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8-Isoprostane, prostaglandin E2, C-reactive protein and serum amyloid A as markers of inflammation and oxidative stress in antiphospholipid syndrome: a pilot study

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Abstarct

Objective

To test the inflammation and oxidative stress hypothesis in antiphospholipid syndrome (APS) patients and to identify possible associations with clinical and laboratory features of the disease.

Methods

Serum amyloid A (SAA), C-reactive protein (CRP), 8-isoprostane and prostaglandin E2 (PGE) were assayed in the sera of 45 APS patients and then compared to control groups made up of 15 antiphospholipid antibody (aPL) negative patients with systemic lupus erythematosus, 15 aPL negative subjects with pregnancy-related morbidity, 15 aPL negative patients with thrombosis, 15 subjects with persistently positive aPL with no signs or symptoms of APS, and 15 healthy volunteers from among the hospital staff.

Results

APS patients showed significantly higher CRP ($p = 0.01$), SAA ($p < 0.01$), 8-isoprostane ($p = 0.05$) and PGE2 ($p = 0.001$) plasma levels as compared to controls. Among APS subjects, significantly higher 8-isoprostane and PGE2 levels were observed in patients with triple positivity for aPL (lupus anticoagulant, anticardiolipin and anti-beta2-glycoprotein I antibodies) compared to APS patients with single or double aPL positivity.

Conclusion

Both inflammation and oxidative stress, as measured by SAA, CRP, 8-isoprostane and PGE2, occur in APS and seem to be related to triple positivity for aPL.

Keywords: Inflammation,Oxidative, stress,Antiphospholipid syndrome

Introduction

Numerous autoimmune-inflammatory rheumatic diseases have been associated with accelerated atherosclerosis or other types of vasculopathies, thus leading to increased cardio- and cerebro-vascular disease risk. Conventional risk factors, as well as systemic inflammation and oxidative stress have been implicated in the development of these pathologies [1]. Antiphospholipid syndrome (APS) is an immune-mediated disease characterized by vascular thrombotic disorders or pregnancy-related morbidity which occurs in patients with persistent autoantibodies directed against phospholipid-binding plasma proteins [antiphospholipid antibodies (aPL)]. The clinical significance of persistent aPL positivity in patients without any clinical events is not fully understood.

Conventional risk factors may be less important in APS than in rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE), since the involvement of dyslipidemia, diabetes mellitus, smoking, obesity, hypertension and sedentary lifestyle in the development of atherosclerosis in APS patients is similar to that of the general population [2–6].Nonetheless, the levels of circulating pro-inflammatory cytokines are elevated in APS [7, 8] and some novel pro-inflammatory genes are upregulated in endothelial cells stimulated with IgG anti-beta2-glycoprotein I (β2GPI) antibody [9]. A slight increase in CRP levels has also been detected [10–13]. At the moment, evidence proving that APS can be considered an inflammatory disease are still elusive.

Reports that β2GPI is a ligand for oxidized low-density lipoprotein (oxLDL) [14] and that the resulting oxLDL–β2GPI complex binds CRP [15] prompted us to evaluate the clinical relevance of oxidative stress. We focused on the family of prostaglandins (PG) and their isomers (isoprostanes) (which are the result of the oxidative modification of arachidonic acid through a free radical–catalysed mechanism) in patients with APS [16, 17].The aim of our preliminary study was to investigate the oxidative stress and inflammatory status in APS patients as compared to five control groups: group 1, aPL negative patients with SLE; group 2, aPL negative subjects with pregnancy-related morbidity; group 3, aPL negative patients with thrombosis; group 4, subjects with persistent positive aPL with no signs/symptoms of APS; group 5, healthy, hospital staff volunteers. All the enrolled subjects were tested for serum amyloid A (SAA), C-reactive protein (CRP), 8-isoprostane and prostaglandin E2 (PGE).

Methods

Patients

Consecutive patients fulfilling the “Sydney” criteria for APS [18] and being followed-up by the Research Centre of Immunopathology and Rare Diseases of the San Giovanni Bosco Hospital and Umberto I Hospital in Turin, Italy were enrolled. Exclusion criteria were: acute or chronic hepatic, renal or pulmonary disease, diabetes, recent history of acute infection (within the 6 weeks prior to the study), post-thrombotic syndrome with venous ulcerations, positive urinary dipstick for nitrates on the day of sampling, treatment with statins and fibrates.

In order to evaluate only aPL-related phenomena, all the SLE patients (both in the APS group and in control group 1, were selected from those with stable disease (no sign/symptoms of flare in the 3 previous months) and with a systemic lupus erythematosus disease activity index (SLEDAI) ranging from 1 to 3 (remission to mild disease).

We enrolled 45 APS patients and 75 control subjects. The control groups were made up as follows: group 1, 15 aPL negative patients with SLE (classified according to the ARA criteria); group 2, 15 aPL negative subjects with pregnancy-related morbidity (consistent with obstetric manifestations of APS); group 3, 15 aPL negative patients with thrombosis; group 4, 15 subjects

with persistent, positive aPL with no signs/symptoms of APS; group 5, 15 healthy, hospital staff volunteers.

Demographic characteristics, aPL profile and ongoing therapy are shown in Table 1.

Blood samples were drawn between 8:00 a.m. and 10:00 a.m. by venipuncture into 5 ml citrate vacutainers, immediately centrifuged at room temperature at 4,000rpm× 6 min. Supernatant plasma was centrifuged again at room temperature at 12,000rpm × 4 min to obtain platelet-poor plasma. Aliquots of the latter were frozen at −80 °C and thawed on the day of testing. Markers were assayed from different aliquots of the same sample.

The study was done in accordance with the Declaration of Helsinki and the Principles of Good Clinical Practice.

aPLs

We searched for LAC, anticardiolipin antibody (aCL, ELISA kit, Phadia, Uppsala, Sweden, EliA Cardiolipin IgG/IgM) and anti-beta2-glycoprotein I antibody (aβ2GPI, ELISA kit, Phadia, Uppsala, Sweden. EliA β2 Glycoprotein I IgG/IgM) in the samples of all the enrolled patients. Plasma samples were tested for the presence of LA according to the recommended criteria from the ISTH Subcommittee on lupus anticoagulant-phospholipid-dependent antibodies [19]. LA measurement included dilute Russell's viper venom time as described by Thiagarajan et al. [20] (dRVVT, Hemosil, LA-screen/confirm, Instrumentation Laboratory, Lexington,USA) and partial thromboplastin time-LA (PTT-LA, Diagnostica Stago, Asnieres, France). If PTT-LA was prolonged, the hexagonal phospholipid neutralization test was performed as confirmation (STACLOT-LA, Diagnostica STAGO, Asnières, France).

Measurement of inflammatory and oxidative stress parameters

Levels of SAA and CRP in the sera were measured by nephelometry (kits from Beckman Coulter, Brea, CA, US).

8-isoprostane concentrations were measured in duplicate using a specific enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI, USA). The detection limit was 5 pg/mL and the intra-assay and inter-assay variabilities were 5 and 6 %, respectively. PGE2 was measured in duplicate using a specific enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI, USA).

Statistical analyses

Data are shown as mean ± SD. Analysis of variance (ANOVA), analysis of covariance (ANCOVA), Mann–Whitney test and Student's *t*-test were used to assess differences between groups where appropriate; multiple regression models assessed associations between variables.

Results

Average plasma levels of CRP, SAA, 8-isoprostane and PGE2 were higher in APS patients than in the other groups we evaluated (Table 2; Fig. 1). Age was the only significant confounder for CRP ($p = 0.05$), SAA ($p < 0.01$), and 8-isoprostane ($p = 0.05$) that was identified by ANCOVA from among age, gender, menopause, oral contraception, smoking and obesity. Age-adjusted significances between groups were $p < 0.01$ for SAA, $p = 0.01$ for CRP, $p = 0.05$ for 8-isoprostane and $p = 0.001$ for PGE2. Interestingly, in the APS group, higher SAA levels were observed in subjects with higher PGE2 levels (1.483 ± 0.2165 vs. 2.213 ± 0.2695 mg/dl, $p = 0.0435$) (Fig. 2). No differences were found in this group with regard to 8-isoprostane and CRP levels. Among the APS patients, 13 subjects showed triple positivity for aPL for lupus anticoagulant, aCL IgG and/or IgM and aβ2GPI IgG and/or IgM. These patients had higher values of 8-isoprostane and PGE compared to patients with single or double positivity alone ($p < 0.05$ and$p = 0.001$, respectively). A further analysis of data was performed taking the

aPL profile into account (patients and carriers were subdivided into groups; triple aPL positivity, double positivity with negative LAC, and single positivity). No statistical differences were observed among the various sub–groups of APS patients and aPL carriers, as shown in Fig. 3.

No statistically significant differences were found between primary APS and APS associated with other immunological diseases. Of interest, no differences were found in vascular APS (31 subjects) compared to obstetric APS (14 subjects).

Discussion

CRP and SAA are acute-phase plasma proteins of inflammation, mainly synthesized by the liver triggered by the pro-inflammatory cytokines IL-1 and IL-6. An increase in such markers may be observed in several chronic inflammatory diseases, such as RA and atherosclerosis [15]. Our data show that APS patients may display a low-grade inflammatory state that might be caused by the disease itself. According to the so-called 'two hit hypothesis', the aPL (representing the first hit) induces a thrombophilic state, but clotting takes place only in the presence of another thrombophilic condition (the second hit) [21]. It has been suggested that infectious processes might constitute the second hit; in fact, infections may precede full-blown APS by the potential involvement of pattern recognition receptors (such as TLRs) triggering an inflammatory response [22] 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 [23].

In detail, among aPL, aβ2GPI antibodies have been shown to induce NF-κB nuclear translocation, thus resulting in a pro-inflammatory endothelial cell phenotype [24]. It has been suggested that aβ2GPI antibody binding could upregulate the expression of cytokines, adhesion molecules [25] and acute phase proteins, such as SAA and CRP. Our data are consistent with this hypothesis, since SAA and CRP plasma levels were higher in APS patients than in all control groups. It is noteworthy that the isolated presence of aPL, with no signs/symptoms of the syndrome, is not associated with an increase in inflammation markers. This observation supports the idea that aPLs are needed, but alone are not enough to induce thrombosis or pregnancy-related morbidity, as previously reported [26].

To our knowledge, this is the first time that 8-isoprostane and PGE2 have been used to investigate oxidative stress in APS.

F2-isoprostanes are arachidonic acid products formed on membrane phospholipids by the action of ROS, and thereby represent a quantitative measurement of oxidative stress in vivo [27].

Among F2-isoprostanes, particular attention has been focused on 8-isoprostane, which, thanks to its stability, specificity for lipid peroxidation and relative abundance in biological fluids, is a reliable marker of lipid peroxidation and oxidative stress. In the present study, higher values of 8-isoprostane were observed in the APS group, especially in subjects with triple positivity for aPL.

It is now widely recognized that multiple positivity in tests exploring the presence of aPL [28,29] and, notably, triple positivity is associated with thrombosis and identifies high-risk patients with APS [30, 31]. In our study, no statistical differences were observed among the different sub–groups of APS patients and aPL carriers (triple aPL positivity, double positivity with LAC negativity, and single positivity). Nonetheless, aPL triple carriers presented higher levels of 8-isoprostane and SAA compared to the other subgroups of carriers, even though this trend did not reach statistical significance, likely because only two patients in group 4 were found to have triple positivity.

It was reported that 8-isoprostane induces a dose-dependent increase in the changes in platelet shape [32] and in calcium release from intracellular stores [33, 34]. The ability of 8-isoprostane to amplify the aggregation response to sub-threshold concentrations of platelet agonists may be

relevant to settings where platelet activation and enhanced free-radical formation coincide, as is the case in APS. This condition may predispose to a pro-thrombotic phenotype leading to clinical features of the syndrome.

This could be consistent with the elevated concentration of PGE2 values we observed in our APS subjects. Under inflammatory conditions, the synthesis of prostanoids in endothelial cells and smooth muscle cells is highly increased. Biosynthesis of PGE2 is predominantly enhanced in vascular smooth muscle cells and macrophages [32] by inflammatory mediators.

Prostanoids are involved in haemostasis by differentially influencing platelet aggregation. PGE2 has long been known to inhibit platelet aggregation at higher concentrations. At lower concentrations, however, PGE2 promotes platelet aggregation induced by ADP or collagen [35–39]. Therefore, the primary action of PGE2 was hypothesised as being pro-aggregatory [32] through the activation of EP3, i.e., one of the four PGE2 receptors. PGE2 eventually facilitates thrombosis by decreasing the activation threshold of platelets, thus making them more sensitive to their agonists [40].

Our data suggest that immune activation and low-grade inflammation occur in APS. As the inflammatory component of APS surfaced, it was hypothesised that statins might prove to be beneficial in APS patients. Statins were shown to prevent aPL-mediated endothelial cell activation [41]. Moreover, in a pilot proteomics study, it was shown that the aPL-mediated over-expression of inflammatory mediators could be reversed by one month of fluvastatin therapy [42].

Our report presents some limitations, mainly related to the preliminary nature of this study. First, data regarding PGE2 measurements need to be confirmed by further analyses using a chromatographic-mass spectrometric approach, both in serum and urine, as previously described [43]. Second, patients were not divided into different subgroups according to disease activity (acute or quiescent state). Third, these results need to be confirmed in a larger cohort.

A further study is ongoing to verify the findings presented here, investigating also cytokines' dosages, malondialdehyde levels, total antioxidant capacity and free radical serum levels.

In addition, future research is needed to confirm these observations and to evaluate whether they are also associated with accelerated atherosclerosis and other manifestations of APS.

From a speculative point of view, in the future, an approach based on the assay of proinflammatory reactants might be needed in deciding the pharmacological treatment of these difficult patients.

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Table 1: Demographic characteristics and ongoing therapy

Group	APS patients	Group 1	Group 2	Group 3	Group 4	Group 5
Age	47 ± 11	41 ± 12	37 ± 9	59 ± 8	44 ± 9	42 ± 15
M/F	21/24	6/9	0/15	8/7	7/8	6/9
Smoking	5 (11 %)	3 (20 %)	3 (20 %)	3 (20 %)	2 (13 %)	5 (33 %)
BMI	27 ± 4	24 ± 4	26 ± 4	31 ± 6	27 ± 4	26 ± 5
Menopause	2	2	0	3	1	1
Hypertension	9 (20 %)	5 (33 %)	3 (20 %)	4 (26 %)	3 (20 %)	2 (13 %)
Oral contraception	0	0	3 (20 %)	0	0	3 (20 %)
Ongoing therapy	29 OAT 5 ASA 1 LMWH	5 ASA 3 OAT	4 ASA	12 OAT 1 LMWH	3 ASA	0
aPL profile						
LAC +ve *n* (%)	33 (73 %)	0	0	0	10 (66 %)	0
aCL mean titre (range)	32 (2–102)	4 (2–7)	2 (0–2)	2 (2–5)	29 (2–87)	3 (2–5)
aβ2GPI mean titre (range)	27 (2–89)	3 (0–5)	3 (2–6)	2 (0–2)	28 (2–91)	2 (0–2)
Thrombosis *N* (%)	31 (69 %)	1 (6.5 %)	0 (0 %)	15 (100 %)	0 (0 %)	0 (0 %)
Pregnancy morbidity	14 (31 %)	1 (6.5 %)	15 (100 %)	0 (0 %)	0 (0 %)	0 (0 %)

OAT oral anticoagulant therapy, *ASA* acetylsalicylic acid, *LMWH* low molecular weight hepatin

Table 2: Plasma levels of CRP, SAA, 8-isoprostaneand PGE2 in APS patients and in the control groups

Group	APS patients	Group 1	Group 2	Group 3	Group 4	Group 5
CRP (mg/l) mean (range)	1.76 (0.08–3.9)	0.9 (0.05–1.3)	1.2 (0.05–1.7)	1.6 (0.057–2.1)	0.5 (0.057–0.7)	0.4 (0.01–0.9)
SAA (mg/dl) mean (range)	1.88 (0.40–4.10)	1.28 (0.4–2.83)	0.6 (0.02–1.1)	0.5 (0.02–1.2)	0.8 (0.04–1.7)	0.5 (0.08–1.1)
8-isoprostane (pg/ml) mean (range)	917.1 (496–1,280)	78.45 (23–146)	209.2 (60–566)	145 (17–558)	127.37 (11–456)	183.1 (111–249)
PGE28 (pg/ml) mean (range)	567.6 (56–969)	114.3 (23–200)	289 (75–677)	150.82 (29–269)	163.25 (20–273)	75.4 (10–600)

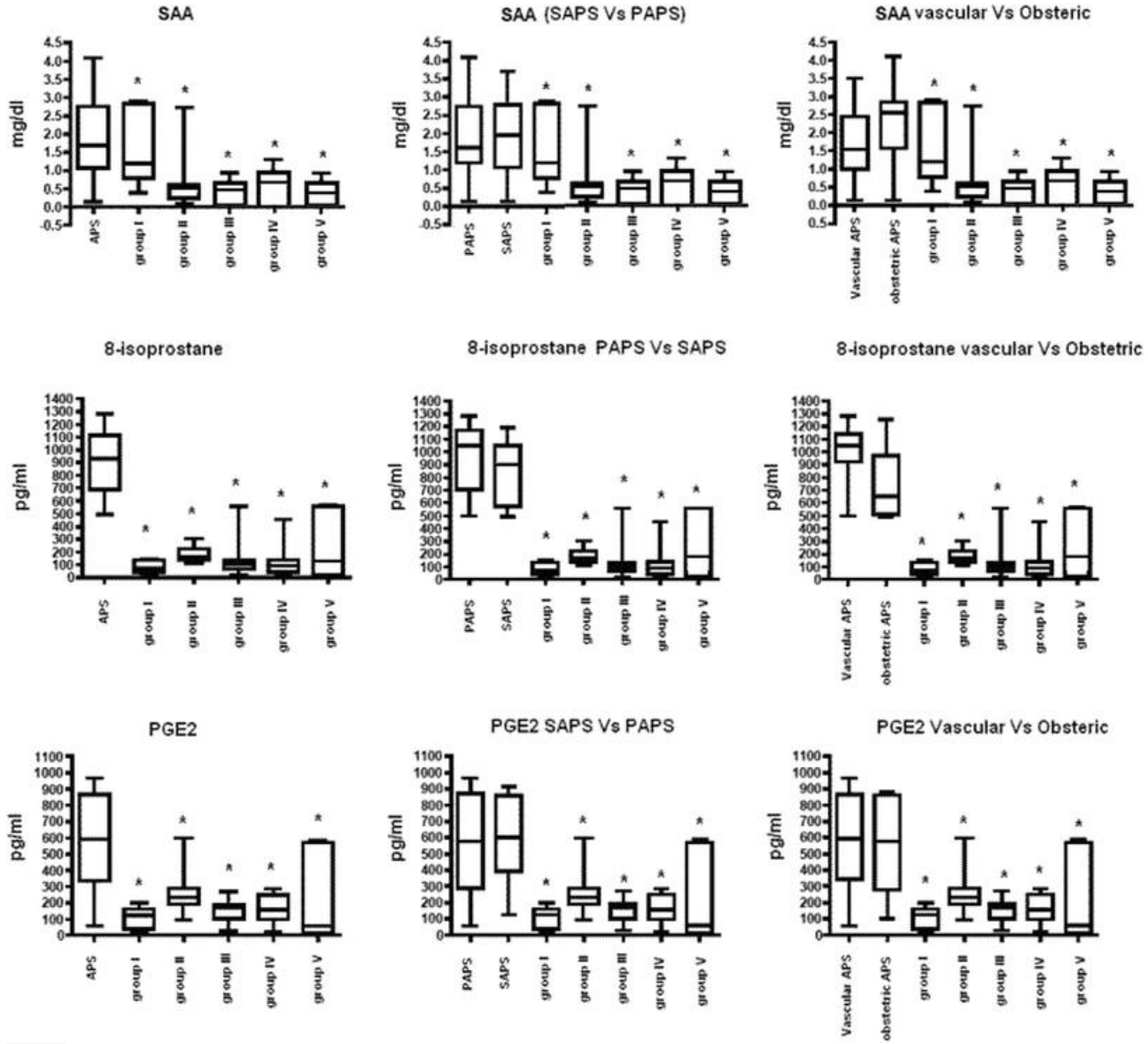

Fig. 1

Average plasma levels of CRP (1.76 mg/l; ranging from 0.08 to 3.9), SAA (1.88 ranging from 0.40 to 4.10 mg/dl), 8-isoprostane (917.1 pg/ml, 496-1280 pg/ml) and PGE2 (567.6 pg/ml, 56-969 pg/ml) were higher in APS patients than in the other groups we evaluated. The control groups included: group 1, 15 aPL negative patients with Systemic Lupus Erythematosus (classified according to the ARA criteria); group 2, 15 aPL negative subjects with pregnancy-related morbidity (consistent with obstetric manifestations of APS); group 3, 15 aPL negative patients with thrombosis; group 4) 15 subjects with persistently positive aPL with no signs/symptoms of APS; group 5, healthy, hospital staff volunteers. *$p < 0.05$, *PAPS* primary antiphospholipid syndrome; *SAPS* secondary antiphospholipid syndrome

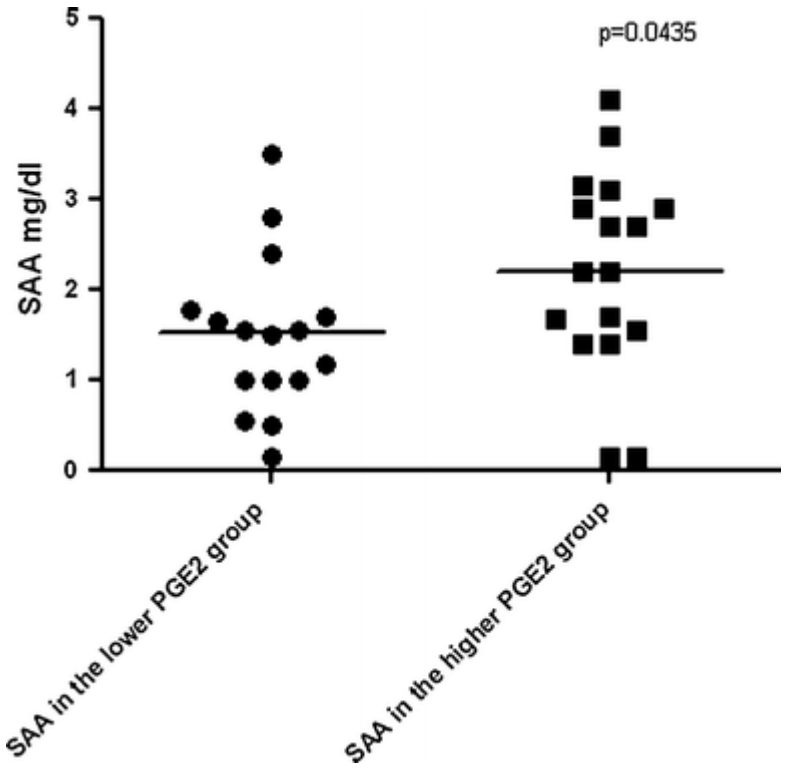

Fig. 2
Higher levels of SAA were observed in APS subjects with higher levels of PGE2 (1.483 ± 0.2165 vs. 2.213 ± 0.2695 mg/dl, $p = 0.0435$). Median value of PGE2 in the APS group was used as a cut off to discriminate between lower and higher measurements

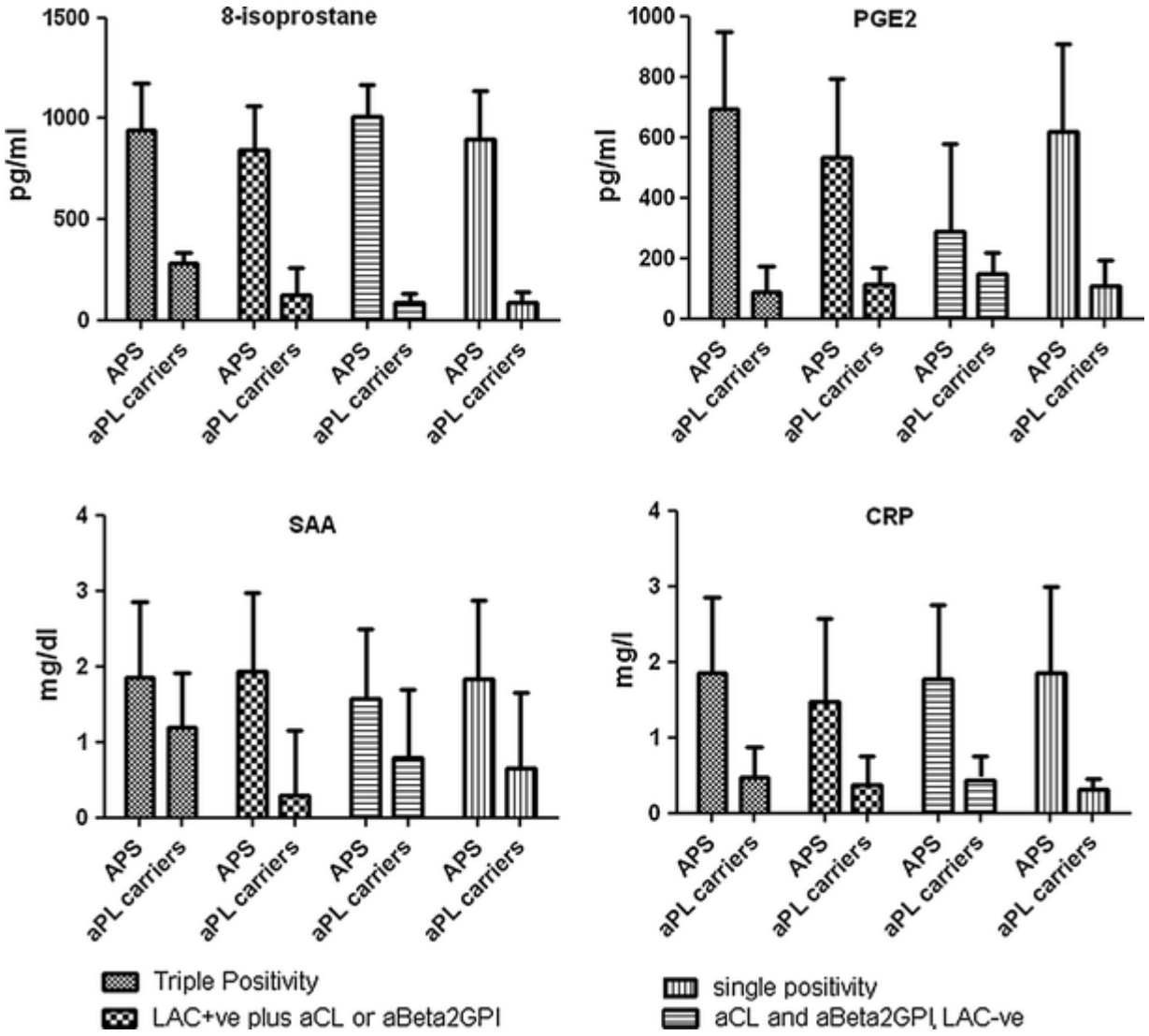

Fig. 3

Plasma levels of CRP, SAA, 8-isoprostane and PGE2 in APS patients and aPL carriers subdivided according to aPL profile. APS patients profile: 15 (33.33 %) with triple positive, 7 (15.5 %) with double positive with LAC negative, 16 (35.55 %) with LAC positive plus aCL or aβ2GPI, and 9 (20 %) with single positive test. aPL carriers' profiles: 2 (13.33 %) with triple positive, 3 (20 %) with double positive with LAC negative, 6 (40 %) with LAC positive plus aCL or aβ2GPI, and 4 (26.66 %) with single positive test