS (n = 41) patients were compared with patients with inherited thrombophilia (IT, n = 44) and controls (CTR, n = 39). IgG aCL, IgG anti-β2-glycoprotein I (β2-GPI), high-sensitivity CRP (hs-CRP), serum amyloid A (SAA), CRP bound to oxidized low-density lipoprotein–β2-GPI complex (CRP–oxLDL–β2-GPI) (as inflammatory markers) neopterin (NPT), soluble CD14 (sCD14) (as immune activation markers) were measured by ELISA.

**Results.** After correction for confounders, PAPS showed higher plasma levels of hs-CRP (P = 0.0004), SAA (P < 0.01), CRP–oxLDL–β2-GPI (P = 0.0004), NPT (P < 0.001) and sCD14 (P = 0.007) than IT and CTR. Two regression models were applied to the PAPS group: in the first, IgG aCL and IgG β2-GPI were included amongst the independent variables and in the second they were excluded. In the first model, SAA (as the dependent variable) independently related to thrombosis number (P = 0.003): NPT (as the dependent variable) independently related to thrombosis type (arterial, P = 0.03) and number (P = 0.04); sCD14 (as the dependent variable) independently related to IgG β2-GPI (P = 0.0001), age (0.001) and arterial thrombosis (P = 0.01); CRP–oxLDL–β2-GPI (as the dependent variable) independently related to IgG β2-GPI (P = 0.0001). In the second model, sCD14 and NPT independently related to each other (P = 0.002) (this was noted also in the IT group, P = 0.0001) and CRP–oxLDL–β2-GPI independently predicted SAA (P = 0.002).

**Conclusion.** Low-grade inflammation and immune activation occur in thrombotic PAPS and relate to clinical features and aPL levels.

**Key words:** Anti-phospholipid syndrome, C-reactive protein, Serum amyloid A, Neopterin, Soluble CD14.

**Introduction**

The primary APS (PAPS) is characterized by the occurrence of single- or multiple-vessel occlusion in the presence and persistence of aPLs tested by immune or by clotting assays in isolation or in combination over a minimum period of 3 months [1].

The presence of antibodies and T-cell clones against β2-glycoprotein I (β2-GPI) in the peripheral blood of affected patients define PAPS as an autoimmune disorder [1–3]. Antibodies against β2-GPI promote endothelial cell, monocyte and coagulation activation: the vessel wall shifts to a pro-adhesive and pro-thrombotic phenotype [4], monocytes express and release TNF and tissue factor [5] and thrombosis may ensue.

Circulating levels of pro-inflammatory cytokines are elevated in APS [6–8] and some novel pro-inflammatory genes are up-regulated in endothelial cells stimulated with IgG β2-GPI [9], but PAPS is not an inflammatory disorder in the traditional sense as only modest increases of CRP have been detected [10–13]. Despite the presence of auto-reactive T-cell clones [2, 3] in vivo markers of immune activation have not been investigated in PAPS. Stimulated T helper cells release IFN-γ [14] that activates monocytes with the release of neopterin (NPT) and modulation of their surface component CD14 [15]. Therefore, NPT is a specific marker of immune cooperation, whereas soluble CD14 (sCD14) may also reflect monocyte activation in the absence of IFN-γ as it can behave as an acute-phase reactant [16].

Moreover, the observation that β2-GPI is a ligand for oxidized low-density lipoprotein (oxLDL) [17] and that the resulting oxLDL–β2-GPI complex binds CRP [18] prompted us to evaluate the clinical relevance of CRP, serum amyloid A (SAA) (inflammatory markers), NPT and sCD14 (immune activation markers) and their relationship with oxLDL–β2-GPI–CRP in thrombotic patients with PAPS.

**Materials and methods**

**Patients and blood sampling**

The study was conceived as a cross-sectional case double-control to serve as a baseline for an interventional trial. Consecutive thrombotic patients fulfilling recent criteria for PAPS [1] and patients with IT attending the Coagulation Unit of the Cardarelli Hospital (Naples, Italy) were invited to participate in the study that was carried out according to the revised Declaration of Helsinki, with approval of the Ethics Board of the Hospital and the written consent of all participants. Exclusion criteria were acute or chronic hepatic, renal, lung disease, diabetes, a recent history of acute infection (within 6 weeks), post-thrombotic syndrome with venous ulcerations, a positive urinary dipstick for nitrates on the day of sampling, treatment with steroids, statins and fibrates. Patients are seen on average every 3–4 weeks for their oral anti-coagulation monitoring and are instructed to self-report any illness they might incur during the intervening periods. Every year, their lipid profile, kidney and liver function tests are checked. Of the PAPS attendees (n = 50), two were excluded because they had gradually developed AS and SLE, one had developed kidney cancer, two were pregnant, one had suffered a recent recurrent event, one had post-thrombotic syndrome and two were evasive regarding their smoking and contraceptive status. Of the IT (n = 46) attendees, two were excluded for venous ulcerations in lower limbs. The study therefore included 41 thrombotic PAPS patients, 44 IT patients and 39 control subjects. The laboratory criteria for diagnosis of APS define Category IIa as lupus anti-coagulant (LA) alone, Ib as aCL alone, Ic as anti-β2-GPI alone and Category I as any combination of the previous [1]; of our PAPS patients, eight were in laboratory Category IIa and the remaining in Category I. From the therapeutic viewpoint, this was carried out according to the revised Declaration of Helsinki, with approval of the Ethics Board of the Hospital and the written consent of all participants. Exclusion criteria were acute or chronic hepatic, renal, lung disease, diabetes, a recent history of acute infection (within 6 weeks), post-thrombotic syndrome with venous ulcerations, a positive urinary dipstick for nitrates on the day of sampling, treatment with steroids, statins and fibrates. Patients are seen on average every 3–4 weeks for their oral anti-coagulation monitoring and are instructed to self-report any illness they might incur during the intervening periods. Every year, their lipid profile, kidney and liver function tests are checked. Of the PAPS attendees (n = 50), two were excluded because they had gradually developed AS and SLE, one had developed kidney cancer, two were pregnant, one had suffered a recent recurrent event, one had post-thrombotic syndrome and two were evasive regarding their smoking and contraceptive status. Of the IT (n = 46) attendees, two were excluded for venous ulcerations in lower limbs. The study therefore included 41 thrombotic PAPS patients, 44 IT patients and 39 control subjects. The laboratory criteria for diagnosis of APS define Category IIa as lupus anti-coagulant (LA) alone, Ib as aCL alone, Ic as anti-β2-GPI alone and Category I as any combination of the previous [1]; of our PAPS patients, eight were in laboratory Category IIa and the remaining in Category I. From the therapeutic viewpoint,
all were taking warfarin, three were on carbamazepine for post-ischaemic epilepsy, two on β-blockers six on folic acid. Of the IT patients, all were on warfarin, two were on β-blockers five on folic acid and two on angiotensin-converting enzyme inhibitors. The control group included 19 blood donors, 9 healthy hospital personnel and 11 subjects on warfarin for mitral valve replacement (mitral valve prolapse, n=4; childhood rheumatic fever, n=4; ventricular septal defects with congenital mitral incompetence, n=3) that occurred on average 22±8 yrs earlier. This partially accounts for the lifelong warfarin intake of the other two groups. Demographic and clinical features of all participants are shown in Table 1. Blood samples were drawn between 8:00 and 10:00 a.m. by neat venepuncture into 5 ml citrate vacutainers, spun immediately at room temperature at 4000 r.p.m. 

**aPLs**

All PAPS patients had their LA screened by activated partial thromboplastin time (aPTT), dilute Russell’s viper venom time (DRVVT) and kaolin clotting time (KCT) according to established guidelines [1]. A clotting time ratio between sample and control plasma >1.2 for aPTT, >1.18 for DRVVT and >1.3 for KCT indicated an abnormal result. After demonstrating the presence of an inhibitor by mixing studies, the platelet neutralization procedure confirmed the presence of a lupus inhibitor in aPTT and DRVVT and high phospholipid concentration in KCT. During follow-up, persistence of LA in aPTT was confirmed by comparing sensitive and insensitive reagents to LA [19]. IgG aCL (Cambridge Life Sciences, UK) and IgG anti-β2GPI (Corgenix, Westminster, CO, USA) were measured according to the manufacturer instructions by ELISA. IgG aCL was measured yearly since inception of the PAPS cohort (1994) whereas IgG anti-β2GPI has been measured yearly since 2004.

**ELISA for oxLDL–β2GPI complexes**

Capture anti-β2GPI mAb, WB-CAL-1, was adsorbed on a microtitre plate (Immulon 2HB, Dynex Technologies, Inc., Chantilly, VA, USA) by incubating at 8 μg/ml (dissolved in Hepes buffer, 50 μl/well) at 4°C overnight. The plate was blocked with 1% skim milk for 1 h. Serum samples (100-fold diluted) were added to the wells (100 μl/well) and incubated for 2 h. The wells were subsequently incubated with biotinyl-anti-apoB-100 antibody (1D2) for 1 h and HRP-labelled avidin for 30 min. Colour was developed with o-phenylenediamine and H₂O₂. The reaction was terminated by 2N sulphuric acid, and the OD at 490 nm was measured. Between each step, extensive washing was performed with Hepes buffer containing 0.05% Tween-20. The mean OD of blank wells corrected the raw OD of samples in individual assays. When 1.0 U/ml was adjusted to 3 S.D. above the mean of serum blanks, the cut-off for the upper normal level of all variables in the study was set at the mean + 5 S.E.M of the control group. Throughout the manuscript, CRP stands for the high-sensitivity measurement.

**Statistical analyses**

Data are shown as mean ± s.d. Analysis of variance (ANOVA), analysis of covariance (ANCOVA) and Student’s t-test assessed differences between groups where appropriate, multiple regression models assessed associations between variables and chi-square analysis assessed differences between proportions. Sensitivity was calculated as true positives divided by the sum of true positives and false negatives multiplied by 100. Specificity was calculated as true negatives divided by the sum of true negatives and false positives multiplied by 100. All statistical analyses were done using SPSS (Chicago, Illinois, USA).

**Results**

**Comparison of inflammatory and immune markers across groups**

Average plasma levels of CRP, SAA, NPT, sCD14, CRP–oxLDL–β2GPI and oxLDL–β2GPI were higher in PAPS than other groups considered (Fig. 1). Amongst age, gender, menopause, oral contraception, smoking and obesity, ANCOVA buffer containing 0.05% Tween-20. Further steps were performed as described above for oxLDL–β2GPI complexes.

**Measurement of inflammatory and immune parameters**

The hs-CRP, NPT (Biosupply Ltd, Bradford, UK), SAA (Europa Bioproducts, Ely, UK) and sCD14 (R&D Systems, Abingdon, UK) were measured by ELISA according to the manufacturer’s instructions. The cut-off for the upper normal level of all variables in the study was set at the mean + 5 S.E.M of the control group. Throughout the manuscript, CRP stands for the high-sensitivity measurement.

**Table 1. Demographics of participants**

<table>
<thead>
<tr>
<th>Age at thrombosis</th>
<th>Thrombosis number</th>
<th>IgG aCL</th>
<th>IgG β2GPI</th>
<th>Smoking (cigarettes/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>2GPI</td>
<td>1.25</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td></td>
<td>1.0 U/ml</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td></td>
<td>1.0 U/ml</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td></td>
<td>1.0 U/ml</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>No.</th>
<th>No.</th>
<th>No.</th>
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<tr>
<td>41</td>
<td>44</td>
<td>28</td>
<td>11</td>
</tr>
<tr>
<td>18/23</td>
<td>19/25</td>
<td>10/18</td>
<td>4/7</td>
</tr>
<tr>
<td>35 ± 11</td>
<td>39 ± 14</td>
<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Age (mean±SD)</th>
<th>M/F</th>
<th>Age at thrombosis (mean±SD)</th>
<th>Thrombosis type</th>
<th>Arterial</th>
<th>Venous</th>
<th>Arterial + venous</th>
<th>Thrombosis number</th>
<th>IgG aCL (mean±SD)</th>
<th>IgG β2GPI (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 ± 12</td>
<td>51 ± 14</td>
<td>37 ± 6.9</td>
<td>90 ± 10</td>
<td>13</td>
<td>9</td>
<td>4</td>
<td>1</td>
<td>131 ± 143</td>
<td>119 ± 67</td>
</tr>
</tbody>
</table>

PAPS: primary antiphospholipid syndrome; IT: inherited thrombophilia; CTR: controls; aCL: anticardiolipin; β2GPI: beta-2-glycoprotein-I; BMI: body mass index; H-CTR: healthy controls; W-CTR: warfarin controls.
identified only age as a significant confounder for NPT ($P < 0.001$), sCD14 ($P = 0.001$), oxLDL–$\beta_2$-GPI ($P = 0.02$) and SAA ($P = 0.04$). Age-adjusted significances between groups were $P < 0.01$ for SAA, $P < 0.0001$ for NPT, $P = 0.007$ for sCD14, $P = 0.0001$ for oxLDL–$\beta_2$-GPI. Within PAPS, IT and CTR, the proportion of participants positive for CRP was 31, 13 and 10%, respectively ($P = 0.02$), for SAA 31, 6 and 2%, respectively ($P < 0.0001$), for NPT 68, 27 and 15%, respectively ($P < 0.0001$), for sCD14 48, 34 and 23%, respectively (0.054), for CRP–oxLDL–$\beta_2$-GPI 53, 31 and 18%, respectively ($P < 0.003$), oxLDL–$\beta_2$-GPI 41, 9 and 10%, respectively ($P = 0.0002$). Sensitivity and specificity of CRP–oxLDL–$\beta_2$-GPI was 51 and 100%, respectively with regards to thrombotic PAPS, but 53 and 64%, respectively with regards to arterial thrombosis.

**Relationship amongst variables in the PAPS group**

Two regression models were employed: the first assessed the predictive effect of IgG aCL, IgG $\beta_2$-GPI, gender, event type, event number, age at event and smoking status on plasma levels of CRP, SAA, NPT, sCD14, CRP–oxLDL–$\beta_2$-GPI and oxLDL–$\beta_2$-GPI; the second assessed the relationship between immune activation markers and inflammatory markers after correction for gender, event type, event number, age at event, smoking status in the absence of IgG aCL and IgG $\beta_2$-GPI.

Remarkably, in the first model, IgG $\beta_2$-GPI was an independent predictor of SAA, NPT, sCD14 and CRP–oxLDL–$\beta_2$-GPI. The number of thrombotic events predicted SAA and NPT, whereas arterial thrombosis predicted NPT and sCD14 (Table 2). Indeed, the average plasma concentration of NPT was greater in patients with arterial than venous thrombosis (16.7 ± 2.4 vs 11 ± 0.9 nmol/ml, $P = 0.02$) but that of sCD14 did not reach significance (data not shown). On the other hand, average plasma concentration of SAA was almost 50% higher in patients with arterial than venous thrombosis (85 ± 18 vs 45 ± 9 mg/ml, $P = 0.04$). In the second regression model, NPT and sCD14 correlated with each other as did CRP–oxLDL–$\beta_2$-GPI and SAA (Table 3).

**Relationship amongst variables in the IT group**

Multiple regression analysis strongly revealed interrelations between SAA and CRP as well as between NPT and sCD14 (Table 4).

**Relationship amongst variables in the control group**

Multiple regression analysis identified oxLDL–$\beta_2$-GPI an independent predictor of CRP, SAA and sCD14, whereas the inflammatory markers were strongly related (Table 5).
CRP and SAA are acute-phase plasma proteins principally expressed and induced in the liver under the regulation of the pro-inflammatory cytokines IL-1, IL-6 and TNF-α though non-acute-phase elevations may occur in several chronic inflammatory diseases such as RA and atherosclerosis [20]. The current survey reveals that a significant number of thrombotic PAPS patients display a low-grade inflammatory state that must be due to the disease itself, as IT patients did not show similar elevations of CRP and SAA. Thus, we support a relationship independent of thrombosis between LA and the inflammatory markers, CRP [12], factor VIII and fibrinogen [11], but failed to confirm our previous data on higher CRP in PAPS with arterial occlusions, probably due to the larger sample and different patients herein [10]. However, elevated levels of CRP independently predicted residual or recurrent symptoms in a cohort of aPL-positive patients with neurological manifestations, mostly cerebral infarctions and transient ischaemic attacks [13].

Contrary to the lack of clinical relevance of SAA in a very small PAPS group [21], SAA was elevated in our patients particularly in relation to multiple thrombotic events. Unfortunately, the cross-sectional nature of the study cannot define whether elevated SAA and CRP are cause or effect of previous occlusive events since both molecules are involved in the expression of tissue factor on monocytes and endothelial cells [22, 23]. Therefore, their value as thrombotic risk markers in PAPS may only be assessed with regards to re-thrombosis as most PAPS patients will be on oral anti-coagulation after their first occlusive event.

Compared with IT and controls, our PAPS patients displayed higher plasma concentrations of NPT and of sCD14 that correlated strongly in both patient groups. In PAPS, NPT was higher in patients with multiple thromboses and both markers related to arterial occlusions: monocytes are more likely to be involved in the pathogenesis of arterial than venous thrombosis [24]. The independent predictive power of IgG β2-GPI vs sCD14 and NPT reflects the in vitro work describing monocyte activation including tissue factor expression by IgG β2-GPI [5, 25] that adds to the T-helper activation pathway.

In a similar context, monocytes may differentiate into foam cells after the uptake of an immune complex consisting of β2-GPI, oxLDL and a monoclonal aP (25). Though oxLDL-β2-GPI complex is formed as an attempt by β2-GPI to prevent LDL oxidation and its uptake by monocyte scavenger receptors, it is recognized by specific antibodies and up-taken by monocytes via Fc receptors [26]. Monocytes play a pivotal role in APS: the direct effect of IgG β2-GPI promotes tissue factor expression [5, 25] and hence clotting in the bloodstream, whereas phagocytosis of oxLDL-β2-GPI bound to their specific antibody probably favours atherosclerosis [27].

OxLDL-β2-GPI complexes are found in primary and secondary APS, SLE [27] and chronic nephritis [28], disorders characterized by enhanced oxidative stress [29, 30]. In PAPS, we and others had noted a correlation between plasma IgG aCL and isoprostanes, sensitive markers of in vivo oxidative stress [31, 32], and in the light of the above this could explain the relationship between IgG aCL and oxLDL-β2-GPI in the present PAPS population.

Experimental work reveals that in analogy with certain natural antibodies and scavenger receptors, CRP binds phosphorylcholine (PC) moieties on apopotic cells and on oxLDL in what appears a very primitive form of innate immunity [17]. More recent in vitro data demonstrate that over a 24-h incubation period CRP can bind oxLDL-β2-GPI via PC in a ternary complex [8]. Given the very short half-life (10 min) oxLDL when injected into mice and the co-localization of CRP, oxLDL and β2-GPI with foamy macrophages in carotid artery plaques CRP-oxLDL-β2-GPI would fulfill the requirements for a specific marker of atherosclerosis [8], a hot topic in APS.

In this study CRP-oxLDL-β2-GPI was 100% specific for PAPS but did not differentiate arterial and venous thrombosis. Interestingly, higher CRP-oxLDL-β2-GPI was predicted by elevated IgG β2-GPI, indicating that an enhanced level of LDL oxidation requires buffering from both β2-GPI and CRP. LDL oxidation is prevented by paraoxonase (PON), the enzyme that accounts for most of the anti-oxidant activity of HDL: in PAPS, decreased PON activity associates with elevated IgG β2-GPI titres and a monoclonal aP inhibited PON activity (PONa) in vitro [33]. On the other hand, β2-GPI buffering of oxLDL would lead to exposure of cryptic auto-antigenic epitopes on β2-GPI with...
persisting antibody stimulation [34]. In the case of the oxLDL-β2GPI complex, the antibody response would mostly be IgG aCL; once the CRP-oxLDL-β2GPI is formed, the highly specific IgG β2GPI would be stimulated.

In the control group, no patient had elevated CRP-oxLDL-β2GPI, but some had elevated oxLDL-β2GPI that was an independent predictor of CRP, SAA and sCD14: a possibility is that CRP-oxLDL-β2GPI and oxLDL-β2GPI are formed at high and low LDL oxidation levels, as would occur in PAPS and normal subjects, respectively. In the latter case, only β2GPI is required to buffer oxLDL—though the resulting oxLDL–sclerotic lesions [35–37]. Indeed, CRP–oxLDL–complex still associates with CRP.

In conclusion, immune activation and low-grade inflammation occur in PAPS. The former was not unexpected but the latter raises further issues: are the inflammatory markers measured of liver or vascular origin? Endothelial cells, smooth muscle cells and monocytes can produce CRP as well as SAA in atherosclerotic lesions [35–37]. Indeed, CRP-oxLDL-β2GPI related to SAA in this survey. Outside the acute setting, mild plasma elevations of CRP, SAA and NPT represent an inflammatory signature of advanced atherosclerosis [38, 39] but do not add discriminatory diagnostic power over established cardiovascular risk factors [40]. The picture may be different in PAPS where atherosclerosis may not necessarily involve traditional cardiovascular risk factors [41]: as number and type of events were linked to plasma levels of CRP, SAA and NPT these may represent useful markers alongside CRP-oxLDL-β2GPI to predict vascular disease progression and to monitor monocyte and vascular activity in intervention trials [42–44].

Rheumatology key messages

- Low-grade inflammation and immune activation occur in primary thrombotic antiphospholipid syndrome.
- Immune activation in vivo reflects monocyte activation by IgG β2GPI in vitro.
- Such markers could aid monitoring drug efficacy in intervention trials.

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Inflammation in primary APS