Impaired platelet activation in patients with hereditary deficiency of p47^{phox}

Chronic granulomatous disease (CGD) is a rare primary immunodeficiency affecting the innate immunological system, which is characterized by impaired reactive oxidant species (ROS) generation and, eventually, defective bacteria killing (van den Berg *et al*, 2009). NADPH oxidase (Nox)2 (also termed CYBB) hereditary deficiency (X-linked CGD) is the most frequent form of CGD and is complicated by life-threatening infections (Martire *et al*, 2008); conversely, hereditary deficiency of the p47^{phox} subunit (also termed NCF1) is less frequent and characterized by partial ROS generation impairment and milder infectious disease (Kuhns *et al*, 2010).

Experimental and clinical studies demonstrated that Nox2 is expressed in platelets and exerts pro-thrombotic effects via isoprostane formation and/or nitric oxide (NO) generation inhibition (Pignatelli *et al*, 2011; Carnevale *et al*, 2014a). So far, no data have been reported on platelet activation behaviour in patients with p47^{phox} hereditary deficiency. Analysis of platelet behaviour in this setting might be useful to assess if, as compared to Nox2 deficiency, a less marked impairment of oxidative stress, as detected in patients with hereditary deficiency of p47^{phox}, is also associated with lowered platelet activation.

This study was performed in collaboration with the Italian Primary Immuno-deficiencies Network. Two researchers visited to each centre at different times to take blood. Fifteen CDG patients (10 X-CGD and 5 with p47^{phox} hereditary deficiency), and 10 healthy subjects (HS), matched for age and sex, were recruited. CGD was diagnosed as previously described (Martire *et al*, 2008). CGD patients were excluded in case of acute infection, critical physical condition or unwillingness to participate in the study. All patients with CGD were under treatment with itraconazole, trimethoprim and sulfamethoxazole. HS were screened at routine visits. Subjects were excluded from the study if they had liver insufficiency, serious renal disorder (serum creatinine >247·5 μmol/l), cancer, myocardial infarction, unstable angina, acute cerebrovascular disease, deep venous thrombosis or were being treated with statins, antioxidant, vitamins or antiplatelet drugs or if they were current smokers. The study was conformed to the declaration of Helsinki and approved by the Ethical Committee of Sapienza University of Rome.

For each patient and control fasting period of at least 12 h, platelet poor plasma and platelet rich plasma (PRP) were prepared as previously described (Carnevale *et al*, 2014b). Plasma levels of glycoprotein IIb/IIIa (GPIIb/IIIa, also known as integrin $\alpha_{IIb}\beta_3$, ITGA2B) and soluble CD40 ligand (sCD40L) were measured with use of a commercial immunoassay (Abcam, Cambridge, UK; DRG International, Marburg, Germany). Platelet production of NO and H_2O_2 by collagen (4 μg/ml)-stimulated PRP was measured by colourimetric assay (Arbor Assays, Ann Arbor, MI, USA).

Distribution of variables was assessed by Kolmogorov–Smirnov test. Categorical variables are reported as number and percentage, continuous variables as means ± standard deviation unless otherwise indicated. Independence of categorical variables was tested by chi-square test. Comparisons between the three groups (i.e. X-CGD patients, p47^{phox}-deficient patients and HS) were analysed by Mann–Whitney test. A value of $P < 0·05$ was considered statistically significant. All analyses were performed with SPSS V.18.0 (SPSS Statistics v. 18.0, SPSS Inc., Chicago, IL, USA).

As previously reported, there were no differences in terms of age, gender and risk factors of atherosclerosis between CGD and HS. None of the CGD patient had a clinical history of bleeding. Table I reports the distribution of antibiotic treatment between X-CGD and p47^{phox}.

Compared to HS, X-CGD patients had lower platelet activation as shown by reduced plasma levels of GPIIb/IIIa and sCD40L (Fig 1A–B). Compared to patients with p47^{phox} deficiency, X-CGD patients had lower levels of GPIIb/IIIa and sCD40L (Fig 1A–B). In the overall population, bivariate analysis showed a significant correlation between sCD40L and GPIIb/IIIa levels (Rs 0·694, $P < 0·0001$).

Table I. Subject characteristics.

	X-CGD ($n = 10$)	HS ($n = 10$)	p47^{phox} deficiency ($n = 5$)
Age, years	17 ± 5	17 ± 5	17 ± 5
Male, *n*	0	1	1
Treatment, *n*	Itraconazole, trimethoprim and sulfamethoxazole, 10	–	Itraconazole, trimethoprim and sulfamethoxazole, 5

X-CGD, X-linked chronic granulomatous disease; HS, healthy subjects.

doi: 10.1111/bjh.14347

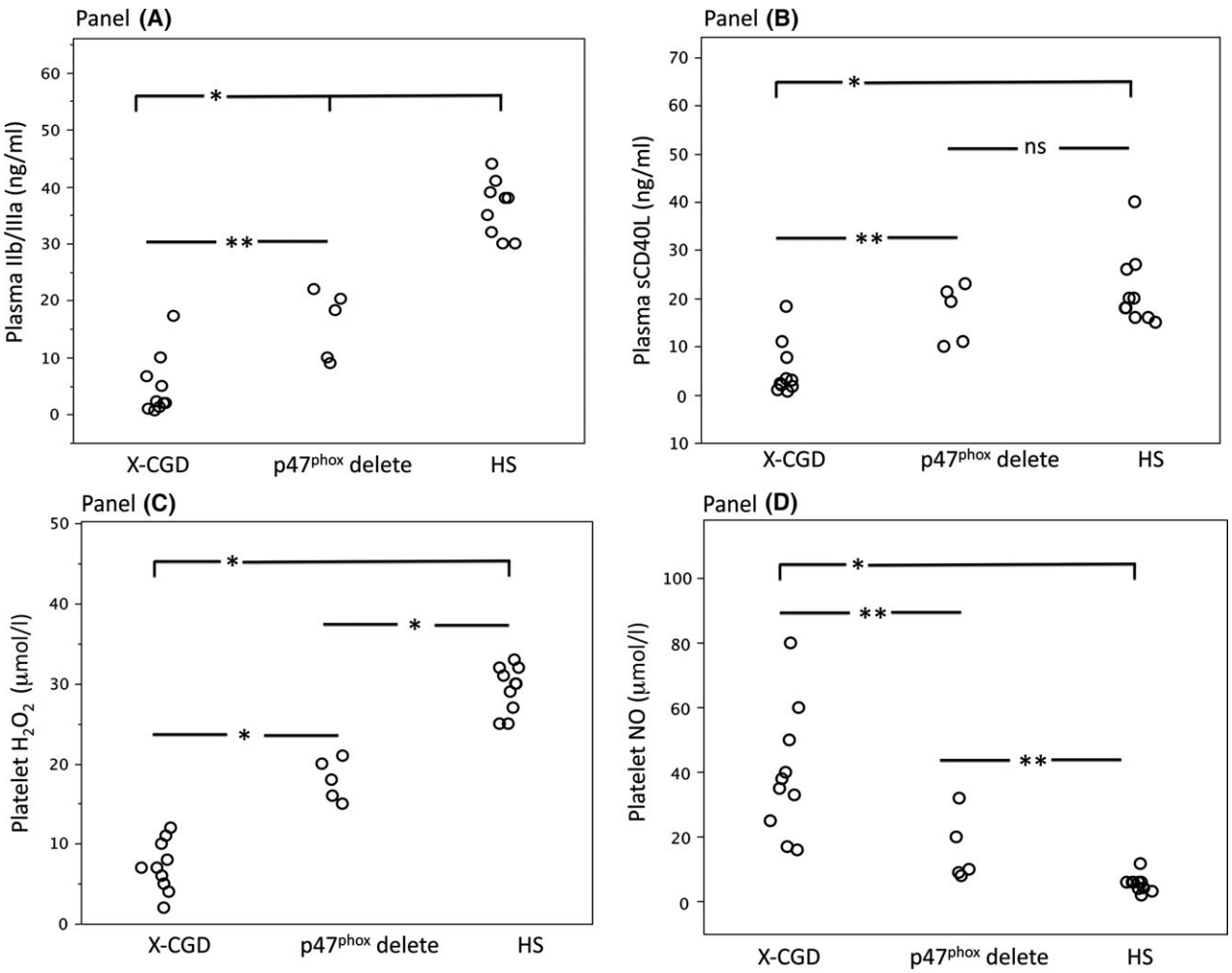

Fig 1. Platelet activation. Plasma levels of glycoprotein IIb/IIIa (IIb/IIIa) (A) and soluble CD40 ligand (sCD40L) (B) in healthy subjects (HS, $n = 10$), X-linked chronic granulomatous disease (X-CGD) ($n = 10$) and p47^{phox} deficiency ($n = 5$). Platelet H_2O_2 production (C) and NO bioavailability (D) levels in HS ($n = 10$), X-CGD ($n = 10$) and p47^{phox} deficiency ($n = 5$). *$P < 0{\cdot}005$, **$P < 0{\cdot}05$.

Ex-vivo studies showed that, compared to HS, CGD patients had lower platelet H_2O_2 production and higher NO bioavailability (Fig 1C–D). Compared to patients with p47^{phox} deficiency, X-CGD patients had significantly lower platelet H_2O_2 and higher platelet NO (Fig 1C–D).

The study provides the first evidence that CGD patients with hereditary deficiency of p47^{phox} display reduced platelet activation *in vivo* and *ex vivo*, further reinforcing the pivotal role of ROS in platelet activation.

This study compared two CGD groups with marked (Nox2 deficiency) and less marked (p47^{phox} deficiency) ROS formation. Both groups showed reduced sCD40L and GPIIb/IIIa circulating levels compared to controls but impairment of platelet activation was greater in patients with hereditary Nox2 deficiency. In accordance with our hypothesis, this behaviour may be attributed to the different impaired platelet production of oxidant species, such as H_2O_2, which, in fact, was more seriously compromised in X-CGD compared to hereditary p47^{phox} deficiency. In this regard, it is noteworthy that H_2O_2 serves to activate platelets via intracellular calcium mobilization and, in turn, thromboxane A_2-dependent and -independent mechanisms (Pignatelli *et al*, 2011). Experiments in animals over-expressing glutathione peroxidase 1, which degrades H_2O_2, further supported the role for this oxidant species in eliciting platelet-related thrombosis (Dayal *et al*, 2013). Enhanced NO generation may be another mechanism accounting for platelet inhibition in both CGD groups; thus, impaired superoxide production results in lower inhibition of biosynthesis and/or activity of NO, which is a powerful antiplatelet molecule (Carnevale *et al*, 2014a).

The study has limitation and potentially clinical implication. The small sample size of p47^{phox} deficient group is a limitation; however, recruitment of these patients is difficult as the prevalence of p47^{phox} hereditary deficiency is more rare than X-CGD prevalence (1:250 000) (Winkelstein *et al*, 2000). Even if experimental studies suggest Nox2 as a novel target for antiplatelet treatment, its inhibition should be

carefully considered for the potentially negative effects in innate immune system and bacterial killing (Delaney *et al*, 2016). Inhibition of $p47^{phox}$, which contributes to Nox2 activation along with the other cytosolic subunits, may be an interesting alternative as indicated by a less negative impact on the innate immune system by $p47^{phox}$ deficiency (Loffredo *et al*, 2013). Furthermore, pharmacological intervention with apocynin, which inhibits $p47^{phox}$ assembly to Nox2, demonstrated an antiplatelet effect in experimental atherosclerosis. Of note, none of the patients included in the study had a clinical history of bleeding.

In conclusion, patients with hereditary $p47^{phox}$ deficiency show reduced platelet activation suggesting a role for this Nox cytosolic subunit in platelet activation.

Authorship contributions

R.C., L.L. and F.V. designed the research, wrote the paper, analyzed the results and made the figures; C.N. and S.B. performed experiments; V.S., A.S. C.A., B.M. and C.P. recruited patients; A.P. contributed to discussion and interpretation of the data; all authors read and approved the final manuscript.

Conflict of interest disclosures

The authors declare no competing financial interests.

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Keywords: chronic granulomatous disease, platelet activation, $p47^{phox}$, Nox2, oxidative stress

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