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Low-grade endotoxemia and risk of recurrent thrombosis in primary antiphospholipid syndrome. The multicenter ATHERO-APS study

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ABSTRACT

Introduction: Low-grade endotoxemia is associated with systemic inflammation, enhanced oxidative stress and cardiovascular events in different clinical settings, but its possible role as "second hit" in patients with primary antiphospholipid syndrome (PAPS) has never been investigated.
Purpose: To evaluate the relationship between plasma lipopolysaccharide (LPS) levels, oxidative stress markers and risk of thrombosis in the prospective multicenter ATHERO-APS study.
Methods: Baseline LPS, soluble NADPH-oxidase 2-derived peptide (sNOX-dp), H_2O_2 production, hydrogen peroxide breakdown activity (HBA), and nitric oxide (NO) bioavailability were compared in 97 PAPS, 16 non-thrombotic aPL carriers and 21 controls (CTRL) matched for age and sex. Correlations among laboratory variables were explored by Rho Spearman's correlation (rS). Cox-regression analysis was performed to assess the association between LPS and risk for a composite outcome of cardiovascular death, venous and arterial thromboembolism.
Results: In the whole cohort (median age 51 years (IQR 43–60), 72 % female), PAPS demonstrated higher levels of LPS, sNOX-dp and H_2O_2 and lower levels of NO and HBA compared to non-thrombotic aPL carriers and CTRL. LPS levels were inversely correlated with HBA (rS: −0.295, $p = 0.001$) and NO (rS: −0.322, $p < 0.001$) and directly correlated with sNOX-dp (rS:0.469, $p < 0.001$) and H_2O_2 (rS:0.282, $p < 0.001$). PAPS showed higher levels of LPS, sNOX-dp and H_2O_2 and lower levels of NO and HBA compared to aPL carriers and CTRL. After a 4.7 years follow-up of, 11 composite outcomes were reported in PAPS (2.5 per 100 patient-years) while none was observed in aPL carriers. On Cox-regression analysis, patients with LPS above the median (>23.1 pg/ml) had a 5-

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fold increased risk of composite outcome compared to those with LPS below the median, after adjustment for sex, age, diabetes, and global antiphospholipid syndrome score.
Conclusion: Low-grade endotoxemia is associated with an increased oxidative stress and a higher risk of thrombosis in PAPS. Its prognostic value in carriers needs to be investigated in larger cohorts.

1. Introduction

Antiphospholipid syndrome (APS) is an autoimmune disease characterized by recurrent arterial (ATE) and venous thromboembolic events (VTE) in the persistent presence of antiphospholipid antibodies (aPL), mainly represented by IgG/IgM anticardiolipin (aCL), IgG/IgM anti-β_2-glycoprotein-I (aβ2GPI) and lupus anticoagulant (LAC) [1], that contribute to enhanced oxidative stress in APS [2,3]. Growing evidence shows that aPL are a necessary but insufficient stimulus to induce thrombotic events [4,5]. Via their interactions with platelets, endothelial cells, and leukocytes, aPL induce a systemic pro-inflammatory and pro-coagulant state, characterized by increased expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase that through the production of superoxide anion leads to the formation of hydrogen peroxide (H_20_2) and to decreased nitric oxide (NO) bioavailability. This chain of events may precipitate thrombosis only during the occurrence of a second hit, realizing thus the thrombotic APS [6]. In experimental models of APS, lipopolysaccharide (LPS), through its binding to Toll Like receptor 4 (TLR-4), may act as a second inflammatory stimulus [7]. TLR-4 is expressed on the surface of endothelial cells, platelets and leukocytes and when activated by LPS, leads to production of large amount of reactive oxygen species (ROS), that could facilitate coagulation activation and thrombus formation [8]. The possible synergistic effect between aPL and LPS seems confirmed by clinical studies showing that gram negative infections are responsible for 40 % of catastrophic APS, a life-threatening condition characterized by a disseminated thrombotic microangiopathy leading to fatal multiorgan failure [9].

Moreover, LPS plays a pivotal role in different clinical conditions characterized by an enhanced oxidative stress and a high risk of thrombotic events, even in absence of a clinical evident infection [10]. Hypothesizing a possible involvement of LPS in the vascular pathogenesis of APS, we investigated the relationship among LPS, oxidative stress and thrombosis in a cohort of patients with thrombotic primary APS (PAPS) and persistent non-thrombotic aPL carriers devoid of any other autoimmune or inflammatory disease.

2. Materials and methods

2.1. Aim of the study

The aims of the present study were as follows: i) to perform a baseline cross-sectional comparison of plasma LPS concentration and oxidative markers in PAPS patients, aPL carriers and healthy controls (CTRL); and ii) to prospectively evaluate the association between plasma LPS concentrations and the risk of thrombosis or re-thrombosis during a 5-year follow-up.

2.2. Patient cohort

Our Multicenter ATHERO-APS study cohort focuses on vascular involvement and includes 97 PAPS patients and 16 aPL carriers attending the: U.O.C. Medicina Interna e Studio dell'Aterosclerosi – A.O.U. Umberto I – Universita' Sapienza di Roma; U.O.C. Malattie Allergiche e del Sistema Immunitario – A.O.U. San Giovanni di Dio e Ruggi D'Aragona - Università di Salerno; Centro Emostasi - A.O.R.N. "SG Moscati", Avellino; Multimedica S.R.L, Napoli. Details of this cohort have been previously published [11,12]. In brief, the enrolment into the cohort was initiated in 2015 and finished in 2017. Inclusion criteria were age above 18 years and i) to fulfill all the clinical and laboratory diagnostic APS Sydney criteria [1] for PAPS or ii) two consecutive, positive aPL tests carried out at least 12 weeks apart for the aPL carriers. aPL were then re-assessed on a yearly basis. Exclusion criteria were pure obstetric PAPS without vascular occlusions because the focus of our interest was vascular involvement, systemic lupus erythematosus and other autoimmune disorders characterized by oxidative stress that would have added to the oxidative stress of PAPS, acute and chronic liver and renal disease, acute and chronic infections, pregnancy, recent surgery and active cancer. The same inclusion and exclusion criteria were applied to our non-thrombotic aPL carrier group. The diagnosis of diabetes mellitus [13], arterial hypertension [14], heart failure [15] and dyslipidemia [15] were made according to the current international guidelines. When a patient reported a thrombotic event in his personal medical history during the first visit or during the follow-up, the medical records (i.e., discharge letter, report, and images of radiology exam) were reviewed to confirm the type of the event, and a copy was stored.

The cross-sectional analysis of this study compared plasma concentrations of LPS, oxidative stress markers, and NO concentration in 97 PAPS, 16 non-thrombotic aPL carriers and 21 CTRL matched for age and sex. The oxidative stress markers considered were as follows: 1) the catalytic core of NADPH oxidase (sNox2-dp); 2) plasma concentrations of Hydrogen Peroxide (H_2O_2); and 3) plasma scavenging activity (HBA) of serum H_2O_2. CTRL were subjects selected from the hospital personnel without cardiovascular risk factors or previous cardiovascular events enrolled in the ATHERO-APS study and followed for a median time of 5.7 years.

The prospective aim of this study investigated the relationship between the plasma LPS concentration at baseline and the risk of thrombotic events during the follow-up in PAPS and non-thrombotic aPL carriers.

2.3. Ethical considerations

The study protocol was approved by the Ethics Committee of the University "Sapienza" of Rome, Italy (Reference No. 4417, March 02, 2017) then adopted and approved by the other centers. The study was conducted in accordance with the principles embodied in the Declaration of Helsinki. All participants to the cohort gave written informed consent.

2.4. Blood sample collection

All blood samples were collected at the enrolment visit, between 9.00 and 12.00 a.m.; they were drawn in EDTA, trisodium citrate and serum tubes according to the required tests; full blood counts were performed on the same morning, while citrate and serum samples, after centrifugation, were aliquoted in volumes of 0.5 ml, frozen at −80 °C and thawed before use.

2.5. Antiphospholipid antibodies detection

LA was detected according to ISTH guidelines [16,17], by dilute Russel's viper time (DRVVT), run on an ACL TOP-500 coagulometer (Instrumentation Laboratory, Milano, Italy). The upper cut-offs for each assay were set at the 99th percentile from testing 142 plasmas from 91 females and 51 males (mean age 39 ± 17 years) who were healthy hospital and laboratory personnel. The same cut-offs were utilized both in PAPS and non-thrombotic aPL carriers. A clotting time ratio between patient and control sample >1.18 for the DRVVT (range 0.90–1.18)

indicated an abnormal result. IgG/IgM aCL and IgG/IgM aβ2GPI antibodies were detected by chemiluminescent assays (Menarini Diagnostica, Milano, Italy). Normal ranges were established using the same 142 healthy hospital personnel as above, with a cut-off for positivity at the 99th percentile (5,6). The inter and intra coefficient of variability for all the immune assays ranged between 3.0 % and 3.9 %. Patients were defined as triple positive if they had the simultaneous presence of IgG/IgM aCL, and IgG/IgM aβ2GPI and LAC. All other blood analytes were laboratory grade reagents.

2.6. Serum LPS

LPS levels in serum were measured using a commercial sandwich ELISA kit (Cusabio, Wuhan, China). The standards and samples were plated for 2 h at room temperature onto a micro-plate pre-coated with the capture antibody specific for LPS. After incubation with the detection antibody, plates were read at 450 nm. Values were expressed as pg/ml; intra-assay and inter-assay coefficients of variation were <10 %.

2.7. Serum sNox2-dp evaluation

Serum Nox2 were measured as soluble Nox2-derived peptide (sNox2-dp) with an ELISA method as previously reported [18]. Briefly, the peptide is recognized by a specific monoclonal antibody that binds the amino acid sequence (224–268) that corresponds to the extracellular membrane portion of Nox2 (catalytic core of NADPH oxidase), released during platelet activation. The enzymatic activity was measured spectrophotometrically by the increased absorbance at 450 nm. Values were expressed as pg/ml; intra-assay and inter-assay coefficients of variation were 8.95 % and 9.01 %, respectively.

2.8. Determination of H_2O_2 production

The Hydrogen Peroxide (H_2O_2) was measured by a colorimetric assay according to manufacture instruction (Abcam, #ab272537). The method utilizes the chromogenic Fe-xylenol orange reaction, in which a purple complex is formed when Fe provided in the reagent is oxidized to Fe by peroxides present in the sample. The intensity of the color, measured at 540-610 nm, is an accurate measure of the peroxide level in the sample. The values were expressed as μM and intra- and inter-assay coefficients of variation were both <10 %.

2.9. Serum hydrogen peroxide scavenging activity

To assess the antioxidant capacity, we measured the plasma hydrogen peroxide (H_2O_2) break-down activity (HBA) by HBA assay kit (Aurogene, code HPSA-50). The % of HBA was calculated according to formula below reported: % Of HBA = [(Ac − As) / Ac] × 100 where Ac is the absorbance of H_2O_2 1.4 mg/mL and As is the absorbance in the presence of the plasma sample.

2.10. Serum NO bioavailability

NO bioavailability was evaluated in serum by a colorimetric assay kit (Abcam, Cambridge, UK) used to determine the metabolites of NO (nitrites and nitrates; NOx). Intra-assay and inter-assay coefficients of variation are 2.9 % and 1.7 %, respectively.

2.11. Adjusted global antiphospholipid syndrome score

The risk of thrombosis in PAPS patients was stratified via the adjusted global antiphospholipid syndrome score (aGAPSS), a well-known tool validated in larger cohorts of APS [19,20]. For each included patient, the aGAPSS was calculated as follows: a score 3 was assigned for dyslipidemia, 1 for arterial hypertension, 5 for aCL IgG/IgM, 4 for aβ2GPI IgG/IgM and 4 for LA.

2.12. Study outcomes

Adverse outcomes were registered during all the observation periods. The primary endpoint of the study was a composite outcome of ATE, VTE and cardiovascular death. ATE was defined any type of arterial thrombotic events (ischemic stroke, myocardial infarction, or peripheral arterial thrombosis/thromboembolism). VTE was defined as any type of deep vein thrombosis with or without pulmonary embolism. Cardiovascular death was defined as death due to acute coronary syndrome, ischemic or hemorrhagic stroke, or pulmonary embolism.

2.13. Statistical analysis

Categorical variables were reported as frequencies and continuous variables were reported as medians with interquartile ranges. Normal distribution was assessed by the Kolmogorov-Smirnov test. Categorical variables were compared by using $\chi 2$ tests. The Student unpaired *t*-test and the Mann Whitney test were used, when appropriate, to explore the differences between cardiovascular, clinical and laboratory continuous variables. Kruskal Wallis test was utilized to compare LPS and oxidative stress markers among PAPS, aPL carriers and CTRL. Correlations among laboratory variables were explored by Rho Spearman's correlation. Only PAPS and aPL carriers were considered for the survival analysis. The incidence rate of adverse outcomes was calculated as number of events/total person years ratio and reported as incidence for 100 persons-year. Cox proportional hazards regression time to the first event analysis was used to calculate the unadjusted and adjusted relative hazard ratios (HRs) and 95 % Confidence Interval (95 % CI) of composite outcome. The risk of composite outcome in PAPS and aPL carriers was investigated utilizing two different models: in the first, LPS was utilized as a continuous variable (Model A); in the second, LPS was used as a dichotomized variable utilizing the median value as cut point and taking as reference patients with LPS levels below the median value (Model B). All the Cox-regression multivariable analyses models were adjusted for age, sex, diabetes and aGAPSS. All tests were 2-tailed, and analyses were performed using computer software packages (SPSS-25.0, SPSS Inc., Chicago, IL). A p value <0.05 was considered as statistically significant.

3. Results

3.1. Baseline comparisons

We studied 97 PAPS patients (median age 51 (interquartile range (IQR): 45–61 years), 71.1 % females), 16 non-thrombotic aPL carriers (median age 52 (IQR: 40–64) years, 87.5 % females) and 21 CTRL (median age 53 (IQR: 42–57) years, 60.0 % females), matched for age and sex (Table 1). Compared to aPL carries, PAPS patients showed higher aCL IgG levels, and LAC and triple positivity, whereas no significative difference was found for aCL IgM, aβ2GPI IgG/IgM, hypertension, diabetes, dyslipidemia, and smoking (Table 1).

On group comparisons, PAPS had significant higher plasma concentrations of LPS, sNOX-dp, and H_20_2, and lower concentrations of NO and HBA compared to aPL carriers and CTRL (Table 1, Fig. 1, Panel A-E). The inter group analysis found that aPL carriers had higher concentrations of sNOX-dp (Fig. 1, Panel B) and H_20_2 (Fig. 1, Panel C), a lower HBA (Fig. 1, Panel D), but not a significant different concentration of LPS and NO (Fig. 1, Panel A and E) compared to CTRL. In the whole cohort, plasma LPS levels were directly correlated with sNOX-dp (rS: 0.469, $p < 0.001$) and H_2O_2 (rS: 0.282, $p < 0.001$) and inversely correlated with HBA (Rs: −0.295, $p = 0.001$) and NO (rS: −0.322, $p < 0.001$) (Table 2).

Plasma LPS levels did not relate to aCL IgG/IgM, aβ2GPI IgG/IgM and/or LAC neither across the whole population nor in PAPS and aPL carriers considered separately (Supplementary Table 1).

Table 1
Baseline comparison among patients with primary antiphospholipid syndrome, antiphospholipid *carriers,* and healthy controls.

	CTRL $n = 21$	aPL $n = 16$	PAPS $n = 97$	*p*-Value
Age years, median (IQR)	53.1 (42.1–57.2)	52.4 (39.8–63.5)	51.1 (45.0–60.5)	0.915
Female sex, n (%)	12 (57.1)	14 (87.5)	69 (71.1)	0.131
BMI (IQR) kg/m2	23.1 (20.8–25.5)	24.3 (22.7–28.2)	28.2 (22.7–28.2)	0.155
HBA %, median (IQR)	64.0 (54.1–70.3)	46.9 (35.2–56.7)	30.7 (26.3–38.4)	<0.001
LPS pg/ml, median (IQR)	14.3 (11.9–17.0)	19.7 (11.8–21.7)	26.3 (17.1–32.0)	<0.001
sNOX-dp pg/ml, median (IQR)	15.4 (12.1–19.4)	21.9 (20.1–25.3)	28.6 (20.9–30.4)	<0.001
H_2O_2 μM, median (IQR)	15.4 (11.5–17.9)	21.1 (18.9–23.9)	25.2 (20.9–30.4)	<0.001
NO μM, median (IQR)	31.7 (24.7–36.7)	28.0 (20.8–34.3)	18.5 (14.8–23.4)	<0.001
aCL IgG, (IQR) GPL U/ml	–	26.6 (20.1–44.1)	109 (32.5–205.4)	0.004
aCL IgM, (IQR) GPL U/ml	–	56 (24.8–130.0)	41.8 (28.6–112.3)	0.963
aβ2GPI IgG, (IQR) AU/ml	–	72.0 (39.1–187.8)	135.2 (35.9–294.6)	0.492
aβ2GPI IgM, (IQR) AU/ml	–	82.3 (31.5–303.0)	34.6 (25.3–98.5)	0.236
LAC, n (%)	–	2 (12.5)	38 (39.2)	0.001
Triple positivity, n (%)	–	2 (12.5)	34 (35.1)	0.006
Hypertension, n (%)	–	4 (25.0)	48 (49.5)	0.060
Diabetes, n (%)	–	0 (0.0)	9 (9.3)	0.200
Dyslipidemia, n (%)	–	2 (12.5)	18 (18.6)	0.546
Smoking, n (%)	–	1 (6.3)	22 (22.7)	0.125

CTRL: healthy controls, aPL: antiphospholipid carriers, PAPS: primary antiphospholipid syndrome, IQR: interquartile range, LPS: lipopolysaccharide, HBA: plasma scavenging activity, sNox2-dp: catalytic core of NADPH oxidase, H_2O_2: Hydrogen Peroxide, NO: Nitric Oxide, aCL: antibodies anti cardiolipin, aβ2GPI: antibodies anti beta-2-glycoprotein-I.

3.2. Demographic and clinical variables according to median LPS concentration

To investigate the presence of clinical characteristics according to the LPS levels we stratified the combined PAPS and aPL populations in two groups according to the median LPS levels: 1) patients with LPS levels > 23.1 pg/ml (above the median) and 2) patients with LPS levels ≤ 23.1 pg/ml (below the median). Patients with LPS levels below the median were mostly aPL carriers while patients with LPS levels above the median were mostly PAPS patients with previous recurrent thrombotic events, higher plasma concentration of sNOX-dp and lower concentration of NO (Table 3). No significative differences were found for aCL IgG/IgM, aβ2GPI IgG/IgM, LAC, triple positivity, and inflammatory markers between these two groups (Table 3).

3.3. Survival analysis

After a median follow-up of 4.7 (IQR: 4.1–5.6) years, 11 composite outcomes were reported: 1 cardiovascular death, 3 ischemic strokes, 2 myocardial infarctions, 3 peripheral arterial thrombosis and 2 VTE. 9 composite outcomes occurred in patients with basal LPS levels above the median value (>23.1 pg/ml) and 2 composite events occurred in patients with LPS levels below the median value (1.5 per 100 patient-year vs 0.6 per 100 patient-year, respectively. $p = 0.013$). All composite outcomes occurred in PAPS (2.5 per 100 patient-years) while no cardiovascular events were observed in aPL carriers.

On univariable Cox-regression analysis the only factors associated with the composite outcome were diabetes (HR 4.25, 95%CI 1.15–15.7)

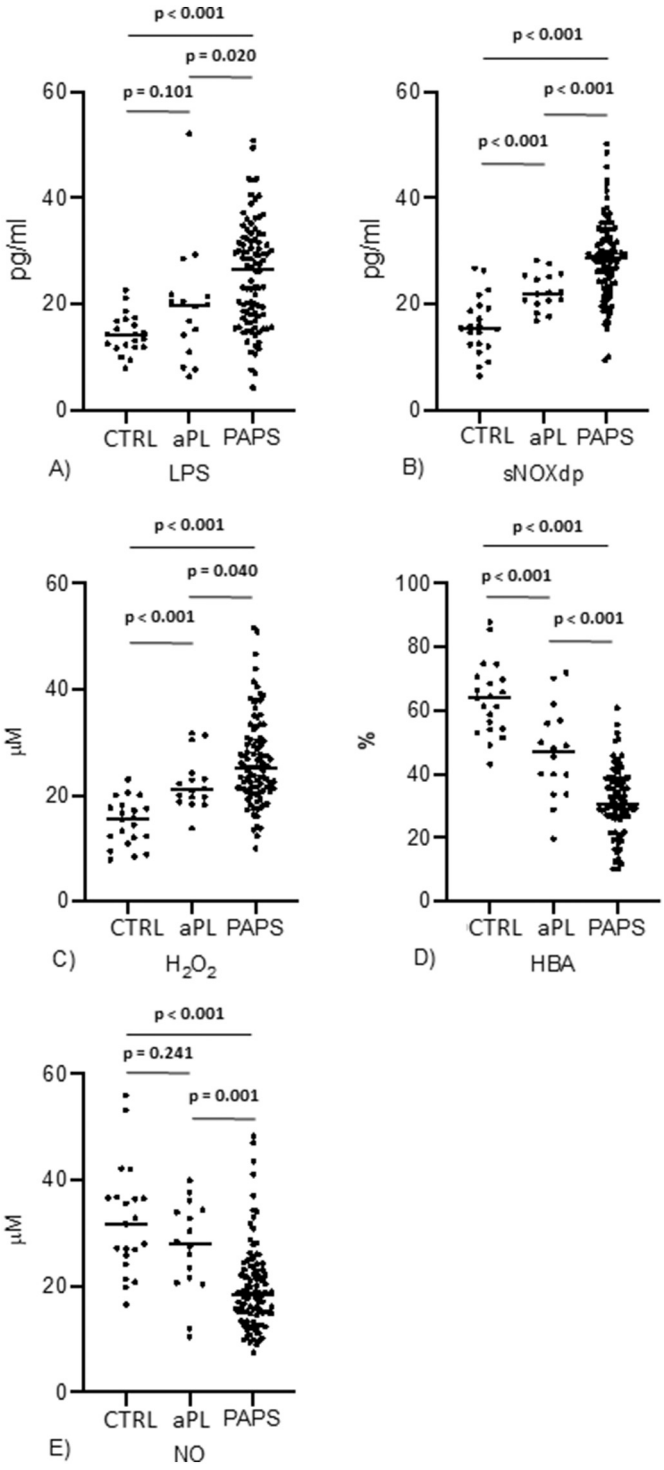

Fig. 1. Comparison of lipopolysaccharide and oxidative stress markers among groups.
Legend: CTRL: healthy controls, aPL: antiphospholipid antibodies carriers; PAPS: primary antiphospholipid syndrome, LPS: lipopolysaccharides, sNox2-dp: catalytic core of NADPH oxidase, H_2O_2: hydrogen peroxide, HBA: H_2O_2 break-down activity, NO: nitric oxide.

and LPS both when considered as a continuous variable (HR 1.07, 95% CI 1.02–1.13) or as a dichotomic variable based on the LPS median level (HR 5.26, 95%CI 1.14–24.36) (Supplementary Table 2).

On multivariable Cox-regression analysis adjusted for age, sex, diabetes and aGAPSS, LPS was still significantly associated with the composite outcome both when considered as a continuous variable (HR

Table 2
Bivariate correlations among lipopolysaccharide and oxidative stress markers.

			HBA (%)	LPS (pg/ml)	sNOX-dp (pg/ml)	H_2O_2 (μM)	NO (μM)
Rho Spearman	HBA (%)	Correlation coefficient	1000	−0,295	−0,360	−0,495	0,394
		p-Value		0,001	<0,001	<0,001	<0,001
	LPS (pg/ml)	Correlation coefficient		1000	0,469	0,282	−0,322
		p-Value			<0,001	<0,001	<0,001
	sNOX-dp (pg/ml)	Correlation coefficient			1000	0,466	−0,352
		p-Value				<0,001	<0,001
	H_2O_2 (μM)	Correlation coefficient				1000	−0,382
		p-Value					<0,001
	NO (μM)	Correlation coefficient					1000
		p-Value					

LPS: lipopolysaccharide, HBA: plasma scavenging activity, sNox2-dp: catalytic core of NADPH oxidase, H_2O_2: Hydrogen Peroxide, NO: Nitric Oxide.

Table 3
Baseline characteristic of patients with antiphospholipid antibodies.

	All patients ($n = 113$)	LPS below the median value ($n = 56$)	LPS above the median value ($n = 57$)	p-Value
Age years (IQR)	51.3 (43.5–60.9)	53.6 (43.7–63.6)	50.3 (42.8–58.6)	0.391
Female, n (%)	82 (73.2)	42 (76.4)	40 (70.2)	0.460
BMI, (IQR) kg/m^2	24.4 (22.5–29.4)	24.4 (21.9–29.4)	24.3 (22.7–19.4)	0.932
White blood cells, (IQR) 10^9/l	6.5 (5.5–8.3)	6.4 (5.4–8.3)	6.5 (5.5–8.3)	0.737
ESR, (IQR) mm/h	23 (10–36)	24 (9–35)	21 (10–37)	0.690
CRP, (IQR) mg/l	1 (0.3–3.2)	1 (0.3–3.1)	1 (0.3–3.2)	0.923
Hypertension, n (%)	52 (46.8)	28 (50.9)	24 (42.9)	0.395
Diabetes, n (%)	9 (8.1 %)	4 (7.3 %)	5 (8.9 %)	0.749
Dyslipidemia, n (%)	20 (17.9 %)	9 (16.4 %)	11 (19.3 %)	0.685
Smoking, n (%)	23 (20.7)	10 (18.2)	13 (20.7)	0.513
Arterial thromboembolism, n (%)	33 (29.5)	15 (27.3)	18 (31.6)	0.617
Recurrent thrombotic events, n (%)	25 (22.3)	7 (12.7)	18 (31.6)	0.017
Carriers, n (%)	16 (14.3)	13 (23.6)	3 (5.3)	0.005
aCL IgG/IgM, n (%)	73 (67.0)	38 (69.1)	35 (64.8)	0.635
aβ2GPI IgG/IgM, n (%)	68 (63.0)	30 (56.6)	38 (69.1)	0.179
LAC, n (%)	55 (50.5)	24 (44.4)	31 (56.4)	0.213
Triple positivity, n (%)	36 (33.0)	15 (27.8)	21 (38.2)	0.248
aGAPSS, (IQR)	10 (5–13)	9 (5–13)	10 (5–13)	0.616
Antiplatelet drugs, n (%)	27 (24.3)	11 (20.0)	16 (28.6)	0.293
Statins, n (%)	20 (18.0)	10 (18.2)	10 (17.9)	0.965
Hydroxychloroquine, n (%)	14 (12.6)	9 (16.4)	5 (8.9)	0.238
Beta-Blockers, n (%)	28 (25.2)	15 (27.3)	13 (23.2)	0.623
ACE-inhibitors/ARBs; n (%)	39 (35.1)	22 (40.0)	17 (30.4)	0.027
Calcium channel blockers, n (%)	14 (12.7)	5 (9.3)	9 (16.1)	0.284
HBA, (IQR) %	32.6 (26.8–39.6)	33.5 (26.8–39.8)	32.4 (27.0–39.9)	0.779
sNOX-dp, (IQR) pg/ml	27.7 (22.2–31.7)	25.0 (20.6–28.7)	29.4 (25.8–34.3)	<0.001
H_2O_2, (IQR) μM	23.8 (20.6–30.2)	23.5 (19.9–29.6)	25.2 (21.0–30.5)	0.440
NO, (IQR) μM	18.9 (15.0–25.0)	22.1 (16.1–30.5)	17.8 (13.3–21.8)	0.001

CTRL: healthy controls, aPL: antiphospholipid carriers, PAPS: primary antiphospholipid syndrome, LPS: lipopolysaccharide, IQR: interquartile range, BMI: body mass index, ESR: Erythrocyte sedimentation rate, CRP: C-Reactive Protein, aCL, anticardiolipin antibodies, aβ2GPI: β2-Glycoprotein-I antibodies, LAC: lupus anticoagulant, ACE: angiotensin converting enzyme, HBA: plasma scavenging activity, sNox2-dp: catalytic core of NADPH oxidase, H_2O_2: Hydrogen Peroxide, NO: Nitric Oxide.

Table 4
Multivariate Cox regression analysis for thrombotic events.

	HR	95%CI	p-Value
Model A			
Age	1.01	0.96–1.07	0.637
Female sex	0.72	0.18–2.83	0.638
Diabetes	4.61	0.98–21.7	0.053
aGAPSS	1.01	0.89–1.15	0.892
LPS (continuous)	1.08	1.02–1.14	0.013
Model B			
Age	1.01	0.95–1.06	0.843
Female sex	1.23	0.24–5.24	0.877
Diabetes	5.90	1.12–31.1	0.036
aGAPSS	1.00	0.87–1.14	0.970
LPS (above the median)	5.06	1.05–24.5	0.044

HR: hazard ratio, CI: confidence interval, aGAPSS: adjusted antiphospholipid syndrome score, LPS: lipopolysaccharide.

1.08, 95%CI 1.02–1.14, Table 4, Model A) or as a dichotomic variable based on the median LPS level (HR 5.06, 95%CI 1.05–24.50) (Table 4, Model B, Fig. 2).

4. Discussion

Our data suggest that LPS is intimately involved in the recurrent thrombogenicity of PAPS. Indeed, LPS levels: i) positively related to markers of oxidative stress and reduced NO bioavailability; ii) were maximally raised in PAPS; and iii) were associated with a higher risk of thrombotic events during the follow-up.

LPS is the main component of the outer membrane of gram-negative bacteria and through the interaction with TLR-4 is responsible for the release of large amount of ROS. Indeed, LPS activates endothelial [21], monocyte [22] and platelet [23] NADPH oxidase, inducing the production of superoxide anion (O_2^-) and H_2O_2: the former is quenched by superoxide dismutase and the latter by catalase. The relation between the plasma concentrations of sNOX-dp and H_2O_2, reflects the activation of this pathway in vivo that is additive to the aPL induced activation of NAPDH oxidase demonstrated in vitro. Either way, the released superoxide anion introduces a O_2 moiety in the ring structure of arachidonic acid independently of the cyclo-oxygenase pathway to generate F2-isoprostanes [24], specific markers of oxidative stress, the plasma and urinary concentrations of which are increased in APS [2,3]. In our population the median LPS levels (23.1 pg/ml) were lower than those detected during a full-blown infection but nevertheless sufficient to trigger a state of enhanced oxidative stress, suggesting thus that low-grade endotoxemia in PAPS can act as possible second pro-thrombotic hit by further worsening the oxidant/antioxidant balance. The mechanism(s) by which the ROS production, induced by LPS, is associated with an increased risk of thrombosis could be partially explained by the NO bioavailability. In our patients, NO metabolites were lower in PAPS than

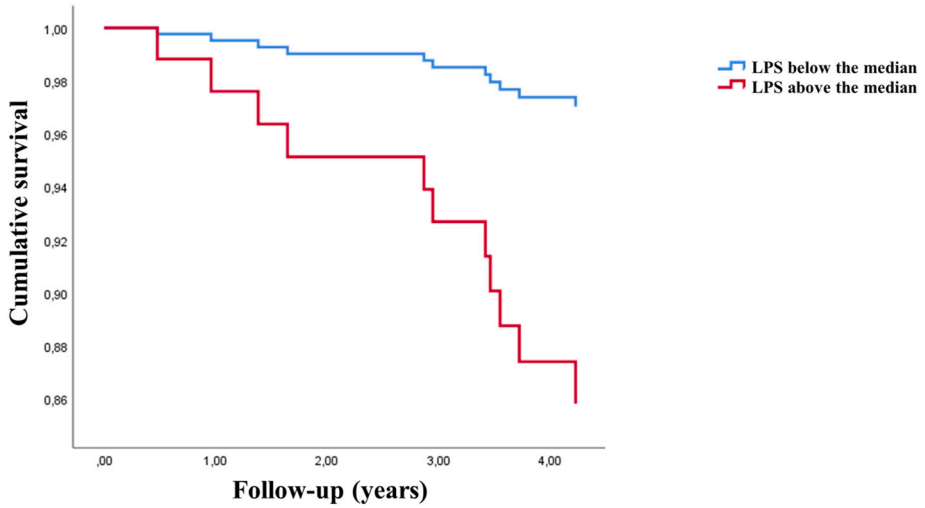

Fig. 2. Multivariate Cox-regression analysis for the risk of composite outcome. Legend: LPS: lipopolysaccharides.

in the other groups and was inversely correlated not only to LPS but also to sNOX-dp, indicating a likely transformation of NO into peroxynitrite. These results confirm a previous study on PAPS, in which elevated nitrative stress was related to a reduced bioavailability of NO and an increased risk of arterial thrombosis [25]. Indeed, NO contributes to the endothelial antithrombotic phenotype and any reduction is associated with vasoconstriction, platelet activation and clot initiation [26].

Over a median follow-up of 4.7 years, we found that 9 patients suffered recurrent ATE (one fatal) and 2 suffered a recurrent VTE. In those patients, LPS levels, either as a continuous or a dichotomous variable, were the strongest predictor of recurrent thrombosis independently from age, sex, diabetes and aGAPSS, a validated risk score for thrombosis in PAPS based on the type of antibodies profile and the presence of dyslipidemia and hypertension [19]. In previous studies, low-grade endotoxemia was associated with the risk of cardiovascular events both in the general population [27] and in pathological conditions characterized by a high thrombotic risk such as acute coronary syndrome [28] and atrial fibrillation [29]. Growing evidence suggests that low-grade endotoxemia could be related to an increased gut permeability that in turn facilitates the LPS translocation from the intestinal lumen to the bloodstream [30]. In this context, several exogenous and endogenous factors influencing gut permeability have been investigated, opening future antithrombotic approaches based on microbiota and gut permeability modulation [31].

In this study we have provided first time evidence that LPS is present in patients with aPL and that the higher concentration was predictive of recurrent thrombosis in PAPS. Indeed, in our population, thrombotic events during follow-up occurred only in anticoagulated PAPS patients while none were reported in aPL carriers. This apparent contradiction leads to the question as to whether LPS is cause or effect of recurrent thrombosis. In fact, in PAPS the persistent aPL activation of the vascular inflammasome [32] may render bowel micro-vessels more permeable to LPS that once in the bloodstream contributes to perpetuation of the inflammasome activation and to a second oxidative hit [33] that culminates in a recurrent clot. In this scenario, we cannot exclude that the lower median aCL IgG titre, the lower proportion of LAC and triple positivity, representing a lower thrombotic risk profile per se, accounted for the lower median LPS and the reduced oxidative stress in aPL carriers compared to the PAPS group. Although we did not find any statistically significant association between aPL and LPS levels, the small sample size hence a poor statistical power may have missed an association between aPL and LPS. Thus, a larger prospective study on non-thrombotic aPL carriers would be required to understand whether LPS may represent a second hit for this group as well.

Besides LPS, diabetes was the only other factor independently associated with the risk of the composite thrombotic outcome during the follow-up. In PAPS the risk of ATE has been associated with the presence of classic cardiovascular risks factors such as hypertension, diabetes, and dyslipidemia [11,12]: the increased oxidative stress characteristic of these conditions [34] adds to that of PAPS [25] enhancing the chance of atherosclerosis [35] and recurrent thrombosis [36].

4.1. Limitations

There are some limitations to consider when interpreting these results. First, the small sample and the low representation of the non-thrombotic aPL carriers group prevents a proper evaluation of the risk of ex novo thrombosis. Second, the relatively low rate of events during the follow-up period does not let us to consider each component of the aGAPS score separately as well as the presence of other possible thrombotic risk factors because the multivariable regression analysis would be highly underpowered. Third, we did not measure any marker of gut permeability that would explain the endotoxemia in PAPS. Fourth, we could not define whether the low NO is due to its biotransformation to peroxynitrite or due to nitric oxide synthase uncoupling whereby NO generation is reduced in favor of increased superoxide production [37]. Furthermore, we could neither exclude the occurrence of subclinical infections nor assess the risk associated with the LPS levels immediately before the recurrent event because these were assessed only at baseline. Finally, we did not investigate the possible role of anti-inflammatory or anti-thrombotic drugs such as statins [38] and/or hydroxychloroquine (26) in preventing the detrimental effect of LPS, as only 18 % and 12.6 % respectively were receiving these medications.

5. Conclusion

Low-grade endotoxemia is associated with increased oxidative stress and a high risk of recurrent thrombosis in PAPS. The possible role of LPS in predicting first thrombotic events in aPL carriers needs further investigation in larger cohorts.

CRediT authorship contribution statement

Tommaso Bucci: Writing - original draft, Investigation, Formal analysis, Conceptualization. Paul R.J. Ames: Writing - original draft, Investigation. Vittoria Cammisotto: Investigation. Chiara Cardamone: Investigation. Antonio Ciampa: Investigation, Resources. Bianca Mangoni: Investigation. Massimo Triggiani: Supervision, Validation. Roberto

Carnevale: Supervision, Validation. Gregory Y.H. Lip Supervision, Validation, Daniele Pastori: Investigation, Validation, Supervision. Pasquale Pignatelli: Supervision, Validation, Resources. All authors approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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Appendix A. Supplementary data

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