Targeting thrombogenicity and inflammation in chronic HIV infection

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INTRODUCTION

Persons with HIV infection (PWH) have increased risk for cardiovascular disease (CVD), but the underlying mechanisms remain unclear. Coronary thrombosis is known to provoke myocardial infarctions, but whether PWH have elevated thrombotic propensity is unknown. We compared thrombogenicity of PWH on antiretroviral therapy versus matched controls using the Badimon chamber. Measures of inflammation, platelet reactivity, and innate immune activation were simultaneously performed. Enrolled PWH were then randomized to placebo, aspirin (81 mg), or clopidogrel (75 mg) for 24 weeks to assess treatment effects on study parameters. Thrombogenicity was significantly higher in PWH and correlated strongly with plasma levels of D-dimer, soluble TNF receptors 1 and 2, and circulating classical and nonclassical monocytes in PWH. Clopidogrel significantly reduced thrombogenicity and sCD14. Our data suggest that higher thrombogenicity, interacting with inflammatory and immune activation markers, contributes to the increased CVD risk observed in PWH. Clopidogrel exhibits an anti-inflammatory activity in addition to its antithrombotic effect in PWH.

RESULTS

Characteristics of participants

A total of 29 participants were enrolled (15 PWH and 14 seronegative controls). One participant with HIV enrolled in the pilot trial did not complete the study treatment due to hospitalization for a surgery, deemed unrelated to the study. Baseline characteristics are summarized in Table 1. The percentage of smokers (current and past) was higher in the PWH group. Of the PWH, two (13%) were on an abacavir-containing ART regimen and six (40%) were on an integrase inhibitor–containing regimen. Self-reported adherence to study treatments was high, with approximately 90% of study participants reporting 100% adherence. Study drugs were safe and well tolerated; there were no serious adverse events. In the PWH randomized to study drugs, plasma HIV-1 RNA levels did not change during treatment and remained below the limits of quantification.

Thrombogenicity is increased in ART-treated HIV infection

PWH had significantly higher thrombus formation than seronegative controls at both low (median interquartile range, IQR) size of 6349 (1547) μm²/mm versus 5237 (1929) μm²/mm, P = 0.006 and high shear rates [9256 (3192) μm²/mm versus 7421 (3513) μm²/mm, P = 0.013] (Fig. 1A). Excluding smokers from the analysis did not change these findings (table S1). Breakdown by gender showed that women accounted more for the higher thrombogenicity in PWH versus controls at both low [7047 (1326) μm²/mm versus 4717 (2321) μm²/mm, P = 0.006] and high shear rates [10,268 (4724) μm²/mm versus 7557 (3572) μm²/mm, P = 0.012]. Differences in thrombus size among male patients and controls did not achieve statistical significance at either low [6071 (1574) μm²/mm versus...
Platelet reactivity is higher in ART-treated HIV infection

As previously described, submaximal concentrations of platelet-activating agonists have been used to identify a hyperreactive platelet phenotype (7, 13). PWH exhibited higher median spontaneous platelet aggregation (SPA) (3% versus 2%, P = 0.008) and also higher median aggregation in response to submaximal agonist concentrations than seronegative controls [collagen (0.05 μg/ml), 3% versus 2%, P = 0.019; arachidonic acid (500 μM), 86% versus 73%, P = 0.013]. Differences in aggregation to low doses of epinephrine and adenosine diphosphate (ADP) between HIV-infected participants and seronegative controls were not significant in this study. At higher agonist concentrations, much of the differences observed between PWH and seronegative controls were attenuated (Table 2).

Thrombogenicity in ART-treated HIV infection

Coagulation plays a major role in thrombosis; the impact of HIV infection on thrombus kinetics was assessed using thromboelastometry in this study. PWH exhibited trends toward shorter coagulation times [161.0 (17.0) s versus 168.5 (20.0) s, P = 0.257] and clot formation times [63.0 (35.0) s versus 66.0 (24.0) s, P = 0.328], but the differences were not statistically significant. Maximum clot firmness [62.0 (10.0) mm versus 61.5 (6.0) mm, P = 0.780] and α angles [78.0° (6.0°) versus 76.5° (4.0°), P = 0.207] were similar between PWH and controls. Breakdown by gender showed that the tendency for higher coagulability in PWH appeared to be more marked among females (table S2).

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sCD14 levels are elevated in ART-treated HIV infection, but other markers of inflammation are similar to seronegative controls

PWH showed significantly higher mean values of sCD14 than did seronegative controls [2094 (420) pg/ml versus 1771 (543) pg/ml, P = 0.014]. There were no differences between the two groups in plasma sCD163 [549 (192) versus 463 (333), P = 0.562], D-dimer [1766 (1321) versus 1969 (917), P = 0.601], soluble tumor necrosis factor receptor 1 (sTNFR1) [693 (149) versus 746 (478), P = 0.984], sTNFR2 [4452 (805) versus 4418 (1886), P = 0.581], and soluble interleukin-6 (sIL-6) [2.0 (2.0) versus 2.1 (4.1), P = 0.936]. The two groups also did not differ significantly in monocyte-platelet aggregate levels [22.0% versus 21.6%, P = 0.97] or in monocyte subsets defined as classical (CD14+CD16-: 66.4% versus 57.4%, P = 0.14), intermediate (CD14+CD16-: 25.6% versus 29.5%, P = 0.07), or nonclassical (CD14dimCD16+: 6.8% versus 7.6%, P = 0.99).

Thrombogenicity in ART-treated HIV infection strongly correlates with D-dimer, sTNFR1 and sTNFR2, and classical and nonclassical monocytes

While no significant associations between thrombus size at low shear rate and other variables were observed, there were significant positive correlations between high shear rate thrombus and D-dimer (r = 0.67, P = 0.017), sTNFR1 (r = 0.75, P = 0.005), sTNFR2 (r = 0.61, P = 0.037), and nonclassical monocytes (r = 0.62, P = 0.031) and a significant negative association between high-shear thrombus and classical monocytes (r = -0.71, P = 0.005; Fig. 2) in PWH. These correlations were not observed in the control group (table S3).

Clopidogrel, but not aspirin, reduced thrombogenicity and sCD14

Treatment with clopidogrel significantly reduced blood thrombogenicity at low shear rate, with a reduction in median thrombus size of 11.3%, from 6743 (1223) µm²/mm to 5981 (1030) µm²/mm (P = 0.047). At high shear rate, there was a marked trend toward reduced thrombogenicity that did not reach statistical significance [19.6% reduction in median thrombus from 8788 (1325) µm²/mm to 7066 (2516) µm²/mm, P = 0.176] (Fig. 3A). Plasma sCD14 levels were also reduced with clopidogrel treatment by 16.3% at 12 weeks [from 2245 (292) pg/ml to 1879 (326) pg/ml, P = 0.010] and 9% at 24 weeks [from 2245 (292) pg/ml to 2045 (143) pg/ml, P = 0.049]. Of interest, neither placebo nor aspirin reduced thrombogenicity or sCD14 (Fig. 3B).

None of the three treatment groups displayed significant changes in clot kinetics; sCD163; D-dimer; sTNFR1; sTNFR2; sIL-6; classical (CD14+CD16-), intermediate (CD14+CD16-), or nonclassical (CD14dimCD16+) monocyte subsets; or monocyte-platelet aggregates from beginning to end of treatment period. Platelet reactivity was significantly reduced by both aspirin and clopidogrel. As expected, aspirin practically abolished arachidonic acid–induced platelet aggregation (76 to 2%, P < 0.001), and clopidogrel significantly reduced platelet aggregation in response to ADP (97 to 68%, P = 0.04). Epinephrine-induced aggregation was significantly inhibited by aspirin (88 to 28%, P = 0.03) but not clopidogrel, and neither treatment significantly affected collagen-mediated aggregation.

Table 1. Baseline demographics of study participants. LDL, low-density lipoprotein; HDL, high-density lipoprotein; NSSTI, integrase strand transfer inhibitors; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

|                      | Serum (n = 14) | PWH (n = 15) |
|----------------------|---------------|-------------|
| Age (years)          | 44 (21)       | 46 (13)     |
| Male, no. (%)        | 7 (50%)       | 8 (53%)     |
| Race                 |               |             |
| White non-Hispanic   | 5 (35.5%)     | 3 (20.0%)   |
| Black non-Hispanic   | 4 (29.0%)     | 6 (40.0%)   |
| Hispanic             | 4 (29.0%)     | 6 (40.0%)   |
| Body mass index (kg/m²) | 25.6 (2.5)      | 27.9 (10.7) |
| CD4 T cell count     | –             | 708 (380)   |
| Smoking              |               |             |
| Never                | 11 (78.6%)    | 7 (46.7%)   |
| Past                 | 1 (7.1%)      | 4 (26.7%)   |
| Current              | 2 (14.3%)     | 4 (26.7%)   |
| ART, no. (%)         |               |             |
| Abacavir-based       | –             | 2 (13.0%)   |
| INSTI + 2 NRTI       | –             | 6 (40.0%)   |
| NNRTI + 2 NRTI       | –             | 6 (40.0%)   |
| PI + 2 NRTI          | –             | 6 (40.0%)   |
| LDL (mg/dl)          | 114 (30)      | 99 (21)     |
| HDL (mg/dl)          | 52 (20)       | 47 (22)     |
| Total cholesterol (mg/dl) | 181 (53)      | 169 (55)   |
| Platelet count (x10⁹/liter) | 221 (95)  | 232 (124) |

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DISCUSSION

It is estimated that currently there are more than 37 million people worldwide living with HIV (14). In high-income countries, 50% of the HIV-infected population is aged 50 years or older (15). ART has had a major impact on the life span of PWH, with some studies now estimating that the life span of PWH who achieve virologic suppression may approximate that of the general population (16, 17). However, there is evidence of increased immune activation and resultant residual inflammation contributing to excess non-AIDS conditions and comorbidities including CVD even among those with complete virologic suppression. As in the general population, CVD event rates increase with age in PWH, and given the increasing longevity of the HIV population, the prevalence of CVD is likely to rise unless effective therapeutic strategies are promptly developed. A better understanding of the specific mechanisms responsible for the higher cardiovascular risk reported in PWH on ART will yield specific and effective therapeutic interventions to ameliorate that risk.

Given the critical role of thrombosis in the onset and severity of ACS, we investigated the blood thrombogenicity of ART-treated PWH versus age- and sex-matched HIV-seronegative controls in this study. Our findings of significantly higher levels of thrombus formation in PWH as compared to seronegative controls strongly suggest the presence of a hyperthrombotic state in HIV infection. The increased blood thrombogenicity was observed at both low- and high-shear rate conditions. An interesting finding was that women with HIV accounted for the greatest differences; however, given the limited sample sizes available for gender analysis, it would be prudent to exercise caution in interpreting this result.

We measured clot formation kinetics, platelet reactivity, soluble markers of inflammation, and percent of circulating monocyte subsets
and monocyte platelet aggregates to determine which of these variables might be associated with blood thrombogenicity. While baseline measurements of these variables, comparing HIV-infected with seronegative participants in our study, differed only when comparing platelet reactivity to low-dose platelet-activating agents and plasma sCD14, as we previously demonstrated (7), we newly observed that high–shear rate thrombogenicity in PWH strongly correlated with D-dimer, sTNFR1, sTNFR2, and percent nonclassical monocytes. Both IL-6 and D-dimer have been shown to be independently associated with serious non-AIDS conditions among PWH with suppressed virus. D-dimer is a fibrin degradation product present in the blood after a blood clot is degraded by fibrinolysis; therefore, elevated D-dimer levels suggest increased fibrin formation. sTNFR1 and sTNFR2 have also been reported to predict non-AIDS morbidity and mortality (18) and are associated with carotid atherosclerosis (19). TNF-α has been shown to accelerate thrombus formation in vivo, and its prothrombotic effects require TNFR2 (20). In addition, TNF-α has also been shown to contribute to platelet activation (21). Nonclassical monocytes, which also correlated strongly with high–shear rate thrombogenicity in PWH in this study, have been shown to highly express tissue factor in patients infected with HIV (22), and tissue factor is a highly potent activator of blood coagulation and platelet activation.

Our pilot study evaluating the effects of two of the most commonly prescribed antiplatelet drugs on thrombogenicity and inflammatory markers in PWH showed that only the P2Y₁₂ inhibitor clopidogrel, but not aspirin, significantly reduced low–shear rate thrombogenicity and sCD14 in these participants. The lack of effect observed in the aspirin arm of this study is in concordance with the results of AIDS Clinical Trials Group A5331, which demonstrated no significant benefit on immune activity or vascular health from aspirin in PWH (23).

Two major conclusions can be drawn from our study. First, PWH suppressed on ART have higher blood thrombogenicity than HIV-seronegative controls, as shown by the increased platelet thrombus formation, with no demonstrable differences in coagulation (thromboelastometry). Second, treatment with clopidogrel can reduce blood thrombogenicity in this patient population and therefore could offer potential benefits in reducing both the hyperthrombotic and hyper-inflammatory status that is postulated to be responsible for the higher cardiovascular risk seen in PWH. Our findings of the antithrombotic and anti-inflammatory activities of clopidogrel in the PWH warrants further investigation to confirm these findings.

Limitations
The sample size of our prospective study was small, especially given the interventional nature of the study. However, this was an exploratory pilot study that included an array of technically challenging and highly specialized assays on HIV-infected and control study participants that were closely matched demographically. In addition, despite the small number of participants, a robust difference in thrombus formation at both shear rates in PWH as compared to seronegative control participants was attained. Furthermore, we observed strong correlations between thrombogenicity and markers...
of coagulation and inflammation, thus providing a strong mechanistic link between these variables.

MATERIALS AND METHODS
The study was conducted in accordance with the Institutional Review Board policies of Icahn School of Medicine at Mount Sinai (ISMMS) and with the Declaration of Helsinki. All participants provided written informed consent before the start of any study procedures. The clinical trial was registered with www.clinicaltrials.gov (NCT02578706).

Study design
The study consisted of two parts (Fig. 4): (i) a cross-sectional, case-control study to compare the blood thrombogenicity of PWH with age- and sex-matched seronegative controls, and (ii) a pilot trial (randomized, double-blind, parallel-group) to investigate the effects of two differentially acting antiplatelet drugs on inflammation (sCD14) and thrombus formation in PWH. For the pilot trial, PWH enrolled in the case-control study were randomized to placebo, aspirin, or clopidogrel treatment for 24 weeks.

Study participants
We enrolled 15 adult participants with chronic HIV infection on suppressive ART (HIV RNA below quantification limit for ≥48 weeks, transiently detectable blips <500 copies/ml were allowed if flanked by undetectable values) and 14 age- and sex-matched seronegative controls in the study. All study participants underwent screening tests (for renal and liver function, hemoglobin, platelet count, pregnancy test when applicable, HIV RNA-1 in PWH, and HIV serology testing in participants without known HIV) to confirm eligibility. Major exclusion criteria (established prospectively) included a history of gastrointestinal or central nervous system bleeding, anemia or thrombocytopenia, recent severe illness, liver or kidney disease, uncontrolled diabetes, known CVD, coexisting cancer, pregnancy or breastfeeding, use of statin, use of nonsteroidal anti-inflammatory drug (including aspirin), and use of immunosuppressive medications.

For the pilot trial, the 15 enrolled PWH were randomized using a double-blind, parallel-group, three-arm design to placebo, aspirin (81 mg once daily; Mylan Pharmaceuticals Inc.), or clopidogrel (75 mg once daily; Bristol-Myers Squibb/Sanofi Pharmaceuticals) treatment for 24 weeks. A permuted block randomization algorithm with a 1:1:1 allocation ratio, block size = 6, was generated by the study biostatistician to determine treatment assignments. Unique study identification numbers (SIDs) were allocated to study participants, and the ISMMS Investigational Drug Service (IDS) pharmacist (unblinded) mapped SIDs to their corresponding treatment regimen. Placebo and study drugs were over-encapsulated by IDS to mask identification and were stored at the IDS facility. Participants, care providers, and study team members were blinded to the intervention assignment.
Following enrollment, all study participants underwent the Badimon perfusion study to assess thrombogenicity and blood sampling for the measurement of thrombus kinetics, platelet reactivity, and markers of inflammation. PWH were then randomized into the pilot trial and began their assigned study treatments. At the midpoint of treatment (12 weeks), blood samples were collected for the measurement of inflammatory markers. At the conclusion of the treatment (24 weeks), all study assessments performed at baseline were repeated. Safety assessments including clinical evaluations, detailed bleeding questionnaires, safety laboratory testing, and blood collection for serum and plasma specimen storage for batched assays were performed at all time points.

**Evaluations**

**Blood thrombogenicity**

Assessments of thrombogenicity were made using the Badimon perfusion system, an ex vivo model of thrombosis suitable for such applications (11, 12). The high-shear chambers (shear rate, 1690 s⁻¹) mimic the rheological conditions of a moderately stenosed coronary artery, while the low-shear chamber (shear rate, 212 s⁻¹) simulates venous flow conditions. Surgically dissected porcine aorta was used as the substrate to trigger thrombus formation over its surface. Native (non-anticoagulated) blood was perfused over this tissue directly from an 18-gauge intravenous cannula in the antecubital vein of the participant for 5 min at a rate of 10 ml min⁻¹. Thereafter, perfused segments were fixed in 4% paraformaldehyde and stained with combined Masson trichrome elastin. Total thrombus area (μm²/mm) was quantified by planimetry using a DM5000B microscope (Leica GmbH, Wetzlar, Germany) under ×10 magnification and Image-Pro Plus software (Media Cybernetics, MD, USA) (24).

**Thrombus kinetics**

The viscoelastic properties of thrombus during formation and early phase of autolysis (thrombus retraction) were assessed by thromboelastometry (ROTEM Gamma; Pentapharm GmbH, Munich, Germany).
Germany). The time to 2-mm clot amplitude in seconds (coagulation time), the time from 2- to 20-mm clot amplitude in seconds (clot formation time), the maximum amplitude in clot size in millimeters (maximum clot firmness), and the tangent to the clotting curve through the 2-mm point (α angle) were measured (25, 26).

**Platelet reactivity**
Light transmission aggregometry was performed according to the manufacturer’s specification using a Chrono-log 570 VS aggregometer (Chrono-log Corporation, Havertown, PA). As previously described (7), functional assays of platelet reactivity in response to agonists at various concentrations were performed as follows: arachidonic acid (150 and 500 µM), ADP (0.4, 1.0, and 20 µM), collagen (0.05 and 2.0 µg/ml), epinephrine (0.05, 0.1, and 5 µM), and no agonist (SPA). Maximum percent aggregation at 10 min was recorded. All aggregation studies were completed within 2 hours of blood collection.

**Monocyte subsets and monocyte-platelet aggregates**
Monocyte subsets were measured in whole blood within 1 hour of collection in EDTA tubes using flow cytometry as previously described (27). Blood was incubated for 15 min on ice with FACS Lyse buffer (BD Biosciences) and then washed in wash buffer [phosphate-buffered saline (PBS) with 1% bovine serum albumin and 0.1% sodium azide]. Cells were stained for 30 min in the dark on ice, washed in wash buffer, and then fixed in 1% formaldehde.

Monocyte-platelet aggregates were measured in whole blood using flow cytometry within 30 min of collection in sodium citrate tubes. Blood was incubated for 15 min with 1% buffered formalin and then stained at room temperature in the dark for 10 min. Water was added then to lyse red blood cells, the incubation was continued for 10 min in the dark, and then PBS was added.

Cells were acquired using a Fortessa LSR II flow cytometer (BD Biosciences). Flowjo software and Prism version 5.0 software (GraphPad) were used to organize and analyze the data. For monocyte subset analysis, monocytes were identified by size, granularity, and expression of CD14 and CD16. CD14 expression was based on a population gating strategy, with both the lymphocyte population and the fluorescence minus one (FMO) serving as the lower limit for determining which cells were CD14+ versus CD14−. The upper limit of CD14+ and the lower limit for CD14+ expression are based on the CD14+ population. Expression of CD16 was based on a conservative FMO gating strategy and was confirmed by fluorescence intensity of the CD16+ lymphocyte population. For monocyte-platelet aggregate analysis, monocytes were identified by size, granularity, and expression of CD14 and CD16. CD61 FMO was used to set the gate for CD14+CD16+ cells. Surface molecule expression was monitored by staining cells with the following: fluorochrome-labeled antibodies sTNFR2, and sIL-6 using Luminex.

**Systemic biomarkers**
Assays for systemic biomarkers were run at the end of the study in plasma samples stored at −80°C during the project after centrifuging at 1550g for 5 min. Plasma samples were thawed immediately before testing for sCD14 and sCD163 using sandwich enzyme-linked immunosorbent assays (R&D Systems, UK) and for D-dimer, sTNFR1, sTNFR2, and sIL-6 using LumineX.

**Statistical analysis**
Demographic and baseline characteristics are summarized for all participants. Data are summarized as median (IQR) unless specified otherwise. Unpaired two-tailed Student’s t tests or Mann-Whitney U tests were used for comparing results of PWH and seronegative controls, as appropriate. Two-tailed paired t tests or Wilcoxon matched-pairs signed-rank test were used for comparing results for PWH before and after study treatments, as appropriate. To examine the biological effects of antiplatelet therapy, as-treated analyses were used, limiting the analysis to participants on treatment for the duration of the study (n = 14). Relationships among variables were assessed using Pearson or Spearman correlation, depending on normality of distribution. All statistical tests were two-sided with a nominal α level of 0.05 with no adjustment for multiple testing. Analyses were performed with GraphPad Prism (version 7.0).

**SUPPLEMENTARY MATERIALS**
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/6/eaav5463/DC1

| Table 1 | Thrombus size (median [IQR] µm²/mm) of PWH versus seronegative controls: Subgroup analyses |
|---------|------------------------------------------------------------------------------------------|
| Table 2 | Coagulation parameters [median (IQR)] of PWH versus seronegative controls: Gender analyses |
| Table 3 | Correlation of high-shear thrombus with parameters of inflammation and thrombosis in PWH versus seronegative controls |

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