The value of flow cytometry in the measurement of platelet activation and aggregation in human immunodeficiency virus infection

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Abstract

Human immunodeficiency deficiency virus (HIV) infection is associated with chronic inflammation and an increased risk of thrombotic events. Activated platelets (PLTs) play an important role in both thrombosis and inflammation, and HIV has been shown to induce PLT activation by both direct and indirect mechanisms. P-selectin (CD62P) is a well-described marker of PLT activation, and PLT glycoprotein (GP) IV (CD36) has been identified as a marker of PLT aggregation. Data on PLT function in the context of HIV infection remain inconclusive. Laboratory techniques, such as flow cytometry, enable the assessment of PLTs in their physiological state and environment, with minimal artifactual in vitro activation and aggregation. In this study, we describe a novel flow cytometry PLT assay, which enabled the measurement of PLT function in HIV infection. Forty-one antiretroviral-naïve HIV-positive individuals and 41 HIV-negative controls were recruited from a clinic in the Western Cape. Platelet function was evaluated by assessing the response of platelets to adenosine diphosphate (ADP) at two concentrations (0.04 mM, 0.2 mM). The percentage expression and mean fluorescence intensity (MFI) of CD62P and CD36 was used to evaluate platelet function. These were then correlated with platelet (PLT) count; CD4 count; % CD38/8; viral load and D-dimers. The % CD62P levels were higher in HIV-positive patients (HIV % CD62P 11.33[5.96–29.36] vs. control 2.35 ± 1.31; p = 0.0001). In addition, the HIV group showed higher CD62P MFI levels (HIV CD62P MFI 3.25 ± 7.23 vs. control 2.48[1.56–6.04]; p = 0.0091). However, the baseline CD36 MFI showed no significant difference between the two groups (HIV CD36 MFI 3.09 ± 0.64 vs. control 2.44 ± 0.11; p = 0.4591). The HIV group showed higher levels of % CD36 expression post stimulation with 0.04 mM ADP 43.32 ± 27.41 vs. control 27.47 ± 12.95; p = 0.0214) and no significant difference at 0.2 mM ADP (HIV % CD36 39.06 ± 17.91 vs. control 44.61 ± 18.76; p = 0.3277). Furthermore, the HIV group showed a single phase response to ADP as compared to the control group, which showed a normal biphasic response. We concluded that PLT flow cytometry is valuable in the assessment of levels of PLT activation, and further, that the addition of an endogenous agonist, such as ADP, enabled the measurement of PLT function in HIV infection. We were able to show that, although PLTs are significantly activated in HIV compared to uninfected controls, they retain their functional capacity.

Introduction

Uninterrupted antiretroviral therapy (ART) successfully suppresses viral RNA replication in human immunodeficiency deficiency virus (HIV)-infected individuals [1]; however, there is an emerging trend of non-AIDS related complications, such as cardiovascular disease (CVD) [2, 3], particularly in the aging population of people living with HIV [4]. Mechanisms underlying cardiovascular complications in HIV-infected individuals are multifactorial and more complex than those of their non-infected counterparts [5]. It has become evident that chronic inflammation is a key factor in the pathogenesis of CVD in HIV-infected individuals [6]. Data from the large strategies for management of anti-retroviral therapy (SMART) trial showed that markers of inflammation correlate with adverse outcomes in patients on ART [7]. HIV infection is characterized by increased levels of pro-inflammatory cytokines, dyslipidemia [8] and CD4+ T cell depletion [9]. Both HIV and ART induce impairments in the metabolism of lipoproteins, which may result in increased risks of CVD [10], and inflammation has been identified as an important driving factor in the pathology of thrombosis [11]. However, despite these findings, the underlying mechanisms and contributing factors that link immune activation and inflammation with cardiovascular events remain inconclusive [12, 13].

Previous studies have reported on the thrombo-inflammatory activities of platelets (PLTs) as contributing factors in vascular diseases [14] thereby establishing activated PLTs as a link between inflammation and thrombosis [15, 16].
CD36, a scavenger receptor, is also known as PLT GP IV and is expressed on macrophages, endothelial cells, and most abundantly on the surface and in the alpha granules of PLTs [17–19]. CD36 is a receptor for thrombospondin-1, ox-LDL and collagen and therefore plays a crucial role in PLT adhesion and aggregation [20–22]. The physiological role of PLT CD36 has not been clearly defined [23–26]; however, it is involved in angiogenesis, atherosclerosis, inflammation and lipid metabolism [27]. Although previous studies have reported on the increased expression of CD36 on monocytes in HIV-infected individuals [28], data evaluating PLT CD36 are scarce. P selectin (CD62P) is an adhesion protein that is released from the alpha granules and expressed on the surface of activated PLTs [29]. CD62P interacts with P-selectin GP ligand-1 expressed on the surface of endothelial cells and leukocytes and mediates leukocyte tethering and rolling along the endothelial surface [30]. CD62P is the most frequently reported marker of PLT activation in HIV-infected individuals [16, 31, 32].

Conventional aggregometry has been utilized to evaluate PLT function in HIV [12, 33]; however, various studies have reported contradictory findings of hyper-reactive [12] and hypo-reactive PLTs [33]. PLT aggregometry is time consuming and poorly standardized [34]. Although whole blood PLT aggregometry has been developed, the technique does not allow for the simultaneous evaluation of PLT surface proteins expressed by PLT subpopulations, which may give insight into the observed agonist-induced response patterns [34]. PLT flow cytometry has been previously standardized and utilized for the measurement of PLT GP receptor expression (GPIIb/IIIa and GPIb/IX) in the diagnosis of inherited disorders [34–37]. However, in the context of HIV, PLT aggregometry is still the most commonly used technique [12, 33]. Attempts have also been made to standardize novel PLT flow cytometry-based assays [38, 39] where CD62P was the most commonly used parameter of PLT activation [40].

In this study, we have aimed to build on the current literature by optimizing a whole blood PLT flow cytometry assay that utilizes both CD62P and CD36 to evaluate PLT activation and aggregation in their physiological state and environment, with minimal artifactual in vitro induced loss of PLT subpopulations. A second objective was to evaluate PLT function in HIV by using varying concentrations of the endogenous agonist, adenosine diphosphate (ADP).

Materials and methods

Participants

Eighty-two consenting participants were enrolled in this study. They consisted of 41 consenting adult (antiretroviral [ARV]-naïve) asymptomatic HIV-positive patients and 41 HIV-negative controls. Participants were not on aspirin, anti-inflammatory or anti-tuberculosis drugs. The demographics and baseline characteristics of the study participants are reported in Table I. Patients and controls were recruited from the Emavundleni Voluntary Counseling and Testing Clinic in Crossroads, Cape Town.

Ethical review was performed according to the declaration of Helsinki and approval was obtained from the Stellenbosch University human research ethics committee (HREC) (number: N07/09/197). The study was also registered with the University of Cape Town research ethics committee (number: 417/2006). Informed consent was taken from each patient according to the University of Stellenbosch HREC regulations.

Design of the study

A comparison of the percentage of PLTs expressing CD36 and CD62P was performed among all patients and controls to ascertain baseline levels of PLT activation. In addition, full blood counts, D-dimers, CD4 counts and viral loads were measured and associations between each parameter were assessed. This was followed by stimulation with the PLT agonist ADP at two different concentrations (0.2 mM and 0.04 mM).

Sample collection

A total of 2–3 ml venous blood was collected by venipuncture into 4.5 ml tubes containing 3.2% sodium citrate (BD Vacutainer, San Jose, CA). Sodium citrate was the anticoagulant of choice as blood collected in EDTA has been reported to show artificially elevated levels of the PLT activation marker CD62P [38]. Samples were kept at room temperature during transit from the clinic to the laboratory in order to limit any effects of temperature fluctuations on PLT activation. The time frame between sample collection and analysis was limited to 1–3 hours.

Antibodies and reagents

The anti-human CD31-FITC (clone 5.6E), CD36-APC (clone FA6.152), CD62P-APC (clone AK-4) antibodies were obtained from Beckman Coulter, Miami, FL. Phosphate buffered saline (PBS) without Ca2+ or MgCl2 was obtained from Sigma-Aldrich, St. Louis, MO. ADP was obtained from BIO/DATA Corporation, Holland, The Netherlands.

D-dimer measurements

D-dimer measurements were performed on citrated blood. Sample collection was done using 4.5 ml tubes containing 3.2% sodium citrate. Measurements were performed using the HemosIL™ Dimertest kit (Instrumentation Lab, Bedford, MA) and the ACLTOP platform at the NHLS Hematology coagulation laboratory at Tygerberg Hospital, which is South African National Accreditation System (SANAS) accredited.

Viral load measurements

Five milliliters of blood sample was collected into EDTA tubes, which was centrifuged at 20 °C at 300 g for 12 minutes. One milliliter of plasma was decanted into a Greiner Bio-one cryotube (Greiner Bio-One GmbH, Frickenhausen, Germany) and sent for viral load testing. The viral load assay performed was a NucliSens EasyQ® HIV-1 v1.2 Viral Load Test (BioMerieux Inc., Boxtel, the Netherlands), which has a detection range of 10–10,000,000 copies/ml. Viral load testing was performed at the
SANAS-accredited, Division of Medical Virology, Faculty of Health Sciences, Stellenbosch University.

CD4 T-cell count

The BD MultiTEST CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC reagent (BD Biosciences, San Jose, CA) and BD TruCOUNT tubes (BD Biosciences) were used for CD4 counts. CD4 count measurement was performed at the SANAS-accredited, Division of Medical Virology, Faculty of Health Sciences, Stellenbosch University.

Measurements of CD38 expression on CD8+ T cells

The BD FACSCalibur flow cytometry instrument was used to measure the percentage expression of CD38 on CD8+ T-cells. Briefly, 50 μl of heparinized whole blood was stained with a titrated monoclonal antibody cocktail containing CD8 Per-CP, CD38 APC and CD3 FITC (BD Biosciences). Data analysis were done using the BD Cell Quest Pro, version 2, software (San Jose, CA).

PLT flow cytometry

Instrument set up. Flow cytometry data acquisition was performed using a Beckman Coulter FC500 flow cytometer (Beckman Coulter). Flow check pro fluorescent labelled beads of known size (Beckman Coulter) were used to verify optical path and laminar flow of the cytometer. This ensured the reporting of standardized results that were not influenced by incidental changes or long-term instrumental drifts.

Detector settings. Forward scatter (FS) and side scatter (SS) parameters were set at a log-scale to allow the detection of small particles (Figure 1). An unstained fresh blood sample was used to set the voltages for the FS/SS photomultiplier tubes to enable the separation of red blood cells (RBCs) and PLTs based on cell size and internal complexity or granularity. Stained activated PLTs were used to determine the percentage of spectral overlap and to perform color compensation. Antibody titration assays were then performed to detect optimal antibody concentrations.

Staining. CD31-FITC was used to characterize PLTs (Figure 1B) as this monoclonal antibody does not bind to receptors that directly affect PLT aggregation and may affect in vitro analysis of PLT function [39]. CD62P-APC and CD36-APC were used to monitor PLT activation and aggregation, respectively. Briefly, 50 μl of citrated whole blood was stained using 5 μl of the antibody cocktail and incubated in the dark for 20 minutes at room temperature; 500 μl of PBS (which did not contain Ca²⁺ or MgCl₂) was added and samples were analyzed immediately.

For assessing PLT function, 50 μl of citrated whole blood was incubated with 20 μl of ADP at two different concentrations (0.2 mM and 0.04 mM) for 15 minutes at room temperature. Samples were then stained following the baseline staining protocol mentioned above.

Gating strategy

The use of the PLT-specific marker, CD31, allowed the detection of PLTs (Figure 1A and B). Side-scatter characteristics and CD31 expression were used to discriminate circulating free PLTs from RBCs and PLT-bound RBCs (Figures 1A and 2B).

Statistical analysis

The Mann–Whitney U test was used to compare non-parametric data, and these values were reported as median and interquartile range. For parametric data, an unpaired student t test was performed, and data was reported as mean and standard deviation. In addition, paired data tests were performed to assess the effects of ADP on %CD36 expression. A paired t-test was performed for parametric data, and Wilcoxon matched data test was performed for non-parametric data. Correlations were performed using a spearman rank correlation. A p value of \( p < 0.05 \) represented statistical significance. Graph pad prism 5 for windows, version 5.00 (San Diego, CA).

Results

The study consisted of 41 HIV-negative controls and 41 HIV-positive ARV-naïve individuals. The participant demographics and baseline characteristics are tabulated in Table I. Participants in the two groups demonstrated similar age ranges (20–48 vs. 20–51) and mean age distribution (shown in Table I). None of the participants in both groups were thrombocytopenic, and the PLT counts of all participants were within the normal reference range of 200–400 \( \times 10^9/l \) (shown in Table I). The HIV group as expected showed significantly lower CD4 counts with a mean absolute CD4 cell count 389 ± 41.66 vs. 763.9 ± 53.51 control group, \( p < 0.0001 \).

![Figure 1](image-url). Gating strategy for CD62P expression. The figure illustrates the gating strategy applied. (A) The color dot plot demonstrates the discrimination of platelets from red blood cells (RBCs) based on side scatter (SS) and CD31 expression. (B) demonstrates the baseline expression of CD62P.
Increased levels of circulating activated PLTs in HIV-infected individuals compared to uninfected individuals

The HIV group showed increased baseline percentages of PLTs expressing CD36 compared to the control group (shown in Table I), median 12.41[6.31–21.83] vs. control 6.04; \( p = 0.0661 \). Although the CD36 mean fluorescence intensity (MFI) was higher in the HIV group compared to the control group, there was no significant difference, and no further correlations were done using this parameter (shown in Table I). In addition, the %CD36 positivity in the HIV group showed an inverse correlation with PLT counts \( r = -0.5170 \), \( p = 0.0196 \) and a direct correlation with %CD62P expression \( r = 0.4669 \), \( p = 0.0247 \) and D-dimers \( r = 0.6487 \), \( p = 0.0424 \). The %CD36 levels showed no significant correlations with the other study parameters as shown in Table II.

The baseline levels of %CD62P expression were higher in the HIV group compared to the uninfected controls (median 11.33[5.96–29.36] vs. control 2.48[1.56–6.04]; \( p = 0.0001 \)). In the HIV group, %CD62P levels showed a direct correlation with PLT count \( r = 0.4669 \), \( p = 0.0247 \) and baseline %CD36 expression \( r = 0.4877 \), \( r = 0.0342 \). The baseline levels %CD62P showed no significant correlation with other study parameters as shown in Table III. Furthermore, the HIV group showed an increased CD62P MFI compared to the control group (HIV group mean CD62P MFI 3.25 ± 7.23 vs. control 2.35 ± 1.31, \( p = 0.0292 \)). However, the CD62P MFI showed no correlations with any of the parameters measured in this study.

**Table II. Correlations with % CD36.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>( r )</th>
<th>( p ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count</td>
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<td>0.0196</td>
</tr>
<tr>
<td>CD4 cell count</td>
<td>-0.1950</td>
<td>0.3613</td>
</tr>
<tr>
<td>% CD36</td>
<td>0.4877</td>
<td>0.0342</td>
</tr>
<tr>
<td>% CD38/8</td>
<td>0.3323</td>
<td>0.1925</td>
</tr>
<tr>
<td>Viral load</td>
<td>-0.1643</td>
<td>0.5585</td>
</tr>
<tr>
<td>D-dimers</td>
<td>0.6487</td>
<td>0.0424</td>
</tr>
</tbody>
</table>

Significant values (\( p < 0.05 \)), are shown in boldface.

**Table III. Correlations with % CD62P.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>( r )</th>
<th>( p ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count</td>
<td>0.4669</td>
<td>0.0247</td>
</tr>
<tr>
<td>CD4 cell count</td>
<td>0.0354</td>
<td>0.8824</td>
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<tr>
<td>% CD36</td>
<td>0.4877</td>
<td>0.0342</td>
</tr>
<tr>
<td>% CD38/8</td>
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<td>0.7867</td>
</tr>
<tr>
<td>Viral load</td>
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<tr>
<td>D-dimers</td>
<td>-0.149</td>
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</table>

Significant values (\( p < 0.05 \)) are shown in boldface.

**PLT responses to stimulation with ADP**

The control group showed a significant increase from baseline %CD36 expression, post stimulation with ADP at 0.04 mM ADP (mean %CD36 30.67 ± 14.94 vs. 7.940 ± 7.33, \( p < 0.0001 \)); at 0.2 mM ADP (mean %CD36 59.85 ± 27.06
vs. 7.94 ± 7.33, p < 0.0001. In addition, the control group showed a significant increase in the %CD36 expression between 0.04 mM and 0.2 mM ADP (mean %CD36 59.85 ± 27.06 vs. 30.67 ± 14.94, p < 0.0001) (shown in Figure 2). In comparison, the HIV group showed no significant difference between these two concentrations (Figure 2B).

The HIV group showed a significantly increased overall response to 0.04 mM ADP (HIV group %CD36 mean 43.32 ± 27.41 vs. control 27.47 ± 12.95; p < 0.0214) and no significant difference at 0.2 mM ADP (HIV group mean %CD36 39.06 ± 17.91 vs. control 44.61 ± 18.76; p = 0.3277) (shown in Figure 2). Notably, the fold change from baseline %CD36 expression and post treatment with ADP in the HIV group was only significantly different to that of the control group at 0.02 mM of ADP (shown in Table III). Notably, the difference in fold change at 0.04 mM to 0.2 mM ADP was significant between the two groups (shown in Table IV; Figure 3).

**Discussion**

The aims of this study were to measure levels of PLT activation, aggregation and function in HIV-infected individuals using a whole blood PLT flow cytometry assay. First, we report on a novel approach to PLT flow cytometry that incorporates methodological considerations, which ensure minimal loss of PLT heterogeneity and subpopulations. In addition, we report on PLT function using varying concentrations of ADP that are traditionally used in PLT aggregometry. In attempts to report on a standardized method, the assay design and sample processing were performed as proposed by the British Committee for standards of hematology (BCSH) [34]. This included the consideration of pre-analytical variables such as sodium citrate as an anticoagulant choice – maintaining specimens at room temperature, limiting the time between blood collection and analysis to between 1 and 3 hours – and performing a protocol that does not require centrifugation of samples.

The results of our study demonstrated increased levels of circulating activated PLTs in HIV-infected individuals compared to uninfected controls. These findings are consistent with data reported in previous investigations [16, 31, 32]. Holme et al. demonstrated increased PLT CD62P surface expression in a cohort of 20 HIV-1 infected individuals, of whom 60% were symptomatic. Our study is the first to describe that levels of PLT aggregation and activation are already increased in the asymptomatic, clinically well stage of the disease. Singh et al. reported on a direct association between PLT activation and monocyte cross talk using flow cytometry, by detecting enhanced levels of circulating PLT-monocyte complexes in HIV-infected individuals [41]. In a recent study, Metcalf Pate et al. demonstrated increased levels of activated PLTs in a macaque model, which were associated with decreased PLT counts. [42]. This is in support of our study finding of an inverse correlation between the PLT count and %CD36. In addition Vaughan et al. described

<table>
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<th>ADP concentration</th>
<th>Control group</th>
<th>HIV group</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM vs. 0.04 mM</td>
<td>2.48 [0.65–10.43]</td>
<td>0.79 [0.25–4.57]</td>
<td>0.1331</td>
</tr>
<tr>
<td>0 mM vs. 0.2 mM</td>
<td>2.66 [1.16–7.09]</td>
<td>1.28 [0.11–3.57]</td>
<td>0.0399</td>
</tr>
<tr>
<td>0.04 mM vs. 0.2 mM</td>
<td>0.40 [0.2–0.91]</td>
<td>–0.02 [–0.44–0.53]</td>
<td>0.0204</td>
</tr>
</tbody>
</table>

Significant values (p < 0.05) are shown in boldface.

Figure 3. Platelet response to ADP stimulation. The figure illustrates platelet response to ADP in the control group (A) and in the HIV group (B). (C) illustrates the differences in platelet responses post stimulation with ADP at varying concentrations in both groups.
increased megakaryocyte activity induced by the rapid clearance of activated PLTs in HIV-infected individuals with immune thrombocytopenia. They showed that immature PLT fraction levels correlated inversely with PLT count and directly with HIV viral load [43]. A recent report demonstrated increased levels of activated PLTs in HIV-infected individuals with detectable viremia (HIV RNA >400 copies/ml) compared to HIV-infected individuals with HIV RNA <400 copies/ml [16]. In our study, we could not demonstrate correlations with viral load. In the context of HIV patients with normal PLT counts as in our study, mechanisms that induce PLT activation may not be directly associated with viral RNA levels. In fact, PLTs are able to bind to HIV via C-lectin type DC-SIGN [44], and Tsegaye et al. demonstrated that activated PLTs may reduce viral spread and suppress HIV infection of T cells in a dose-dependent manner [45].

Investigations by Calza et al. also demonstrated elevated plasma levels of CD62P in HIV-infected treatment-naïve individuals [32]. However, in their study, PLT activation was measured using soluble CD62P, rather than flow cytometry.

In this present study, we report on elevated PLT CD36 expression in HIV-infected individuals compared to uninfected controls. Unlike CD62P, which is only released from the alpha granules and localizes on the surface of PLTs upon activation [46], PLT CD36 is expressed ubiquitously on the surface of PLTs [37]. Previous investigations have reported on the pivotal role of PLT CD36 signaling in various physiologic and pathological conditions including innate immunity, atherogenesis and diabetes. Using an animal model, Ghosh et al. simulated low-grade vascular injury in mice and demonstrated the essential role PLT CD36 signaling plays in thrombus formation and PLT activation [25]. In the context of HIV, our findings suggest that increased PLT CD36 signaling may be a key contributing factor in the thrombotic phenotype of HIV-infected individuals. Interestingly, this study reports on a lack of qualitative differences between HIV-infected and uninfected individuals as measured by the CD36 MFI. This may suggest that in HIV-positive individuals, elevated levels of aggregation involve a quantitative (increased levels of PLTs expressing CD36, as measured by %CD36 expression) rather than a qualitative (increased CD36 receptors per cell as measured by the MFI) mechanism. Notably, unlike other PLT receptors that are expressed on the PLT surface and subsequently cleaved into soluble forms [47], the resistance of CD36 to proteolytic cleavage has been previously described [48].

Dyslipidemia is a major cardiovascular risk factor that has been reported as a common clinical feature of HIV infection [49] and is characterized by high levels of LDL cholesterol [50]. In addition, HIV has been established as a cause of dyslipidemia even in untreated (ART naïve) HIV-infected individuals [50]. In vivo, circulating lipoproteins, which include LDL, are susceptible to oxidation [27], and ox-LDL induce PLT activation via the CD36 pathway that involves Syk kinase and calcium signaling [18]. In our experiments, CD36 expression was significantly associated with increasing levels of activated PLTs and decreasing levels of circulating PLTs. In addition, CD36 expression correlated directly with D-dimer levels (shown in Table II), which further supports the association of CD36 with the coagulation pathway. These findings may suggest the potential role of CD36 as a measurable parameter in the thrombotic risk profiling of HIV patients. Previous studies have reported elevated CD36 expression on circulating monocytes during HIV infection [28]; but to our knowledge, this study is the first to report increased expression of PLT CD36 in asymptomatic, treatment-naïve HIV-infected individuals.

The HIV group showed increased D-dimer levels compared to the control group. These findings are consistent with those of Boullware et al. who reported elevated D-dimer levels in ART-naïve individuals. In addition, elevated D-dimer levels persisted 1 month after initiation of ART [51]. The SMART study associated increased D-dimer levels with all-cause mortality [7]. Notably, in our study, the D-dimer levels showed no correlation with PLT counts and viral load, but rather showed good correlations with the marker of PLT aggregation CD36. As expected, the HIV group showed increased levels of activated CD8+ T cells as measured by the expression of CD38 on CD8+ T cells. The expression of CD38 on CD8+ T cells has been established as a marker of immune activation and as an independent marker of disease progression in HIV [52, 53]. However, none of the parameters in our study showed any associations with the levels of CD38 on CD8+ T cells. Therefore, multifactorial and complex mechanisms are likely involved in the regulation of PLT activation and function in HIV. Furthermore, we report that PLTs in HIV-infected individuals have retained their function in response to the endogenous agonist ADP. Upon stimulation with ADP, at concentrations that induce biphasic aggregation, ADP induces PLT activation and aggregation via the P2Y receptors, which play a role in thrombosis [54, 55]. These findings are consistent with those reported recently by O’Brien et al., where the authors used light transmission aggregometry with submaximal agonist concentrations to evaluate PLT response in ART-treated HIV-infected individuals [12]. Although the participants of this particular study had higher mean CD4 counts and suppressed viral RNA replication, PLTs remained reactive to low concentrations of endogenous agonists [12]. Our findings suggest that PLT function is maintained in HIV-infected individuals and that this phenomenon is independent of viral RNA replication. The response patterns observed in our control group were analogous to that reported using conventional aggregometry in which a biphasic response is observed in normal PLT response to ADP [56]. This is classified as reversible (primary wave) and irreversible (secondary wave) aggregation. Although this assay measures a different aspect of PLT function, PLT CD36 expression showed patterns that may be comparable to the primary and secondary wave obtained in aggregometry-based assays. Our study demonstrated a biphasic response in the control group (shown in Figure 2A) that suggests a normal response to ADP, whereas the HIV group showed a single-phase response (shown in Figure 2B). Taken together, our findings suggest that although PLT function is retained in HIV, PLTs are likely to have been activated in vivo and therefore may respond irreversibly to low concentrations of ADP.

Our study has demonstrated an increased percentage of PLTs expressing CD36 at baseline levels, which may be a key factor in the enhanced response to ADP stimulation in HIV. CD36 also plays a role in priming PLTs for activation by suboptimal physiological agonist concentrations [26]. Podrez et al. demonstrated that the PLT surface expression of CD36 results in PLT hyper-reactivity [46]. In contrast, Satchell et al. demonstrated hyporesponsive PLTs in 20 HIV-infected individuals compared to 20 uninfected controls [33]. Importantly, the authors used an aggregometry assay that required the preparation of PLT-rich plasma [33]. The centrifugation and vortexing of PLTs may have induced PLT degranulation and aggregation prior to stimulation with agonists [34, 38]. Taken together, these differences in the cohorts and methodology used in assessing PLT function may explain the contradictory findings. In attempts to overcome these limitations, our study protocol omitted any centrifugation and vortexing methods prior to analysis. In addition, no PLT-enrichment techniques were performed. This ensured that confounding factors that may be introduced by these methods in vitro were kept minimal.
The cross-sectional design of this study limits the conclusive links between the measured parameters and the development of CVD in HIV infection. However, we report on a potentially important association between HIV infection and increased levels of circulating activated and hyper-reactive PLTs, which may be important in understanding the key mechanisms involved in the development of thrombotic complications in HIV-infected individuals. These findings are subject to existing confounding factors known to contribute to increased PLT activation, such as the body mass index (BMI), age and ethnicity. In this study, age and ethnicity could be excluded, as there were good similarities between the mean age and ethnicity of the cohort. However, we cannot exclude gender and BMI as confounding factors, although in a study by Mayne et al. the BMI showed no effect on PLT activation [16].

In conclusion, we have shown heightened levels of both PLT activation and aggregation by flow cytometry in HIV infection, which correlated with D-dimer levels and not with viral load. In addition, we demonstrated the value of flow cytometry in the assessment of PLT function. Numerous studies point to general inflammation rather than direct viral replication as a key factor in the development of thrombotic complications in the context of HIV. The levels of activated PLTs have been shown to persist in ARV-naïve and ARV-experienced individuals. Our findings suggest that PLTs in HIV-infected individuals retain their functional capacity. Furthermore, they are primed to respond to sub-threshold concentrations of physiological agonists, and this may result in hyper-reactive PLTs. The implications of these findings in the thrombotic risk profiling of HIV-infected individuals will require further studies.

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Declaration of interest
The authors declare that there are no financial, personal or professional competing interests that may interfere with this work.

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