Clinical Relevance of Nitric Oxide Metabolites and Nitrative Stress in Thrombotic Primary Antiphospholipid Syndrome

PAUL R.J. AMES, JOANA R. BATUCA, ANTONIO CIAMPA, LUIGI IANNACCONE, and JOSE DELGADO ALVES

ABSTRACT. ***Objective.*** To assess the role of nitrite (NO_2^-), nitrate (NO_3^-), and nitrative stress in thrombotic primary antiphospholipid syndrome (PAPS).

Methods. We investigated 46 patients with PAPS: 21 asymptomatic but persistent carriers of antiphospholipid antibodies (PCaPL), 38 patients with inherited thrombophilia (IT), 33 patients with systemic lupus erythematosus (SLE), and 29 healthy controls (CTR). IgG anticardiolipin (aCL), IgG anti-beta$_2$-glycoprotein I (anti-ß$_2$-GPI), IgG anti-high density lipoprotein (aHDL), IgG anti-apolipoprotein A-I (aApoA-I), crude nitrotyrosine (NT) (an indicator of nitrative stress), and high sensitivity C-reactive protein (CRP) were measured by immunoassays. Plasma nitrite (NO_2^-), nitrate (NO_3^-), and total antioxidant capacity (TAC) were measured by colorimetric spectroscopic assays.

Results. Average plasma NO_2^- was lower in PAPS, PCaPL, and IT ($p < 0.0001$); average NO_3^- was highest in SLE ($p < 0.0001$), whereas average NT was higher in PAPS and SLE ($p = 0.01$). In thrombotic PAPS, IgG aCL titer and number of vascular occlusions negatively predicted NO_2^- ($p = 0.03$ and $p = 0.001$, respectively), whereas arterial occlusions and smoking positively predicted NO_3^- ($p = 0.05$ and $p = 0.005$), and CRP positively predicted NT ($p = 0.004$). In the PCaPL group IgG aCL negatively predicted NO_3^- ($p = 0.03$). In the SLE group IgG aCL negatively predicted NO_2^- ($p = 0.03$) and NO_3^- ($p = 0.02$).

Conclusion. PAPS is characterized by decreased NO_2^- in relation to type and number of vascular occlusions and to aPL titers. Nitrative stress and low grade inflammation are linked phenomena in PAPS and may have implications for thrombosis and atherosclerosis. (J Rheumatol First Release Oct 1 2010; doi:10.3899/jrheum.100494)

Key Indexing Terms:
ANTIPHOSPHOLIPID SYNDROME THROMBOSIS
NITRIC OXIDE NITRITE NITRATE

The primary antiphospholipid syndrome (PAPS) is characterized by venous and arterial thromboses, recurrent miscarriages, and premature atherosclerosis in persistent carriers of antibodies against ß$_2$-glycoprotein I (anti-ß$_2$-GPI) and other coagulation proteins in the absence of any other underlying immune disorder[1,2]. From a biochemical standpoint PAPS is also characterized by an antioxidant/oxidant balance tilted towards the latter, partly due to decreased paraoxonase activity and enhanced oxidative stress[3,4]. Indeed, IgG anticardiolipin (aCL) antibody titers positively correlated to plasma levels of F2-isoprostanes, a marker of increased oxidative stress, and to decreased urinary excretion of nitric oxide (NO·) metabolites in PAPS[3]. NO• is the main endothelial vasodilator agent, and interference with NO• biology induces vascular dysfunction, particularly in the early phases of atherosclerosis[5]. After physiological stimulation of constitutive endothelial nitric oxide synthase (eNOS)[6] or inflammatory activation of inducible (iNOS) enzyme[7], NO• is released at higher rates and behaves as a pathogenic mediator or a cytotoxic molecule.

In the latter case, most NO• mediated pathogenicity depends on formation of secondary intermediates such as peroxynitrite anion ($ONOO^-$) and nitrogen dioxide ($•NO_2$), which are typically more reactive and toxic than NO• per se[8]. In the presence of oxidants such as superoxide radical ($O_2^{•-}$) NO· gives rise to $ONOO^-$, a strong 1-electron and

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2-electron oxidant with such a short biological half-life (10–20 ms) that it cannot be measured directly but must be inferred by indirect methods[9,10]. In fact $ONOO^-$ interacts with CO_2 to give nitrosoperoxycarbonate ($ONOOCO_2^-$) that will nitrate tyrosine residues in proteins[6]: measurement of nitrated proteins therefore represents a fingerprint of the interaction of $O_2^{\bullet -}$ with NO•[6,11].

Possible involvement of NO• in APS has been explored in animal studies[12,13] and in a few patient series whose numbers were too limited to provide a full understanding of its significance[14,15]. We therefore hypothesized that NO• might play a role in the vascular pathogenesis of PAPS, and compared the behavior of NO• metabolites nitrite (NO_2^-) and nitrate (NO_3^-), total antioxidant capacity (TAC) (expressed as $ONOO^-$ quenching), and nitrotyrosine (NT) in patients with thrombotic PAPS, in asymptomatic but persistent carriers of antiphospholipid antibodies (PCaPL), in patients with inherited thrombophilia (IT) with vascular occlusions, in patients with systemic lupus erythematosus (SLE), and in healthy subjects. Possible relationships between NO_2^-, NO_3^-, C-reactive protein (CRP), and several aPL were also investigated.

MATERIALS AND METHODS

Patients. Our study was devised as a cross-sectional case-quadruple control: PAPS patients with vascular occlusions represented cases; IT patients with vascular occlusions represented thrombotic controls PCaPL without vascular occlusions represented nonthrombotic aPL-positive controls; patients with SLE represented inflammatory controls; and healthy subjects represented normal controls. All participants were age- and sex-matched (where possible), except for SLE patients, who were all female. Consecutive patients with thrombotic PAPS, according to recent criteria[1], with IT and persistent aPL attending the Coagulation Unit of the Cardarelli Hospital (Naples, Italy) were invited to participate between January 2008 and July 2008. Our study was carried out according to the revised Declaration of Helsinki, with approval of the Ethics Board of the hospital and written consent of all participants. Exclusion criteria were acute or chronic hepatic, renal, and lung disease; diabetes; acute infection (within 6 weeks); post-thrombotic syndrome with or without venous ulcerations; positive urinary dipstick for nitrites on the day of sampling and treatment with statins or fibrates. PAPS and IT patients are seen on average every 3 to 4 weeks for oral anticoagulation monitoring and are instructed to self-report any illness during the intervening periods; their lipid profiles and kidney and liver function tests are checked annually. PCaPL subjects were diagnosed as such either because of the presence of prolonged clotting tests in routine assays, subsequently confirmed as lupus anticoagulants (LAC), or because of thrombocytopenia or other symptoms that prompted a search for aPL. Of the PAPS attendees (n = 50), 2 were excluded because they had gradually developed ankylosing spondylitis and SLE, one had developed kidney cancer, 2 were pregnant, one had suffered a recent recurrent event, one had post-thrombotic syndrome, and 2 were evasive regarding their smoking and contraceptive status. Of the IT (n = 46) attendees, 2 were excluded for post-thrombotic syndrome and venous ulcerations in lower limbs. Of the PCaPL attendees (n = 27) one was excluded for development of non-insulin-dependent diabetes; one for the development of SLE, hemolytic anemia, nephrotic syndrome, and pulmonary embolism after ovarian hyperstimulation; one for the development of chronic lymphoid leukemia; one for spontaneous onset of ischemic stroke; and 2 had moved to a different town. Of the remaining aPL subjects 4 had moderate thrombocytopenia (platelets < 100×10^9/l) not requiring treatment.

Consecutive patients with SLE fulfilling the American Rheumatism Association (ACR) criteria[16] were enrolled among those attending the Autoimmune Outpatient Clinic of the Curry Cabral Hospital, Lisbon (Portugal) between January 2008 and August 2008. Exclusion criteria included acute or chronic renal impairment that would significantly alter NO metabolites, liver cirrhosis, diabetes, acute infection (within 6 weeks), post-thrombotic syndrome with or without venous ulcerations, positive urinary culture following positive dipstick for nitrite (urinary excretion in SLE may be increased in the absence of infection), and treatment with statins or fibrates.

Of 52 patients with SLE, 16 were excluded on the basis of the above criteria. Of the remaining 36, one was found weeks later to have tuberculosis and 2 were pregnant; their samples were discarded. Therefore 33 SLE patients participated in the study: of these, 9% had visceral involvement without renal disease, 12% cardiac and lung involvement, 9% central nervous system involvement, 66% arthritis, 3% myositis, 9% alopecia, 3% hemolytic anemia, 21% thrombocytopenia, 9% neutropenia, 81% presence of anti-DNA antibodies, and 90% presence of antinuclear antibodies. The average SLE Disease Activity Index (SLEDAI) score was 4.85 ± 3.96 (median 3.5, range 0–16). Their medication intake was: prednisolone in 54%, (< 6 mg/day in 27%, 6–10 mg/day in 18%, > 10 mg/day in 9%) azathioprine in 24% (100 mg/day in 18%, 150 mg/day in 6%), hydroxychloroquine 200 mg/day in 60%, aspirin in 12%, warfarin in 9%. Twenty-nine healthy hospital staff served as normal controls: 15 from Cardarelli Hospital in Naples and 14 from the Curry Cabral Hospital in Lisbon. To minimize dietary influences on nitric oxide metabolite concentrations all participants were asked to refrain from foodstuffs containing high concentrations of nitrate/nitrite (such as lettuce, spinach, beetroot, radish, salamis, and pickled items) for 3 days before blood sampling, which was drawn between 8:00 and 10:00 AM. Blood samples were drawn by neat venepuncture into 5 ml citrate vacutainers, spun immediately at room temperature at 4000 rpm for 6 min; supernatant plasma was spun again at room temperature at 12,000 rpm for 4 min to obtain platelet-poor plasma: aliquots were frozen at –80°C and thawed on the day of testing. The study was therefore carried out on 46 thrombotic PAPS patients, 21 PCaPL subjects, 38 IT patients, 33 SLE patients, and 29 control subjects. Their demographics are shown in Table 1.

Determination of antiphospholipid antibodies. All participants had their aPL determined according to established criteria[17]; LAC screened by activated partial thromboplastin time (aPTT) and dilute Russell's viper venom time (DRVVT)[17]. A clotting time ratio between sample and control plasma > 1.2 for aPTT and > 1.18 for DRVVT indicated an abnormal result. After demonstrating the presence of an inhibitor using mixing studies, the platelet neutralization procedure confirmed the presence of a lupus inhibitor in aPTT and DRVVT. IgG aCL (Cambridge Life Sciences, Ely, UK) and IgG anti-$ß_2$-GPI (Corgenix, Broomfield, CO, USA) were measured by ELISA according to manufacturer's instructions. Since the inception of the PAPS cohort (1994), after initial diagnosis with repeat testing of aPL after 6 weeks, IgG aCL was measured yearly, whereas IgG anti-$ß_2$-GPI was measured yearly only since 2004.

Measurement of IgG anti-high density lipoprotein (aHDL) antibodies, IgG anti-apolipoprotein A-I (aApo A-I) antibodies, plasma nitrotyrosine, and high sensitivity C-reactive protein. aHDL and aApo A-I were measured by ELISA as described[4]; similarly ELISA was employed to measure nitrotyrosine (HyCult Biotechnology, Uden, The Netherlands) and high sensitivity CRP (Biosupply Ltd., Bradford, UK) according to the manufacturer's instructions. "CRP" stands for the high-sensitivity test throughout this article.

Measurement of plasma nitrate and nitrite. Nitric oxide metabolites nitrite (NO_2^-) and nitrate (NO_3^-) were determined using a modified Griess reaction, following the reduction of nitrate to nitrite using nitrate reductase and nicotinamide adenine dinucleotide phosphate (NADPH). Briefly, the assay was performed in a standard flat-bottomed 96-well microtiter plate half divided for simultaneous measurement of nitrite and nitrate concentration. To each well was added 50 μl/well of standard or diluted sample (1 in 4

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Table 1. Demographics of participants in study groups.

	PAPS, n = 46	PCaPL, n = 21	IT, n = 38	SLE, n = 33	CTR, n = 29
Age, yrs (mean ± SD)	43 ± 12	40 ± 9	41 ± 14	40 ± 12	41 ± 7
M/F	18/28	6/15	13/25	0/33	15/14
Disease duration, yrs, median (range)	10 (1–18)	10 (1–16)	12 (1–22)	8 (1–13)	
LAC	44	18	0	6	0
IgG aCL < 40 (GPL)	15	14	38	22	29
IgG aCL 41–80 (GPL)	7	3	0	8	0
IgG aCL > 80 (GPL)	24	4	0	3	0
IgG aCL, median (range)	100 (5–500)	24 (2–309)	8.4 (4–20)	62 (12–112)	8 (2–18)
IgG anti-ß$_2$-GPI U/ml, median (range)	146 (2.6–184)	4 (2–90)	5 (2–12)	12 (3.6–102)	4 (2.5–8.2)
IgG aHDL, %, median (range)	70 (25–436)	112 (28–240)	70 (22–292)	98 (25.4–328)	61 (19–161)
IgG aApoA-I, %, median (range)	0.69 (0.17–6.3)	1.2 (0.17–7.4)	0.14 (0.02–1.2)	1.2 (0.17–6)	0.18 (0.06–0.57)
MTHFR +/+	13	6	5	4	5
PT20210	2	1	4	0	2
FVL	2	0	12	1	1
F-PS deficiency	32	4	8	NA	0
FVL + PT20210	0	0	7	0	0
FVL + PS deficiency	0	0	2	0	0
Thrombosis					
Arterial	14	0	11	4	0
Venous	26	0	27	1	0
Arterial + venous	6	0	0	0	0
No. of occlusions					
1	19	0	38	4	0
2	16	0	0	0	0
3	3	0	0	0	0
4	2	0	0	0	0
Smokers					
< 6 per day	6	3	4	2	4
7–15 per day	5	2	8	5	6
> 15 per day	4	0	1	1	2

PAPS: primary antiphospholipid syndrome; PCaPL: persistent carriers of antiphospholipid antibody with no underlying disorder and no thrombosis; IT: inherited thrombophilia; SLE: systemic lupus erythematosus; CTR: normal controls; LAC: lupus anticoagulant; aCL: anticardiolipin; ß$_2$GPI: beta-2-glycoprotein-1; aHDL: anti-high density lipoprotein; aApoA-I: anti-apolipoprotein A-I; MTHFR: homozygous methylen-tetrahydrofolate reductase C667T thermolabile mutation; PT: heterozygous prothrombin; FVL: heterozygous factor V Leiden; F-PS: free protein S.

with phosphate buffer pH 7.4) in duplicate. The assay was blanked against phosphate buffer. In half plate, 4 μl of nitrate reductase (Sigma-Aldrich) and 10 μl of NADPH (Sigma-Aldrich) were added to each well, giving a final concentration of 6.3 U/l and 550 μmol/l, respectively. The plate was incubated at room temperature for 2 h. Griess reaction was initiated by addition to each well of equal volumes of 2% sulfanilamide (Sigma-Aldrich) in H_3PO_4 5% and 0.2% N-(1-naphthyl)-ethylenediamine dihydrochloride (Sigma-Aldrich) in water, mixed just before use. After 10 min incubation at room temperature the absorbance of the reaction mixture was measured at 540 nm and the levels expressed as μM.

Measurement of total antioxidant capacity of plasma. TAC of plasma was measured by peroxynitrite ($ONOO^-$) quenching: 100 μl of phosphate buffer (50 mM, pH 7.4) containing Pholasin® (1.7 μg/ml) was pipetted into a microcuvette. Plasma or buffer for control (5 μl) was added. The reaction was initiated by adding 3-morpholino-sydnonimine HCl (SIN-1; 2 μl of 2 mg/ml in water), and light emission was measured continuously at 5 min intervals until the maximum reading was obtained. Antioxidant capacity was expressed as the time at which maximum light was emitted. Lower values reflect decreased plasma TAC (peroxynitrite-related).

Statistical analysis. Variables were compared by ANOVA (post-hoc analysis) and ANCOVA with log transformation of variables that did not follow a normal distribution. The assumptions of univariate analysis within groups (not shown) were tested by multiple regression models. All statistical analyses were done using SPSS (SPSS, Chicago, IL, USA).

RESULTS

Comparison of variables in PAPS, PCaPL, IT, SLE, and healthy controls. Average plasma NO_2^- was lower in the PAPS, PCaPL, and IT groups (Figure 1A), whereas NO_3^- was higher in SLE (Figure 1B) and NT was higher in SLE and PAPS (Figure 1C). Mean plasma TAC was lowest in SLE (Figure 2A), where CRP was highest (Figure 2A and 2B). Average TAC was higher in males than in females in all non-SLE groups: in PAPS 11280 ± 3041 versus 9749 ± 2967 μmol/l ($p = 0.02$); in IT 6392 ± 1399 versus 5325 ± 1720 μmol/l ($p = 0.04$); and in healthy controls 7967 ± 991 vs 6194 ± 1265 μmol/l ($p = 0.001$).

Age, sex, and smoking correlated to NO_2^-, NO_3^-, and TAC but had no confounding effect on resulting significance findings by ANCOVA. Age and IgG aCL related to NT and their confounding effect by ANCOVA reduced the comparative significance ($p < 0.02$).

Relationship among variables in PAPS. The effect of antibodies and that of other clinical and laboratory variables on plasma concentrations of NO_2^-, NO_3^-, and NT was tested by

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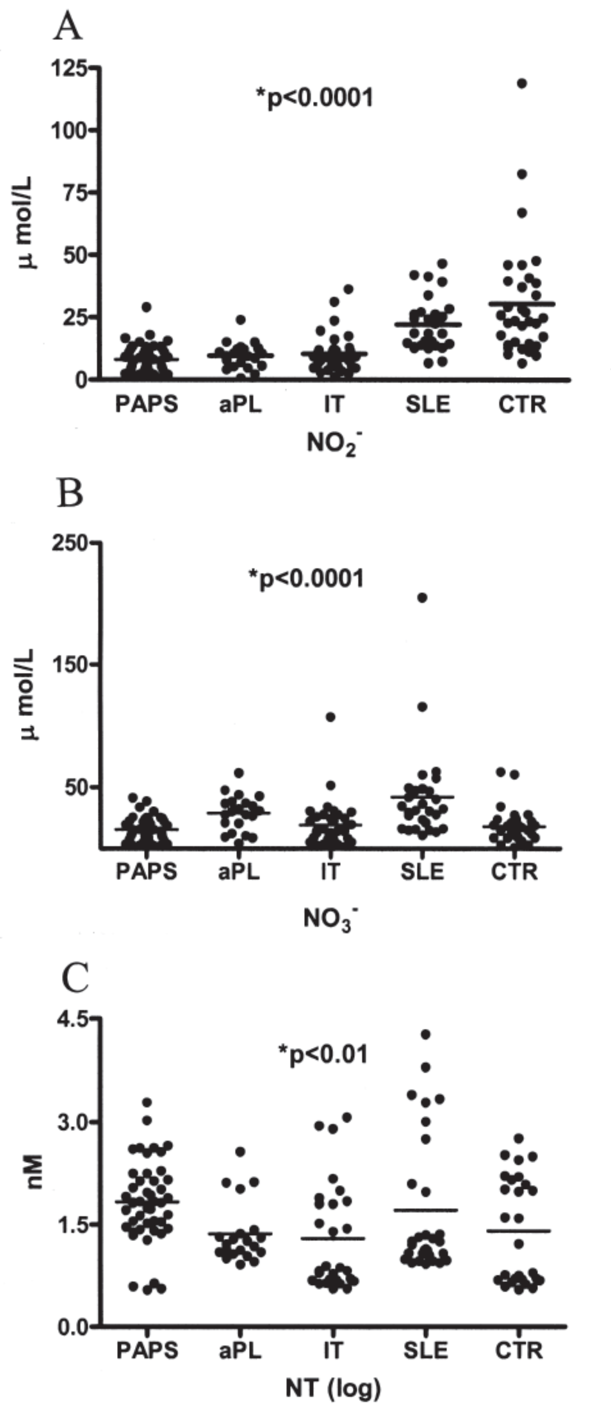

Figure 1. Comparison (ANOVA) of nitrite (NO_2^-), nitrate (NO_3^-), and nitrotyrosine (NT) across study groups: (A) average concentration of NO_2^- (Bonferroni's multiple comparison test: PAPS vs SLE, $p < 0.001$; PAPS vs CTR, $p < 0.001$; PCaPL vs SLE, $p < 0.01$; aPL vs CTR, $p < 0.001$; IT vs SLE, $p < 0.01$; IT vs CTR, $p < 0.001$); (B) average concentration of NO_3^- (Bonferroni's multiple comparison test: PAPS vs SLE, $p < 0.001$; IT vs SLE, $p < 0.001$; SLE vs CTR, $p < 0.001$); (C) average concentration of NT (Bonferroni's multiple comparison test: PAPS vs IT, $p < 0.05$). PAPS: primary antiphospholipid syndrome; PCaPL: persistent carriers of antiphospholipid antibodies without thrombosis or miscarriages; IT: inherited thrombophilia; SLE: systemic lupus erythematosus; CTR: controls.

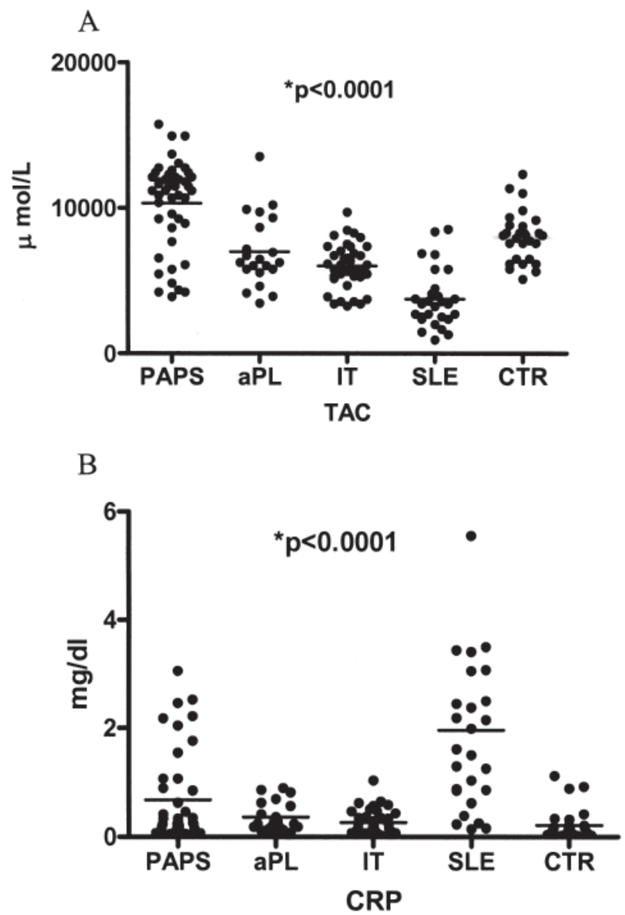

Figure 2. Comparison (ANOVA) of total antioxidant capacity (TAC) and high sensitivity C-reactive protein (CRP) across study groups: (A) average concentration of TAC (Bonferroni's multiple comparison test: PAPS vs IT, $p < 0.001$; PAPS vs SLE, $p < 0.001$; IT vs SLE, $p < 0.05$; IT vs CTR, $p < 0.05$; SLE vs CTR, $p < 0.001$); (B) average concentration of CRP (Bonferroni's multiple copmparison: SLE vs PAPS, SLE vs aPL, SLE vs IT, SLE vs CTR, all $p < 0.001$). PAPS: primary antiphospholipid syndrome; PCaPL: persistent carriers of antiphospholipid antibody positive without thrombosis or miscarriages; IT: inherited thrombophilia; SLE: systemic lupus erythematosus; CTR: controls.

separate multiple regression models. In the model with NO_2^- as the dependent variable and IgG aCL, IgG anti-ß$_2$-GPI, IgG aHDL, and IgG aApoA-I antibodies as independent variables, IgG aCL resulted in the only negative predictor of NO_2^- ($p = 0.03$; Table 2). Average NO_2^- was lower in patients with a history of arterial thrombosis versus those with venous thrombosis (11.41 ± 7.6 vs 18.43 ± 11.06 μmol/l; $p = 0.03$) although in a separate model with NO_2^- as the dependent variable and age at first thrombotic event and thrombosis number and type as the independent variables, thrombosis number negatively predicted NO_2^- ($p = 0.001$; Table 2).

In the model with NO_3^- as the dependent variable and IgG aCL, IgG anti-ß$_2$-GPI, IgG aHDL, and IgG aApoA-I antibodies as independent variables, IgG aCL was the only negative predictor of NO_3^- ($p = 0.03$) (Table 2). In a different model, with NO_3^- as the dependent variable and age,

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Table 2. Regression model predictors of nitric oxide metabolites and nitrotyrosine in primary antiphospholipid syndrome.

Independent Variables	Dependent Variables	Predictors	t	p
IgG aCL, IgG anti-ß$_2$-GPI, IgG aApoA-I	NO_2^-	IgG aCL	–1.87	0.03
Age at first thrombosis, thrombosis number, thrombosis type	NO_2^-	Thrombosis number	–3.24	0.001
IgG aCL, IgG anti-ß$_2$-GPI, IgG aHDL, IgG aApoA-I	No_3^-	IgG aCL	–1.93	0.03
Age, sex, thrombosis type, smoking, TAC	No_3^-	Arterial	1.67	0.05
		Smoking	2.66	0.005
NO_3^-, NO_2^-, TAC, smoking, CRP	NT	CRP	2.74	0.004

IgG aCL: anticardiolipin; IgG anti-ß$_2$-GPI: beta-2-glycoprotein-I; IgG aHDL, anti-high density lipoprotein; IgG aApoA-I, anti apolipoprotein A-I; NO_2^-: nitrite; NO_3^-: nitrate; TAC: total antioxidant capacity; NT: nitrotyrosine; CRP, C-reactive protein.

sex, thrombosis type, smoking, and TAC as the independent variables, arterial thrombosis and smoking independently predicted NO_3^- ($p = 0.05$ and $p = 0.005$, respectively).

In a further model with NT as the dependent variable and the antibodies as the independent variables none of the latter bore any relationship with NT; but in a similar model NT as the dependent variable and with NO_3^-, NO_2^-, TAC, smoking, and CRP as independent variables, CRP was the only independent predictor of NT ($p = 0.004$) (Table 2).

Relationship among variables in PcaPL. In the regression model with NO_2^- as the dependent variable and IgG aCL, IgG anti-ß$_2$-GPI, IgG aHDL, IgG aApoA-I, aPTT, and DRVVT as independent variables, IgG anti-ß$_2$-GPI showed only a negative trend with NO_2^- ($p = 0.07$) (Table 3).

In the model with NO_3^- as the dependent variable and IgG aCL, IgG anti-ß$_2$-GPI, IgG aHDL, IgG aApoA-I, aPTT, and DRVVT as independent variables, negative predictors were IgG aCL ($p = 0.03$) and DRVVT ($p = 0.03$), and a trend was seen for IgG anti-ß$_2$-GPI ($p = 0.06$; Table 3).

In a further model none of the antibodies bore any relationship with NT as the dependent variable but with NO_3^-, NO_2^-, TAC, and CRP set as independent variables, NO_2^- negatively predicted NT ($p = 0.05$; Table 3).

Relationship among variables in IT. In the regression model with NT as the dependent variable and NO_3^-, NO_2^-, TAC, smoking, and CRP as independent variables, only CRP independently predicted NT ($p = 0.0006$; Table 3).

Relationship among variables in SLE. In the regression model with NO_3^- as the dependent variable and IgG anti-ß$_2$-GPI, IgG aHDL, IgG aApoA-I, and IgG aCL as the independent variable, IgG aCL negatively predicted NO_3^- ($p = 0.03$); a similar result was obtained when NO_2^- was substituted for NO_3^- ($p = 0.02$; Table 3). In the model with NT as the dependent variable and NO_3^-, NO_2^-, TAC, smoking, and CRP as independent variables, NO_2^- negatively predicted NT ($p = 0.002$) and NO_3^- positively predicted NT ($p = 0.001$; Table 3). Finally, in the model with SLEDAI as the dependent variable and NO_3^-, NO_2^-, TAC, CRP, smoking,

Table 3. Regression model predictors of nitric oxide metabolites and nitrotyrosine in non-primary antiphospholipid antibody syndrome groups.

Independent Variables	Dependent Variables	Predictors	t	p
Persistent carriers of antiphospholipid antibodies				
IgG aCL, IgG anti-ß-GPI, IgG aHDL, IgG aApoA-I, aPTT, DRVVT	NO_3^-	IgG aCL	–2.06	0.03
		DRVVT	–1.93	0.03
		IgG aß$_2$GPI	–1.68	0.06
NO_3^-, NO_2^-, TAC, smoking, CRP	NT	NO_2^-	–1.74	0.05
Inherited thrombophilia				
NO_3^-, NO_2^-, TAC, smoking, CRP	NT	CRP	3.75	0.0006
Systemic lupus erythematosus				
IgG aCL, IgG anti-ß$_2$-GPI, IgG aHDL, IgG aApoA-I	NO_2^-	IgG aCL	–2.12	0.02
IgG aCL, IgG anti-ß$_2$-GPI, IgG aHDL, IgG aApoA-I	NO_3^-	IgG aCL	–1.84	0.03
NO_3^-, NO_2^-, TAC, smoking, CRP	NT	NO_2^-	–3.25	0.002
		NO_3^-	3.65	0.001
NO_3^-, NO_2^-, TAC, CRP, smoking, NT	SLEDAI	NT	2.55	0.009
		CRP	1.44	0.08
Normal controls				
Age, sex, TAC, CRP, smoking, NT	NO_3^-	Smoking	2.90	0.003
Age, sex, NO_3^-, NO_2^-, TAC, CRP, smoking	NT	Smoking	2.21	0.02
		NO_3^-	1.75	0.04

IgG aCL: anticardiolipin; IgG anti-ß$_2$-GPI: beta-2-glycoprotein-I; IgG aHDL, anti-high density lipoprotein; IgG aApoA-I, anti apolipoprotein A-I; aPTT: activated partial thromboplastin time; DRVVT: dilute Russell viper venom time; NO_2^-: nitrite; NO_3^-: nitrate; TAC: total antioxidant capacity; NT: nitrotyrosine; CRP, C-reactive protein; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index..

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and NT as the independent variables, NT predicted SLEDAI ($p = 0.009$) and a trend was seen for CRP ($p = 0.08$; Table 3).

Relationship among variables in the control group. No effect on NO_2^- was seen in a multiple regression model with NO_2^- as the dependent variable and age, sex, smoking, IgG aHDL, and IgG aApo-I as explanatory variables. In a similar model where NO_3^- was set as the dependent variable, smoking independently predicted NO_3^- ($p = 0.003$; Table 3). Similarly, when NT was set as a the dependent variable with age, sex, smoking, TAC, NO_2^-, and NO_3^- as explanatory variables, smoking independently predicted NT ($p = 0.02$) alongside NO_3^- ($p = 0.04$; Table 3).

DISCUSSION

NO• is synthesized in the vasculature by 2 related nitric oxide synthases (NOS), constitutive eNOS and inducible NOS; both convert L-arginine to NO• and citrulline at different concentrations according to substrate availability[18]. The role of NO• in PAPS is unknown: one study found lower urinary NO_2^- in a small number of patients with PAPS in negative correlation with IgG aCL titer[3]. Of the NO• metabolites, it is widely accepted that in humans, only NO_2^- reflects changes in eNOS activity[19,20] and endothelial dysfunction[21] known to be impaired in APS[22].

To evaluate the clinical significance of NO_2^- and NO_3^- with regard to thrombosis in PAPS we employed as comparator patients with IT who had vascular occlusions, patients with PCaPL who had no vascular occlusions, patients with SLE as an inflammatory disease control group, and healthy subjects. The low average concentration of NO_2^- found in PAPS and IT suggests that reduced NO_2^- may be involved in the vascular events of these patients, although causality cannot be established since vessel occlusion might have led to reduced NO_2^-: in fact, the number of vascular occlusions was a negative independent predictor of NO_2^- in PAPS. Nevertheless NO_2^- was also low in the PCaPL group who never had vessel occlusions, suggesting that impaired NO_2^- generation may precede and hence represent a predisposing factor for thrombosis.

Reduced NO• has a wider importance in the vascular biology of PAPS. NO• maintains vascular homeostasis against the vasopressor effects of endothelin-1, of isoprostanes derived from lipid peroxidation, and of thromboxane generated after platelet activation (as reviewed[23]). Indeed, elevated plasma and/or urinary levels of the aforementioned molecules have all been described in PAPS[15,24,25]: hence loss of the antiplatelet effect of NO• may be relevant to thrombosis[26], whereas loss of its vasodilator effect may be relevant both to thrombosis and to atherosclerosis, as recently confirmed in PAPS[2]. In the latter study, carried out on almost the same cohort of PAPS patients, diastolic blood pressure in patients with arterial thrombosis was higher than in patients with venous thrombosis[2].

Of further interest, aPL negatively predicted NO_3^- and/or NO_2^- in the PAPS, PCaPL, and SLE groups, adding further to the pathogenic potential of aPL. We demonstrated that monoclonal IgG aCL was associated with decreased concentrations of NO• metabolites in a mouse model[12]. Inducible NOS may generate a 1000-fold higher concentration of NO• than eNOS, which is associated with vascular damage and cytotoxic effects, whereas NO• is generated by eNOS for short periods of time to maintain vascular homeostasis[27]. Likely only the latter pathway is impaired in PAPS, whereas the former pathway may be more active in SLE, the group that showed a greater concentration of NO_3^-, although a large difference was not seen because our patients with SLE were mostly clinic attendees devoid of acute or chronic renal disease with low disease activity, hence inflammatory activity. Notwithstanding, our data in SLE would be consistent with possible iNOS activation, cytotoxic release of NO• ultimately leading to tyrosine nitration due to the inflammatory nature of the disease[27].

Among the antibodies that might have had an influence on NO• we included IgG aHDL and IgG aApoA-I because in previous work we demonstrated that their average plasma concentrations were elevated in SLE and PAPS, where they adversely affected the antioxidant system associated with HDL, favoring oxidation[4,28]. In our present study, they failed to show any relation with NO• metabolites, indirectly confirming their specificity in blunting the antiatherogenic and antiinflammatory effects of HDL[4].

To investigate nitrative stress in PAPS we measured crude plasma NT: this was higher in the SLE group, where NT related to disease activity and was predicted by NO_3^-, in keeping with findings from other inflammatory rheumatic disorders[29]. On the other hand, having demonstrated that low grade inflammation characterizes PAPS[30], we found that CRP was an independent predictor of NT in the PAPS group, suggesting that nitrative stress and low grade inflammation may be related phenomena in these thrombotic patients. Interestingly, smoking predicted NO_3^- in PAPS, and it is known that active[31] and passive smoking[32] may induce oxidative stress.

Our study has several limitations: (1) its retrospective design prevented a full appreciation of the role of NO• in thrombosis as most PAPS patients were diagnosed after vascular occlusion; (2) our SLE group comprised female patients of whom only 5 had a history of thrombosis; however, we had opted for inclusion of the SLE group mostly to show the inflammatory behavior of NO• rather than to control for thrombosis, which was provided for by the IT group; (3) the method we employed for the measurement of NO• metabolites is not sensitive enough to detect nanomolar concentrations of NO_2^- and NO_3^- [33], and we did not evaluate eNOS and/or iNOS gene polymorphisms that may have accounted for differences in measured metabolites[34,35], although our groups would have been too small to yield significant data.

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In conclusion, our study, alongside our previous animal data[12], indicates a possible impairment of the vascular biology of NO• in PAPS, the consequences of which may be thrombosis and atherosclerosis. With regard to the former, we cannot define whether decreased NO_2^- is a cause or an effect of previous thromboses, but the low NO_2^- in PCaPL without vessel occlusions and the relationship between NO• metabolites and aPL in the PAPS, PCaPL, and SLE groups indicate that aPL may negatively influence some physiological activities of NO•. With regard to the latter, patients with PAPS exhibit a certain degree of nitrative stress that relates to low grade inflammation, also noted in other settings[36]: given the finding of NT in vessels of atherosclerotic patients[37], this aspect needs to be further explored in PAPS. From a practical point of view, our study provides evidence that smoking should be avoided in patients with PAPS.

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Ames PRJ, Batuca JR, Ciampa A, Iannaccone L, Delgado Alves J. Clinical relevance of nitric oxide metabolites and nitrative stress in thrombotic primary antiphospholipid syndrome. J Rheumatol 2010;37:2523-30. Table 2, under the heading “Predictors”: “Arterial” should be “Arterial thrombosis”. We regret the error.
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