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HIV-1 induces in vivo platelet activation by enhancing platelet NOX2 activity

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KEYWORDS
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Summary *Objectives:* HIV-1 patients show increased platelet activation, but the mechanisms involved are not completely clarified. We speculated that HIV-1 might induce in vivo platelet activation by enhancing platelet NOX2-related oxidative stress.
Methods: We measured soluble CD40 Ligand (sCD40L), a systemic marker of platelet activation, in 36 HIV-1 patients under effective combined antiretroviral therapy (cART) and in 10 naïve HIV-1 subjects. As control, 20 healthy subjects (HS) were included. Platelet oxidative stress was measured by platelet NOX2-derived peptide (sNOX2-dp), $p47^{phox}$ translocation to platelet membrane and platelet prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$).
Results: sCD40L was increased both in HIV-1 naïve and cART patients compared to HS ($p < 0.001$). Platelet sNOX2-dp and 8-iso-$PGF_{2\alpha}$ were significantly higher in HIV-1 naïve subjects compared to those on cART and to HS, and both were mutually correlated ($R = 0.734$, $p < 0.001$). A stepwise multivariable linear regression analysis showed that platelet sNOX2-dp (β: 0.803, $p < 0.001$), HIV-1 infection (β: 0.146, $p = 0.014$) and age (β: 0.166, $p = 0.001$) were independently associated to sCD40L levels.
Conclusions: HIV-1 infection is associated with increased platelet oxidative stress, which was

Abbreviations: AIDS, Acquired Immunodeficiency Syndrome; cART, combined antiretroviral therapy; HIV-1, Human Immunodeficiency Virus type 1; HS, healthy subjects; NADPH, nicotinamide adenine dinucleotide phosphate oxidase; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleos(t)ide reverse transcriptase inhibitor; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PI, protease inhibitor; ROS, reactive oxygen species; sCD40L, soluble CD40 Ligand; sNox2-dp, soluble Nox2-derived peptide.

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related to the activation of NOX2. The independent association between platelet NOX2 activation and plasma levels of sCD40L suggest that in vivo platelet activation may be dependent upon platelet oxidative stress.

Introduction

The use of effective combined antiretroviral therapy (cART) have progressively lengthened the survival of patients with Human Immunodeficiency Virus type 1 (HIV-1) infection and dramatically reduced the disease progression to full-blown Acquired Immunodeficiency Syndrome (AIDS).[1] Prolonged life expectancy revealed a previously unrecognized risk of developing cardiovascular (CV) diseases in HIV-1 patients, with an accelerated atherosclerosis,[2,3] increased coronary heart disease and atherosclerotic plaque vulnerability.[4,5] Ischemic complications may include not only coronary, but also cerebrovascular disease, affecting intracranial vessels and leading to progressive neurological and cognitive impairment.[6,7]

Platelet activation play a crucial role on the onset of atherosclerotic complications,[8] and recent evidences suggest that patients with HIV-1 infection disclose an enhanced platelet activation.[9]

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a key enzyme involved in reactive oxygen species (ROS) production.[10] NADPH oxidase, which was well defined in phagocytic cells, is composed of plasma membrane-bound subunits (gp91^{phox}/NOX2 and p22^{phox}) and several cytosolic subunits.[11] Assembly of the cytosolic subunits with NOX2 leads to enzyme activation and ROS production. Upon activation, NOX2 releases a soluble peptide (sNOX2-dp), which is a direct expression of the enzyme activation.[12]

NOX2 was identified in platelets[13] and its activation was recognized to be a fundamental step in the formation of platelet prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) in response to cell agonists, arachidonic acid in particular.[14,15] NOX2 activation is also a critical step for the release of prothrombotic molecules such as soluble CD40L (sCD40L),[10,13] indicating that NOX2 may be implicated on in vivo platelet activation. Indeed, sCD40L has been proposed as a reliable marker of platelet activation.[16]

Therefore, we speculated that NOX2 activity is upregulated in platelets from HIV-1 patients, so contributing to in vivo platelet activation.

Thus, aim of our study is to investigate the behavior of platelet activation and its interaction with platelet NOX2 in HIV-patients.

Materials and methods

Patients

Forty-six HIV-1 infected patients were enrolled. Subjects were followed in an outpatient basis at the Department of Clinical Medicine, "Sapienza" University of Rome. Thirty-six patients were included according to the following criteria: diagnosis of HIV-1 infection at any stage; effective cART with a viral load <37 copies/mL from at least 12 months; age >18 years; absence of severe obesity, arterial hypertension, diabetes mellitus, and dyslipidemia. Patients with an active opportunistic infection were excluded.

HIV-1 patients were treated with cART according to current guidelines.[17] Medication compliance was assessed with regular drug refill.

Another group of 10 naïve HIV-1 patients with similar demographic and clinical characteristics was enrolled. As control, 20 healthy subjects (HS) from the same geographic area matched for sex and age were included.

The study was approved by local ethic committee and conducted according to the principles of the Declaration of Helsinki.[18] All enrolled subjects gave their written informed consent.

Platelet preparation

Blood samples were obtained after a 12-h fast between 8.00 and 9.00 a.m. from subjects underwent routine biochemical analysis including total cholesterol and glucose. Samples, obtained from healthy subjects after supine rest for at least 10 min, were taken into tubes with 3.8% sodium citrate. To acquire platelet-rich plasma (PRP), samples were centrifuged for 15 min at 180 g. In order to avoid leukocyte contamination, only the top 75% of the PRP was collected according to Pignatelli et al.[12] Platelet pellets were obtained by double centrifugation (5 min, 300 g) of PRPs. Acid/citrate/dextrose (ACD) (1:7 v/v) was added to avoid platelet activation during processing. Samples were suspended in HEPES buffer in presence of 0.1% albumin, pH 7.35. Platelet concentration in samples used for *in vitro* experiments was adjusted at 2×10^5 platelets/mL. Cells were activated with Arachidonic Acid (0.5 mM) for 10 min at 37 °C, separated from the supernatant by centrifugation (5 min, 300 g) and the two fractions, cells and supernatants, were stored at −80 °C until analysis. All materials used were from Sigma Aldrich unless otherwise specified.

Plasma soluble CD40L

Plasma levels of soluble CD40L (sCD40L) were measured with a commercial immunoassay (Tema Ricerca, Bologna, Italy). Values were expressed as ng/ml/2×10^5platelets. Intra-assay and inter-assay coefficients of variation were 5% and 7% respectively. The detection limits is 0.16 ng/ml.

Analysis of platelet sNOX2-dp

Extracellular levels of soluble NOX2-derived peptide (sNOX2-dp), were detected by an ELISA method as previously described by Pignatelli et al.[12] in the supernatant of

platelet suspension. The peptide was recognized by the specific monoclonal antibody against the amino acidic sequence of the extra membrane portion of NOX2 (catalytic core of NADPH oxidase), which was released in the medium upon platelet activation with AA.[12] Values were expressed as pg/ml/2 × 10^5platelets; intra-assay and inter-assay coefficients of variation were 5.2% and 6%, respectively.

Platelet isoprostanes

Prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) was measured by a previously described and validated enzyme immunoassay method[19,20] in the supernatant of platelet suspension. 8-iso-$PGF_{2\alpha}$ values were expressed pmol/L/2 × 10^5platelets. Intra-assay and inter-assay coefficients of variation were 4.4% and 8.8%, respectively.

Membrane and cytoplasmic protein extraction

Briefly, the extraction of membrane and cytoplasmic proteins was performed by using the Proteo JETTM Membrane Protein Extraction Kit (Fermentas International Inc, Maryland, USA) as reported by Fortuño et al.[21]

Western blot analysis of $p47^{phox}$

Platelets from patients who were evaluated for $p47^{phox}$ translocation were analyzed immediately after the blood sample collection. Platelets were suspended in a 2× Lysis buffer (5 mM EDTA, 0.15 mol NaCl, 0.1 mol Tris pH 8.0, 1% triton and protease inhibitor cocktail). Equal amounts of protein (30 μg/lane) estimated by Bradford assay were solubilized in a 2× Laemmli buffer containing 2-mercaptoethanol and loaded in a denaturing SDS/10% polyacrylamide gel. Western blot analysis was performed with monoclonal anti-$p47^{phox}$ (Santa Cruz Biotechnology, Dallas, USA) (1 μg/ml) incubated overnight at 4 °C. Immune complexes were detected by enhanced chemiluminescence. The developed spots were calculated by densitometric analysis on a NIHimage 1.62f analyzer and the values were expressed in arbitrary unit (AU).

In particular, we removed from the densitometric analysis the aspecific spot, and the values were expressed as the ratio between the absolute values of the membranes spots divided to absolute values of the corresponding cytosolic spots (Densitometric Analysis of Membrane Spots − Densitometric Analysis of Aspecific Spots/Densitometric Analysis of Cytosol Spots − Densitometric Analysis of Aspecific Spots).

Statistical analysis

Categorical variables were reported as counts (percentages) and continuous variables as means ± standard deviation (SD) or median and interquartile range (IQR) unless otherwise indicated. Independence of categorical variables was tested by χ^2 test. Normal distribution of parameters was assessed by Kolmogorov–Smirnov test.

Student unpaired t test and Pearson product–moment correlation analysis were used for normally distributed continuous variables. Group comparisons were performed using analysis of variance (ANOVA). Appropriate nonparametric tests (Mann–Whitney U test, Kruskal–Wallis test, Spearman rank correlation test) were employed for all the other variables. Stepwise multivariable linear regression analysis was used to assess factors influencing sCD40L. Only p values lower than 0.05 were considered as statistically significant. All tests were two-tailed and analyses were performed using computer software packages (SPSS-18.0, SPSS Inc.).

Sample size

On the basis of the results from a previous work on healthy subjects,[22] we calculated that a minimum of 10 patients per group were required to have a 90% chance of detecting, as significant at the 5% level, a difference for platelet NOX2 between groups of 6 pg/ml with SD = 4 pg/ml.

Results

Clinical and biochemical characteristics

Table 1 reports clinical characteristics of healthy subjects (HS), HIV-1 naïve subjects, and HIV-1 subjects on cART. No differences on age, systolic and diastolic blood pressure, BMI, fasting blood glucose, total cholesterol, platelet count and smoking were present among groups. None of patients or controls was receiving a treatment with antiplatelet drugs or statins at baseline.

Antiretroviral agents

HIV-1 patients on cART were receiving non-nucleoside reverse transcriptase inhibitor (NNRTI) − or protease inhibitor (PI)-based regimens (23 and 13 patients, respectively), both in association with a nucleos(t)ide reverse transcriptase inhibitor (NRTI) fixed-dose combination (lamivudine/zidovudine or emtricitabine/tenofovir difumarate). Median length of cART was 136.2 [84.4–204.1] months, and nadir of CD4+ T cells was 269.9 ± 131.3 cells/μl. There was no difference in the length of cART between the group with NNRTI-based and PI-based cART (124.8 [91.6–183.3] vs. 164.0 [28.4–214.9] months, p = 0.829). HIV-1 patients receiving cART had higher values of CD4+ T lymphocytes compared to HIV-1 naïve subjects (CD4% 34.0 ± 11.9 vs. 23.5 ± 6.3, p = 0.011; CD4+ T absolute numbers 786.9 ± 354.6 vs. 507.8 ± 390.9 cells/μl, p = 0.037). Viral load was undetectable in all HIV-1 cART treated patients, whereas among HIV-1 naïve subjects, median value of viral load was 1711.0 copies/ml [IQR 129.2–8191.5].

Platelet activation and oxidative stress

Platelet activation

After stimulation with AA, HIV-1 subjects showed increased values of sCD40L (21.1 ± 5.8 vs. 12.6 ± 3.3 ng/ml, p < 0.001) compared to HS (Fig. 1). When we analyzed separately HIV-1 naïve subjects and HIV-1 subjects on cART, we found an increased level of sCD40L in naïve HIV-1 infected group (25.9 ± 4.12 ng/ml), compared to the cART one (19.7 ± 5.57 ng/ml, p = 0.002) (Fig. 1).

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Table 1 Demographic characteristics of seronegative and seropositive subjects.

	HIV-1 negative (n = 20)	HIV-1 cART group (n = 36)	HIV-1 naïve subjects (n = 10)	p value[a]
Age (years)	37.4 ± 12.2	42.6 ± 7.0	43.7 ± 8.6	0.091
Female (n/%)	12 (60.0%)	11 (30.6%)	5 (50.0%)	0.089
High (cm)	169.4 ± 10.8	172.9 ± 8.3	168.8 ± 7.1	0.263
Weight (kg)	64.6 ± 9.9	68.9 ± 10.5	63.8 ± 11.5	0.215
Body Mass Index (kg/m^2)	22.6 ± 3.2	23.1 ± 2.5	22.2 ± 3.0	0.659
Fasting blood glucose (mg/dl)	86.7 ± 9.0	83.3 ± 9.2	81.9 ± 7.7	0.656
Total cholesterol (mg/dl)	185.1 ± 17.5	188.1 ± 51.3	160.7 ± 44.8	0.133
Platelet count (×10^3/μl)	201.7 ± 17.6	189.8 ± 43.1	191.8 ± 12.9	0.447
Smoking (%)	45.0	51.4	60.0	0.736
Systolic blood pressure (mmHg)	111.4 ± 9.7	107.9 ± 7.0	107.5 ± 5.4	0.232
Diastolic blood pressure (mmHg)	66.4 ± 6.3	68.3 ± 7.3	71.0 ± 5.7	0.225

cART: combined antiretroviral therapy.
[a] ANOVA.

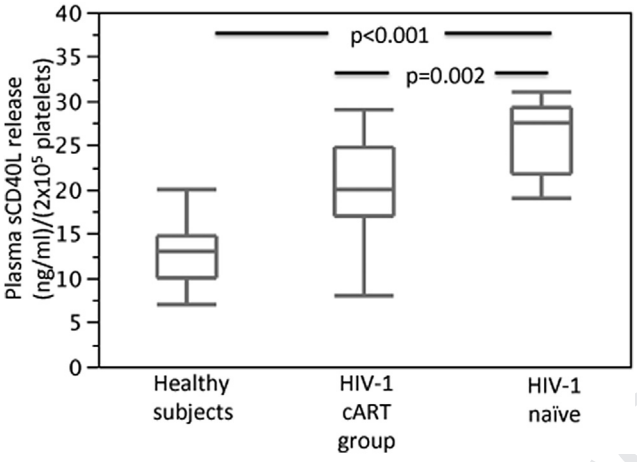

Figure 1 Markers of platelet activation: **box and whiskers plots (median, interquartile range)** of platelet activation, assessed by plasma sCD40L levels in Healthy Subjects, HIV-1 cART group, and HIV-1 naïve subjects.

Platelet oxidative stress

Analysis of unstimulated platelets showed that oxidative stress from HIV-1 subjects was higher compared HS. Thus, we found that platelet sNOX2-dp and 8-iso-PGF$_{2\alpha}$ levels were significantly higher in HIV-1 naïve and HIV-1 cART subjects compared to HS (Fig. 2, Panels A and B).

After stimulation with AA, platelet sNOX2-dp (23.5 ± 7.6 vs. 14.8 ± 3.8 pg/ml, p < 0.001), and platelet 8-iso-PGF$_{2\alpha}$ (148.9 ± 47.9 vs. 120.8 ± 16.8 pmol/l, p = 0.001) from HIV-1 subjects were higher compared to HS. Moreover, HIV-1 naïve patients disclosed higher platelet sNOX2-dp values (31.7 ± 5.4 pg/ml), compared to patients under cART (21.2 ± 6.5 pg/ml p < 0.001, Fig. 2 Panel A).

Similarly values of platelet 8-iso-PGF$_{2\alpha}$ were significantly increased in HIV-1 naïve patients (184.3 ± 28.4 pmol/l) compared to those on cART (139.0 ± 47.8 pmol/l, p = 0.001, Fig. 2 Panel B).

Platelet 8-iso-PGF$_{2\alpha}$ and sNOX2-dp levels were highly correlated (R = 0.734, p < 0.001).

A sub analysis showed a significant difference in platelet oxidative stress between HIV-1 patients treated with PI and those treated with NNRTI (Table 2), both for platelet sNOX2-dp (p = 0.015) and platelet 8-iso-PGF$_{2\alpha}$ (p = 0.018). In addition, patients treated with PI showed increased values of sCD40L, compared to those on NNRTI (p = 0.009). Compared to HS, both the PI and NNRTI group showed higher values of sCD40L and sNOX2-dp, whilst

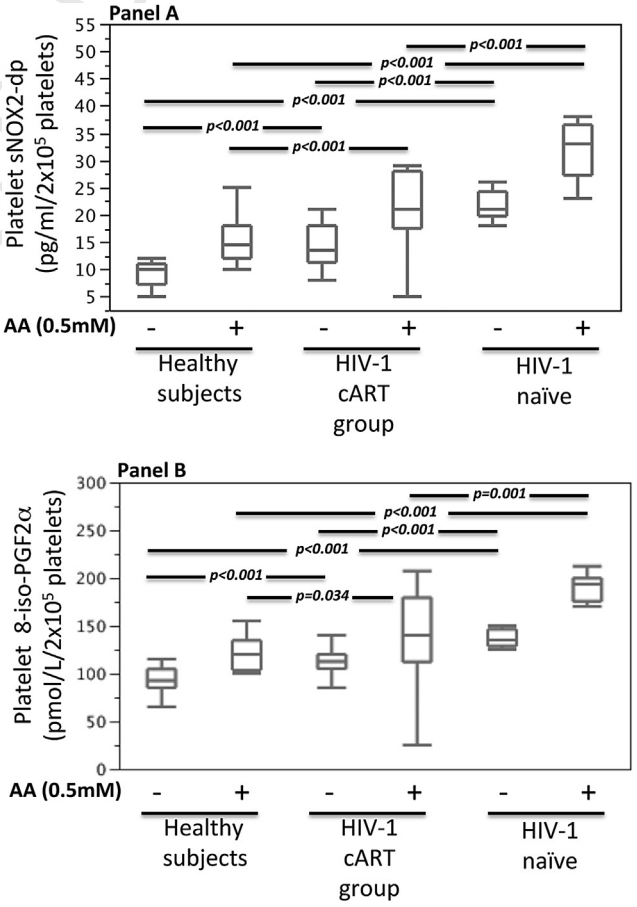

Figure 2 Platelet oxidative stress: **box and whiskers plots (median and interquartile range)** of platelet sNOX2-dp (Panel A) and platelet 8-iso-PGF2α (Panel B) levels in Healthy Subjects, HIV-1 cART group, and HIV-1 naïve subjects.

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Table 2 Differences of platelet activation and oxidative stress in AA-stimulated platelets between Healthy Subjects and HIV-1 patients according to the different cART regimens.

	Healthy Subjects	HIV-1 cART PI-group	p value[a]	HIV-1 cART NNRTI-group	p value[a]	PI vs. NNRTI
sCD40L (ng/ml)	12.6 ± 3.3	22.5 ± 3.6	<0.001	18.2 ± 5.9	0.001	0.009
Platelet sNOX2-dp (pg/ml)	14.8 ± 3.8	24.2 ± 4.0	<0.001	19.5 ± 7.1	0.010	0.015
Platelet 8-iso-PGF2α (pmol/l)	120.8 ± 16.8	162.8 ± 40.3	<0.001	125.6 ± 47.1	0.657	0.018

cART: combined antiretroviral therapy, PI: protease inhibitor, NNRTI: non-nucleoside reverse transcriptase inhibitor.
[a] vs. Healthy Subjects.

platelet 8-iso-$PGF_{2\alpha}$ of patients treated with NNRTI were similar to those of HS (Table 2).

Determinants of sCD40L levels

Table 3 reports correlations of sCD40L levels with selected variables. We also performed a multivariable linear regression analysis which showed that platelet sNOX2-dp (β: 0.803, 95%CI 0.576–0.763, $p < 0.001$), HIV-1 infection (β: 0.146, 95%CI 0.427–3.675, $p = 0.014$) and age (β: 0.166, 95%CI 0.046–0.185, $p = 0.001$) were independently associated to sCD40L levels (adjusted R^2:0.85).

In vitro study

To further investigate the mechanism underlying NOX2 up-regulation, we measured AA-induced platelet $p47^{phox}$ translocation to cell membrane. We used AA as we previously demonstrated that AA directly induces $p47^{phox}$ activation and translocation to platelet membrane.[12] We observed that AA is able to increase the translocation of $p47^{phox}$ to platelet membrane compared to unstimulated platelets, with consequent decrease in the content of the cytosolic fraction (Fig. 3, Panel C).

Western blot analysis showed that membrane levels of $p47^{phox}$ were increased in HIV-1 infected patients compared to HS (13.4 ± 3.7 vs. 27.0 ± 10.4 AU, $p < 0.001$) (Fig. 3, Panels A and B).

Among HIV-1 patients, naïve subjects showed increased value of $p47^{phox}$ compared to those on cART (41.9 ± 7.1 vs. 22.9 ± 6.7 AU, $p < 0.001$), and in naïve subjects (vs. cART group), compared to HS (Fig. 3, Panel A). P47phox membrane translocation was significantly associated to NOX2 activation ($R = 0.806$, $p < 0.001$).

Discussion

Results from our study provide evidence that HIV-1 infection is associated with in vivo platelet activation and suggest platelet NOX2 up-regulation as a potential underlying mechanism.

Patients with HIV-1 infection are characterized by accelerated atherosclerosis[4] and platelet activation is believed to play a major role.[9] A direct interplay between HIV-1 and platelets may be implicated in platelet activation.[23] Thus, platelets have been shown to possess receptors for viruses, including HIV-1,[24] and to be able to internalize HIV-1 acting as phagocytes.[25,26] Furthermore, a relationship between virus replication and platelet activation has been demonstrated.[23,27,28] However, the mechanism potentially accounting for platelet activation in HIV-1 subjects it is still undefined.

Previous studies reported that HIV-1 structural and functional proteins may promote NADPH oxidase activation in different cell lines.[29–31] This finding is of particular interest in the context of platelet function as NADPH oxidase play a relevant role in the process of platelet activation.[13] Thus, studies from our group demonstrated that platelet express this enzyme,[13] which, upon activation, is implicated in the mechanism of platelet activation via formation of isoprostanes, a chemically stable eicosanoids with pro-aggregating properties.[14] Accordingly, patients with up-regulation of the enzyme disclose platelet activation coincidentally with platelet ROS formation and isoprostanes over-production.[10]

In our study, platelets from HIV-1 subjects show enhanced NOX2 activity, as depicted by higher levels of platelet sNOX2-dp. Consistently with this finding platelet 8-iso-$PGF_{2\alpha}$ was higher in HIV-1 patients, especially in HIV-1

Table 3 Correlations of factors associated to sCD40L levels.

	R	p value
Presence of HIV-1 infection	0.604	<0.001
Length of cART	0.164[a]	0.347
Age (years)	0.296	0.016
Body mass index	0.031	0.804
Smoking habit	0.059	0.640
Platelet sNOX2-dp (pg/ml)	0.897	<0.001
Platelet 8-iso-PGF2α (pmol/l)	0.707	<0.001

[a] Spearman correlation.

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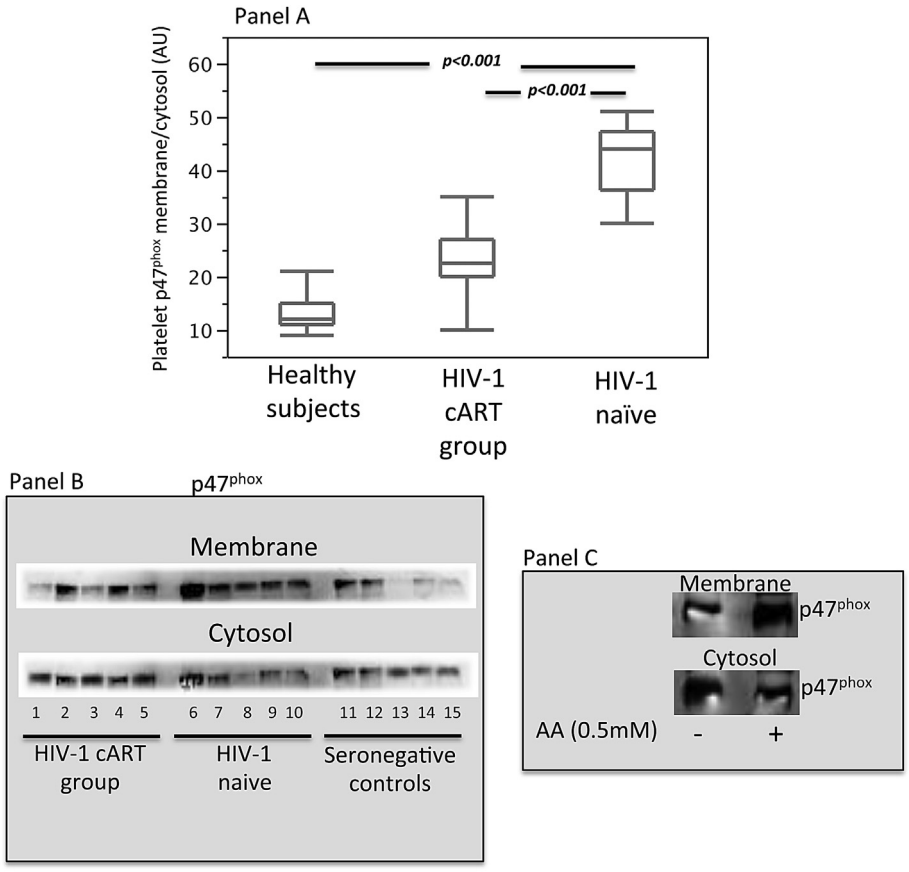

Figure 3 Densitometry evaluation of the membrane p47^{phox} content in platelets of Healthy Subjects, HIV-1 cART group and HIV-1 naïve (Panel A). A representative Western blot analysis of the membrane and cytosol p47^{phox} content in Healthy Subjects (n = 5), HIV-1 cART group (n = 5) and HIV-1 naïve (n = 5) (Panel B). Western blot analysis of the membrane and cytosol p47^{phox} content in HIV-1 naïve (n = 2) (Panel C).

naïve subjects, compared to HS. Platelet 8-iso-PGF$_{2\alpha}$ was highly correlated to sNOX2-dp, confirming, also in this setting, the strong relationship between NOX2 activation and isoprostanes formation. The increased level of isoprostanes is in accordance with previous data reporting increased plasma 8-iso-PGF$_{2\alpha}$ levels in HIV-1 patients in association with viral replication.[32,33] Together these data show enhanced NOX2-derived oxidative stress in HIV-1, which is dependent upon the viral replication in the blood. To further substantiate the role of NOX2 in enhancing platelet oxidative stress, we also performed an *in vitro* study in which we analyzed translocation of the cytosolic p47^{phox} to platelet membrane NOX2. The role of this subunit in NOX2 regulation is evident since subjects with the hereditary deficiency of p47^{phox} showed lower sNOX2-dp level.[34] HIV-1 patients showed significant increase of membrane translocation of the p47^{phox} in association with enhanced sNOX2dp levels. Furthermore, p47^{phox} was more expressed in HIV-1 naïve subjects compared to those receiving cART suggesting a role for viral replication in favoring platelet NOX2 activation.

The results so far reported may provide a mechanistic insight into the increase of in vivo platelet activation detected in HIV-1 patients. Thus, plasma sCD40L, an established marker of platelet activation[35] was more elevated in HIV-1 subjects compared to HS and significantly correlated with NOX2 regulation, indicating a role for NOX2-derived oxidative stress in eliciting in vivo platelet activation.

Another important finding of our study is that, despite being on effective cART, HIV-1 patients still showed an increased platelet oxidative stress. This was demonstrated by the residual significant difference, in platelet sNOX2-dp and 8-iso-PGF$_{2\alpha}$, between HIV-1 subjects under cART and seronegative subjects. This result may be attributable to the persistence of a minimal residual viral load, responsible for the persistent immune activation and chronic inflammation.[36] However, we cannot exclude that antiretroviral agents per se may induce in vivo platelet activation.[37,38] Thus, analyzing the different antiretroviral treatment, we found that patients treated with protease inhibitors showed enhanced platelet oxidative stress and platelet activation compared to patients on non-nucleoside reverse transcriptase inhibitor-based regimens. This finding suggests that antiretroviral agents may have different impact on platelet function.

An implication of the study is that inhibition of NOX2 may represent a target to inhibit platelet activation in HIV-1 patients. In this context, statins may be an interesting option as this drug category is able to down regulate

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NOX2.[39] Small sample size is a limitation of the study, as it does not allow conclusive data on the role of HIV-1 and different antiretroviral agents on platelet function. In particular, we could not exclude that different impact of cART on platelet function and NOX activity might be mediated by modulation of platelet levels of cyclic AMP.

In conclusion, oxidative stress-mediated platelet activation may represent a mechanism potentially responsible for in vivo platelet activation in HIV-1 patients. Antiplatelet treatment including NOX inhibition,[38] in addition to effective cART, may be an useful approach for the prevention of CV complications during HIV-1 infection.

Conflict of interests

None.

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c) Disclosures: None.

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