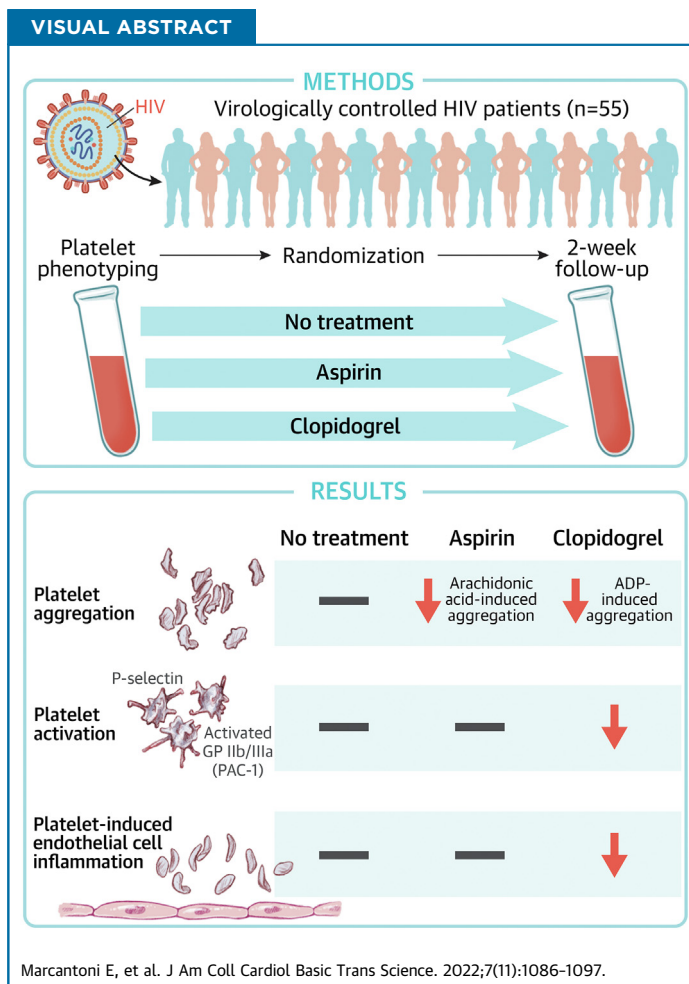**ORIGINAL RESEARCH - CLINICAL**

Antiplatelet Effects of Clopidogrel Vs Aspirin in Virologically Controlled HIV

A Randomized Controlled Trial

Emanuela Marcantoni, PhD,[a],* Michael S. Garshick, MD, MS,[a,b],* Tamar Schwartz, BA,[a] Nicole Ratnapala, MS,[c]
Matthew Cambria, BA,[d] Rebecca Dann, MS,[e] Meagan O'Brien, MD,[f] Adriana Heguy, PhD,[g]
Jeffrey S. Berger, MD, MS[a,b,h,i]

VISUAL ABSTRACT

Marcantoni E, et al. J Am Coll Cardiol Basic Trans Science. 2022;7(11):1086–1097.

HIGHLIGHTS

- In treated HIV, cardiovascular disease is a leading cause of morbidity and mortality, the prevention of which is not fully known.

- Activated platelets are causal in atherosclerotic cardiovascular disease, and platelets in HIV exhibit heightened activity.

- In this study, clopidogrel, as opposed to aspirin, reduced platelet activation, and platelet-induced endothelial inflammation in persons with HIV.

- The use of clopidogrel for the primary prevention of cardiovascular disease in HIV should be evaluated in more extensive clinical trials.

From the [a]Leon H. Charney Division of Cardiology, Department of Medicine, New York University School of Medicine, New York,
New York, USA; [b]Center for the Prevention of Cardiovascular Disease, Department of Medicine, New York University School of
Medicine, New York, New York, USA; [c]Dartmouth Geisel School of Medicine, Hanover, New Hampshire, USA; [d]Division of Vascular
and Endovascular Surgery, Department of Surgery, New York University School of Medicine, New York, New York, USA;
[e]New York Medical College, Valhalla, New York, USA; [f]Regeneron Pharmaceuticals, Inc, Tarrytown, New York, USA; [g]Genome
Technology Center, New York University School of Medicine, New York, New York, USA; [h]Division of Hematology, Department of

SUMMARY

Patients with HIV exhibit platelet activation and increased risk of cardiovascular disease, the prevention of which is not fully known. Fifty-five HIV-positive patients were randomized to clopidogrel, aspirin, or no-treatment for 14 days, and the platelet phenotype and ability to induce endothelial inflammation assessed. Clopidogrel as opposed to aspirin and no-treatment reduced platelet activation (P-selectin and PAC-1 expression). Compared with baseline, platelet-induced proinflammatory transcript expression of cultured endothelial cells were reduced in those assigned to clopidogrel, with no change in the aspirin and no-treatment arms. In HIV, clinical trials of clopidogrel to prevent cardiovascular disease are warranted. (Antiplatelet Therapy in HIV; NCT02559414) (J Am Coll Cardiol Basic Trans Science 2022;7:1086–1097) © 2022 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

ABBREVIATIONS AND ACRONYMS

ADP = adenosine diphosphate

AIDS = acquired immunodeficiency virus

ART = antiretroviral therapy

CV = cardiovascular

CVD = cardiovascular disease

HIV = human immunodeficiency virus

MFI = mean fluorescence intensity

HUVEC = human umbilical vein endothelial cell

PWH = persons with HIV

Effective antiretroviral therapy (ART) has altered the landscape of HIV morbidity and mortality, and nonacquired immune deficiency (AIDS)-related deaths now surpass AIDS-related ones. In persons with HIV (PWH), cardiovascular (CV) disease (CVD) is a significant cause of morbidity and mortality, and confers upwards of a 1.5- to 2-fold higher rate of CV events when compared with uninfected controls.[1-3] Although the pathogenic mechanism(s) for increased risk of CV events in PWH are incompletely understood, platelet activation likely plays an important role.[4] Although platelets are key cellular mediators of atherothrombosis,[5,6] a growing body of evidence supports their role as proinflammatory mediators across autoimmune and proinflammatory conditions.[7,8] Endothelial proinflammatory activation is a key step in atherosclerosis, and activated platelets interacting with the endothelium alters the chemotactic, adhesive, and proteolytic properties of these cells.[5,9-11] Studies exploring the platelet phenotype in PWH do not just reveal an over-activated state, but also highlight the potent effector cell properties of HIV platelets by inducing proinflammatory and chemotactic endothelial transcript expression, suggesting that platelet activation may contribute to HIV-mediated CVD.[12,13]

In non-PWH, yet high CV risk populations, antiplatelet therapies for the prevention of CVD are established.[14,15] However, their utility to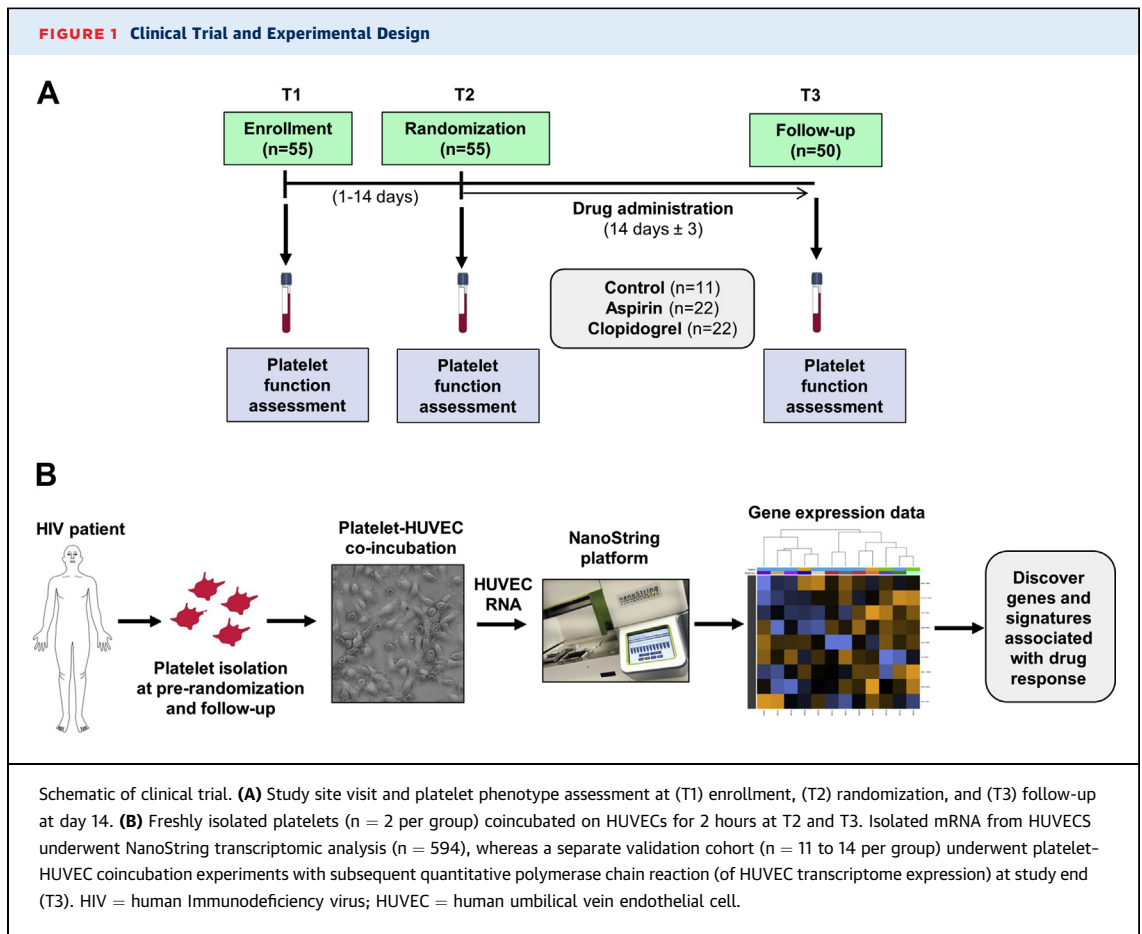 reduce CV risk in PWH who otherwise do not meet a clinical indication for these therapies is uncertain.[16] A prior study from our group found that PWH have increased platelet aggregation (spontaneous and agonist-induced) compared with control subjects. Although urinary 11-dehydro-thromboxane B2 decreased in both PWH and control subjects following aspirin therapy, levels remained higher in PWH than control patients.[17] A randomized controlled trial of aspirin (100 mg, 300 mg) or placebo in PWH found no reduction in biomarkers of systemic inflammation (CD14, interleukin [IL]-6, CD163), coagulation (D-dimer), or improvement in endothelial function after 3 months.[18] Altogether, these studies suggest reduced aspirin efficacy in PWH and the need to investigate alternative antiplatelet strategies.

Clopidogrel, a member of the thienopyridine group of antiplatelet compounds that targets the $P2Y_{12}$ receptor and lowers the risk of CV events in various clinical settings by inhibiting adenosine diphosphate (ADP)-mediated platelet activation and aggregation.[19-23] Data are limited on the effect of clopidogrel on markers of platelet activity and endothelial function in ART-treated PWH. A small study of clopidogrel vs aspirin noted a reduction in thrombogenicity in clopidogrel and not in aspirin.[24] In the non-HIV setting, clinical trials demonstrate reduced CV events in those randomized to clopidogrel when compared with aspirin.[14,15] Platelet activation is causal in atherosclerosis and correlates with CV events.[11,25-27] In addition to their well-described role in thrombosis, platelets are

Medicine, New York University School of Medicine, New York, New York, USA; and the ⁱDivision of Vascular Surgery, Department of Surgery, New York University School of Medicine, New York, New York, USA. *Drs Marcantoni and Garshick contributed equally to this work.

FIGURE 1 Clinical Trial and Experimental Design

Schematic of clinical trial. **(A)** Study site visit and platelet phenotype assessment at (T1) enrollment, (T2) randomization, and (T3) follow-up at day 14. **(B)** Freshly isolated platelets (n = 2 per group) coincubated on HUVECs for 2 hours at T2 and T3. Isolated mRNA from HUVECS underwent NanoString transcriptomic analysis (n = 594), whereas a separate validation cohort (n = 11 to 14 per group) underwent platelet–HUVEC coincubation experiments with subsequent quantitative polymerase chain reaction (of HUVEC transcriptome expression) at study end (T3). HIV = human Immunodeficiency virus; HUVEC = human umbilical vein endothelial cell.

potent effector cells and induce inflammation and immunity via their effects on endothelial cells, monocytes, and macrophages.[11,27] We therefore conducted an open-label randomized controlled trial to evaluate the ability of aspirin and clopidogrel treatments to reduce platelet aggregation and the platelet effector cell properties on endothelial cells in ART-treated PWH.

METHODS

STUDY DESIGN. This study (Antiplatelet Therapy in HIV; NCT02559414) was a randomized, open-label, clinical trial designed to investigate the effects of aspirin and clopidogrel on markers of platelet activity and platelet effector cell functions in ART-treated PWH (**Figure 1A,** Supplemental Figure 1). Eligible participants were between 18 and 80 years of age, who were HIV-positive on ART, with a HIV RNA viral load <200 copies/mL for at least for 3 months. Exclusion criteria included active nonsteroidal anti-inflammatory drug use in the past week (including

aspirin), known CVD, current antiplatelet and antithrombotic drug use, chronic kidney disease, steroids or immunosuppressive agents, active drug or alcohol use, known anemia (hemoglobin <8 mg/dL), or thrombocytopenia ($<100 \times 10^3/\mu L$) or thrombocytosis ($>500 \times 10^3/\mu L$). The protocol was approved by the New York University Langone Medical Center Institutional Review Board, Bellevue Hospital Center, and the central office of the New York City Health and Hospital Corporation. All participants provided written informed consent in line with the declaration of Helsinki. The New York University Investigational Pharmacy was used for study drug procurement, storage, dispensation, and accountability.

RANDOMIZATION, PROCEDURE, AND OUTCOMES. Fifty-five participants were randomized into 1 of 3 groups, control (no treatment), aspirin, or clopidogrel group in 1:2:2 ratios for 14 days of treatment (Supplemental Figure 1). Participants randomized into the aspirin group received a single 325-mg dose of aspirin followed by 81 mg per day for the remaining 13 days. Those randomized to the clopidogrel arm

received a single 300-mg dose followed by 75 mg/day for the remaining 13 days. In the control group, participants received no antiplatelet therapy. Subjects were asked to fast overnight and to not smoke or perform intensive exercise for at least 4 hours before each study visit. A random number generator was used for subject study assignment and performed in block randomization. Visits at each time point included blood and urine collection. During follow-up adverse events were reviewed. The primary outcome(s) were platelet aggregation to arachidonic acid (for those assigned to the aspirin group) and ADP (for those assigned to the clopidogrel arm). Secondary outcomes included other metrics of platelet activity (aggregation and platelet receptor expression), and the platelet's ability to induce endothelial inflammation in vitro.

BLOOD COLLECTION AND PLATELET AGGREGATION.
Blood was collected in 3.8% sodium citrate tubes and centrifuged (200 relative centrifugal force, 10 minutes) to obtain platelet-rich plasma both for platelet function and platelet-cell interaction assays. Using an AggRAM light transmission aggregometer (Helena Laboratories, Beaumont, Texas), the maximal percentage aggregation and percentage of aggregation at 300 seconds was measured in platelet-rich plasma after the addition of the following agonists: ADP at 5 μmol/L and arachidonic acid (1,600 μmol/L). All aggregation measures were completed within 2 hours of phlebotomy. Serum thromboxane B2 was measured via enzyme-linked immunosorbent assay as per manufacturer recommendations with a 1:50 dilution (Cayman Chemical).

FLOW CYTOMETRY ANALYSIS OF PLATELET FUNCTION.
To assess P-selectin, PAC-1, and CD40 expression, citrated whole blood was incubated with CD42b-APC (platelets) and CD62P-FITC (P-selectin) or PAC-1-FITC or CD40 for 30 minutes in the dark at room temperature after stimulation with agonist. Platelet marker expression was recorded as mean fluorescence intensity (MFI) of 10,000 CD42b$^+$ events. All antibodies were purchased from BD Biosciences, and flow cytometry was performed on an Accuri C6 flow cytometer (BD Biosciences). For monocyte platelet aggregates and leukocyte-platelet aggregates, citrate anticoagulated blood was fixed with 1% formaldehyde in a 1:1.2 ratio for at least 15 minutes. Fixed whole blood was stained with CD61-FITC (platelets) and either CD14-APC (monocytes) or CD45-APC (total leukocytes) for 10 minutes at room temperature. Monocytes and leukocytes were collected based on side-scatter properties and positive staining for CD14 or CD45, respectively. Monocyte platelet aggregates

were identified as having a positive stain for CD14 and CD61, and leukocyte platelet aggregates were identified as having a positive stain for CD45 and CD61. Data are expressed as a percentage of all leukocytes or monocytes positive for adherent platelets. In these experiments, 25,000 leukocytes and 2,000 monocytes per sample were analyzed with further gating strategies as noted in Supplemental Figure 2.

PLATELET AGONISTS.
Arachidonic acid, ADP, and epinephrine were purchased from Helena Laboratories. For arachidonic acid (ref 5364), sodium arachidonate was lyophilized at 5 mg/mL and reconstituted in molecular grade water to a stock concentration of 1,600 μmol/L as per manufacturer recommendations. For adenosine diphosphate (ref 5366), adenosine 5-diphosphate was reconstituted to a concentration of 200 μmol/L and diluted to concentrations of 20 μmol/L, 5 μmol/L, and 1 μmol/L. For epinephrine (ref 5367), L-epinephrine bitartrate was reconstituted to a concentration of 3 mmol/L and diluted to 10 μmol/L, 0.4 μmol/L, and 0.1 μmol/L concentrations, which have been previously published.[28] Thrombin (0020301100), used in flow cytometry, was purchased from HemosIL lyophilized from bovine thrombin at 35 U/mL with bovine albumin, calcium chloride, buffer, and stabilizers, and used at a diluted concentration of 0.025 units.

PLATELET PREPARATION AND INCUBATION WITH ENDOTHELIAL CELLS.
As previously described,[12] freshly isolated platelet-rich plasma was added to 1:10 anticoagulant citrate dextrose solution, centrifuged (1,000*g*, 10 minutes) and the platelet pellet resuspended in Tyrode's buffer and 1 μmol/L PGE$_1$ (Sigma-Aldrich). Platelets were counted on a Coulter Ac•T diff2 Hematology Analyzer (Beckman Coulter) and adjusted to the desired concentration by addition of endothelial starvation medium. Washed platelets were incubated with serum-starved human umbilical vein endothelial cells (HUVECs) (0.5% bovine serum albumin in basal medium, overnight) at a 1:100 ratio for 2 hours at 37°C under static conditions, methodology, which has been previously published.[12] Unbound platelets were extensively washed away and HUVECs lysed in Qiazol for RNA extraction. HUVECs (purchased from Lonza) were cultured in endothelial growth medium (Lonza) supplemented with 10% fetal bovine serum (Invitrogen, Waltham, Massachusetts). For all experiments, HUVECs were used between passages 3 to 5 (**Figure 1B**).

NanoString AND QUANTITATIVE POLYMERASE CHAIN REACTION GENE EXPRESSION ANALYSIS AND BIOINFORMATICS.
Total RNA was isolated from HUVECs coincubated with platelets isolated from

HIV-infected subjects using the Direct-zol RNA Mini-Prep (Zymo Research), following the manufacturer's protocols (Figure 1B). For quantitative polymerase chain reaction experiments at study end, RNA was standardized to 100 ng/mL and converted to cDNA using iSCRIPT cDNA Synthesis kit (Bio-Rad Laboratories). The SYBR Green system was used on an Applied Biosystems 7500 Fast Real-Time PCR System. All work was performed using the manufacturers' protocols and as previously published.[29,30] Transcripts were normalized to the housekeeping gene *RNA18S5* and are reported as fold change over control. All primers were obtained from Integrated DNA Technologies.

In the targeted, NanoString transcriptomic studies, analysis was performed on baseline and follow-up samples (n = 2 per study group) using the nCounter Immunology Panel (Catalog # XT-CSO-HIM2-12, NanoString Technologies), which allowed the study of up to 594 genes involved in inflammatory diseases, inflammatory response, cellular development, hematological system development, cell death, and cell-to-cell signaling. Per sample, 100 ng of total RNA was mixed with a 3′ biotinylated capture probe and a 5′ reporter probe tagged with a fluorescent barcode from the custom gene expression code set. Probes and target transcripts were hybridized overnight at 65°C for 12 to 16 hours per the manufacturer's recommendations. Hybridized samples were run on the NanoString nCounter preparation station using the high-sensitivity protocol, in which excess capture and reporter probes were removed and transcript-specific ternary complexes were immobilized on a streptavidin-coated cartridge. The samples were scanned at maximum scan resolution on the nCounter Digital Analyzer. The counts from each hybridization were normalized to the internal positive controls to account for slight differences in assay efficiencies according to nCounter Data Analysis Guidelines. Normalization to 15 housekeeping genes was performed. A cutoff value for expressed transcripts was used based on negative controls and per manufacturer recommendations allowing approximately 246 genes per treatment group. The fold changes were calculated and were subsequently uploaded into Ingenuity Pathway Analysis (Qiagen) and analyzed.

STATISTICS. The sample size was determined based on aspirin's ability to decrease arachidonic acid–induced platelet aggregation by 50% vs control, and clopidogrel to decrease ADP-induced platelet aggregation by 50% vs control. Incorporating multiple comparison adjustment (alpha = 0.025) and a 2:2:1 ratio for aspirin–clopidogrel–control, 50 subjects were needed for >90% power for the 2 coprimary comparisons. Data are expressed as mean ± SD or median (IQR) or count (percentage) as noted within the paper, each table, or Figure legend. Normality was tested using a Shapiro-Wilk normality test. Statistical significance was determined using Student's *t*-test (between groups), paired Student's *t*-test (within group), the Wilcoxon rank-sum test (between groups), or the Wilcoxon signed rank test (within group) as appropriate and is indicated within each Figure legend. An analysis of covariance model was also used to test differences between antiplatelet strategies when platelets were incubated on the endothelial cells in vitro. Analyses were performed using STATA software version 17 (StataCorp), GraphPad Prism software version 9 (GraphPad Solution), and R Core Team (2022, R Foundation for Statistical Computing).

RESULTS

DEMOGRAPHICS. Patient characteristics recruited into the clinical trial (Supplemental Figure 1) are listed in Table 1. The participants were on average middle-aged (median age 53 years [IQR: 50-58 years]), predominantly Black (80%), overweight (median body mass index 25.6 kg/m² [IQR: 23.0-31.2 kg/m²]), and split evenly between men and women. One-half were active smokers, whereas a quarter had hypertension and hyperlipidemia. Approximately 10% had diabetes, and only 16% were on statin therapy (Table 1). Regarding HIV status, participants had a median HIV duration of 19.9 years (IQR: 13.7-24.9 years) and on suppressive ART therapy approximately 14 years on average. Although the median CD4 count was 597.5 (IQR: 417.0-791.8) cell/mm³ at the time of study enrollment, over one-half of the participants had a history of AIDS. The remaining characteristics are listed in Supplemental Tables 1 and 2. During the study period, other medications (including ART therapy) were unchanged.

ASPIRIN AND CLOPIDOGREL DECREASE PLATELET AGGREGATION IN PATIENTS WITH HIV. To determine the effect of 14 days of antiplatelet therapy in ART-treated HIV subjects, ex vivo platelet aggregation in response to platelet agonists was performed. Compared with baseline, arachidonic acid–induced platelet aggregation was inhibited by aspirin (mean change −38.8%, 95% CI: −21.5% to −56.1%), with minimal change in those assigned to clopidogrel (mean change −10.5%, 95% CI: −21.4% to 0.5%) (Figures 2A and 2B). Confirming excellent compliance to aspirin, serum thromboxane B2 was reduced 97.3% (mean change −13.4 ng/mL, 95% CI: −8.5 to −18.4 ng/mL) in the aspirin (but not the control or

TABLE 1   Patient Characteristics

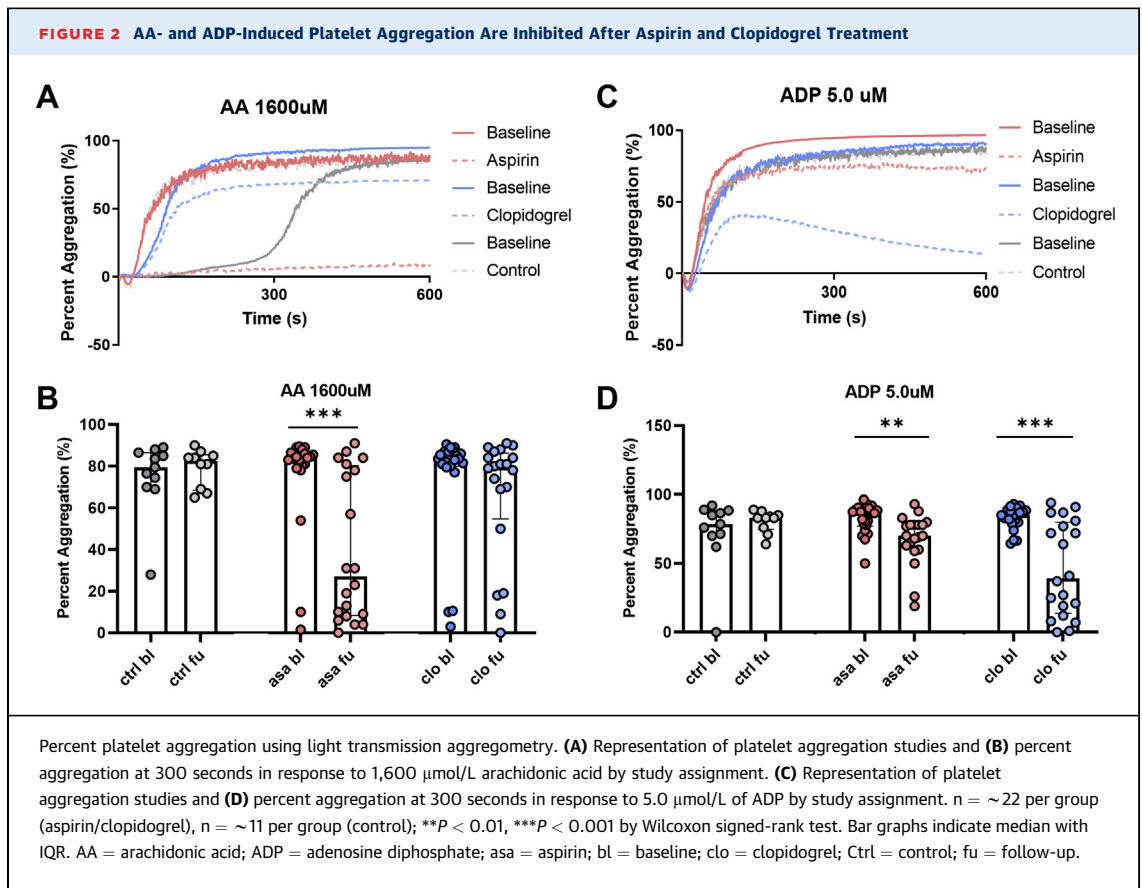|	All (N = 55)	Control (n = 11)	Aspirin (n = 22)	Clopidogrel (n = 22)
Demographics				
Age, y	53 (50-58)	54 (49-57)	52 (49.75-58)	55 (51.75-60.25)
Male	32 (58)	45 (5)	68 (15)	55 (12)
Black	80 (44)	91 (10)	77 (17)	77 (17)
Hispanic	13 (7)	9 (1)	14 (3)	14 (3)
Height, cm	173 (166-180.0)	171.0 (162.0-183.0)	175.0 (167.5-180.8)	171.5 (164.5-178.0)
Weight, kg	81 (70-89)	77 (65-90)	81 (73-86.5)	80.5 (70-89.3)
Body mass index, kg/m^2	25.6 (23.0-31.2)	36.4 (23.0-32.5)	25.5 (23.3-28.4)	25.0 (22.3-33.0)
Medical history				
Active smoking	53 (29)	45 (5)	55 (12)	55 (12)
Hypertension	25 (14)	36 (4)	27 (6)	18 (4)
Diabetes	11 (6)	9 (2)	9 (2)	14 (3)
Hyperlipidemia	24 (13)	9 (1)	23 (5)	32 (7)
Statin	16 (9)	0 (0)	14 (3)	27 (6)
HIV status				
Duration of HIV, y	19.9 (13.7-24.9)	20.0 (10.6-25.6)	21.9 (10.6-25.6)	19.6 (16.6-25.0)
ART duration, y	16 (10-19)	11.5 (9.0-18.8)	16 (9.8-19.5)	16 (12-19)
CD4$^+$ T cell, c/mm^3	597.5 (417.0-791.8)	570 (373-745)	608.0 (340.0-862.5)	598.0 (495.5-791.8)
History of AIDS	58 (32)	64 (7)	59 (13)	55 (12)

Values are median (IQR) or n (%). Active smoking indicates the percentage of patients who were actively smoking within 12 months of study enrollment. *P* value indicates analysis of variance or chi-square between treatment groups as appropriate.

ART = antiretroviral therapy.

clopidogrel) treatment group (Supplemental Figure 3). Similarly, platelet aggregation in response to ADP stimulation (5 μmol/L) was inhibited by clopidogrel (mean change −37.0%, 95% CI: −20.7% to −53.3%), but to a lesser degree by aspirin (mean change 15.2%, 95% CI: −6.9% to −23.4%) (Figures 2C and 2D). No change in platelet aggregation to arachidonic acid or ADP was noted in the control group (Figure 2). Finally, no subject experienced an adverse serious or nonserious bleeding event.

EFFECTS OF CLOPIDOGREL AND ASPIRIN ON PLATELET ACTIVATION MARKERS AND PLATELET-LEUKOCYTE AGGREGATES. Platelet activation (via assessment of activated αIIbβ3 [termed PAC-1]) and platelet P-selectin expression are robust markers of platelet activity,[26,31-33] and directly participate in atherothrombosis.[34-36] After 14 days, the change in platelet P-selectin expression and PAC-1 in basal and stimulated states were not reduced in the aspirin and control groups (Figure 3). By contrast, subjects randomized to clopidogrel resulted in a decreased P-selectin in the basal state (mean change −12.6 MFI, 95% CI: −1.1 to −24.1 MFI), P-selectin after thrombin stimulation (mean change −215.3 MFI, 95% CI: −7.8 to −422.9 MFI), and PAC-1 expression after ADP (mean change −86.4 MFI, 95% CI: −10.4 MFI to −162.3 MFI) and arachidonic acid (mean change −141.7 MFI, 95% CI: −52.3

to −231.1 MFI) stimulation (Figure 3). Consistent reductions of these platelet activation markers were observed when stratified by statin therapy, abacavir, efavirenz, and ritonavir (Supplemental Table 3). Neither aspirin nor clopidogrel had any significant effect on platelet CD40 expression, and leukocyte, neutrophil, monocyte, or lymphocyte platelet aggregates (Supplemental Figure 4).

CLOPIDOGREL REDUCES PLATELET-STIMULATED PROINFLAMMATORY ENDOTHELIAL CELL GENE EXPRESSION. Because platelets induce endothelial cell activation,[12] we investigated the ability of antiplatelet therapies to modulate platelet cell effector functions on endothelial cells. Platelets isolated from the study participants were coincubated with HUVECs before study assignment and at day 14 (Figure 1B) The transcriptomic signature of these endothelial cells was assessed using the immunology panel NanoString code set, which yielded approximately 246 expressed immune- and inflammatory-related expressed genes per study assignment. Based on directionality, a total of 103 unique HUVEC transcripts were down-regulated in those assigned to clopidogrel and 37 up-regulated, whereby only 39 were down-regulated after aspirin therapy and 106 up-regulated (Figure 4A). The average composite proinflammatory transcriptome was significantly decreased after clopidogrel treatment (−3.3%,

FIGURE 2   AA- and ADP-Induced Platelet Aggregation Are Inhibited After Aspirin and Clopidogrel Treatment

Percent platelet aggregation using light transmission aggregometry. **(A)** Representation of platelet aggregation studies and **(B)** percent aggregation at 300 seconds in response to 1,600 μmol/L arachidonic acid by study assignment. **(C)** Representation of platelet aggregation studies and **(D)** percent aggregation at 300 seconds in response to 5.0 μmol/L of ADP by study assignment. n = ~22 per group (aspirin/clopidogrel), n = ~11 per group (control); **$P < 0.01$, ***$P < 0.001$ by Wilcoxon signed-rank test. Bar graphs indicate median with IQR. AA = arachidonic acid; ADP = adenosine diphosphate; asa = aspirin; bl = baseline; clo = clopidogrel; Ctrl = control; fu = follow-up.

95% CI: −1.1% to −5.5%), was increased after treatment with aspirin (11.6%, 95% CI: 6.8% to 16.4%), and the no treatment group (3.6%, 95% CI: 0.2% to 7.1%; $P < 0.001$ between groups) (**Figure 4B**).

To understand the functional significance of these endothelial transcriptomic changes after anti-platelet treatment in PWH, the composite HUVEC transcriptome changes (**Figure 1B**) were investigated using Ingenuity Pathway Analysis. The top 14 differentially expressed canonical pathways (absolute z-score >1) were up-regulated or unchanged in the aspirin and control groups (Supplemental Figures 5A and 5B). However, in platelets from PWH assigned to clopidogrel, a majority (11 of 15) of pathways were downregulated (Supplemental Figure 5C). Specifically, the top 5 down-regulated canonical pathways after clopidogrel treatment (as compared with the aspirin and control groups) included highly inflammatory pathways such as inducible nitric oxide synthase (iNOS), Toll-like receptor, TREM1, interferon signaling, and Th1 pathways (**Figure 4C,** Supplemental Table 4A). Among all of the genes within these down-regulated pathways (Supplemental Table 4B), the top genes down-regulated included those related to the TNF

family, intracellular adhesion (ICAM1), and inflammasome-related mRNA, including CAPS1 and IL-18 (**Figure 3D,** Supplemental Table 4B) and those related to interferon signaling.

To validate these exploratory findings, an expanded analysis utilizing freshly isolated platelet-HUVEC coincubation studies from HIV subjects at study end (n = 10 to 14 per study group) was performed. A reduced ability in the platelet's ability to induce interferon-related HUVEC transcripts was noted in the clopidogrel-treated group, as compared with aspirin and control groups (Supplemental Figure 6). Altogether, our results indicate that in PWH randomized to 14 days of clopidogrel as opposed to aspirin (and control), platelet activation is reduced, and the platelets' ability to promote endothelial proinflammatory and interferon gene expression decreases.

DISCUSSION

The risk of CVD including acute myocardial infarction, ischemic stroke, and heart failure is increased in PWH as compared with the general population.[1,37,38]

FIGURE 3 Platelet P-Selectin and PAC-1 Expression Is Reduced After Aspirin and Clopidogrel Treatment
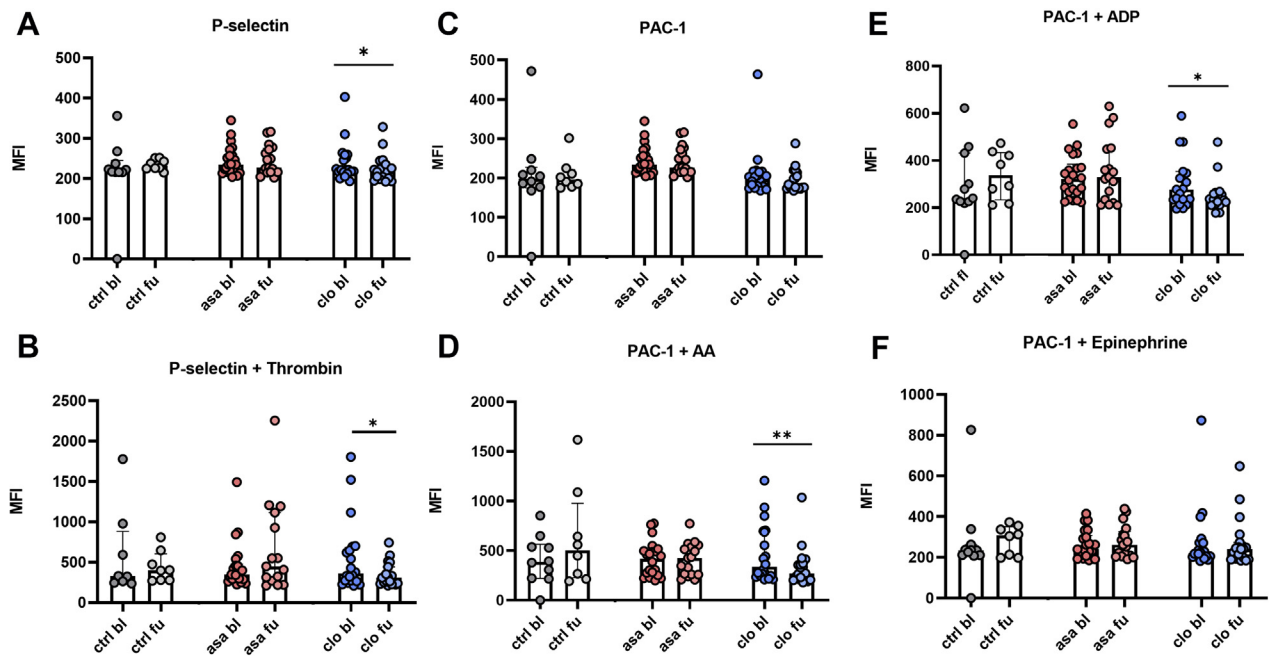
Platelet flow-cytometry (expressed as mean fluorescence units [MFI]). Resting **(A)** and **(B)** thrombin (0.05 U/mL) stimulated platelet p-selectin (CD62p+, CD42b+) expression in each treatment group. Resting **(C)**, arachidonic acid **(D**, 0.60 μmol/L), ADP **(E**, 0.1 μmol/L), and epinephrine **(F**, 0.4 μmol/L) stimulated PAC-1 (CD42b+ PAC-1+) expression. All stimulated treatments occurred over 5 minutes. **P < 0.01, ***P < 0.001 by Wilcoxon signed rank test. Bar graphs indicate median with IQR. Abbreviations as in **Figure 2**.
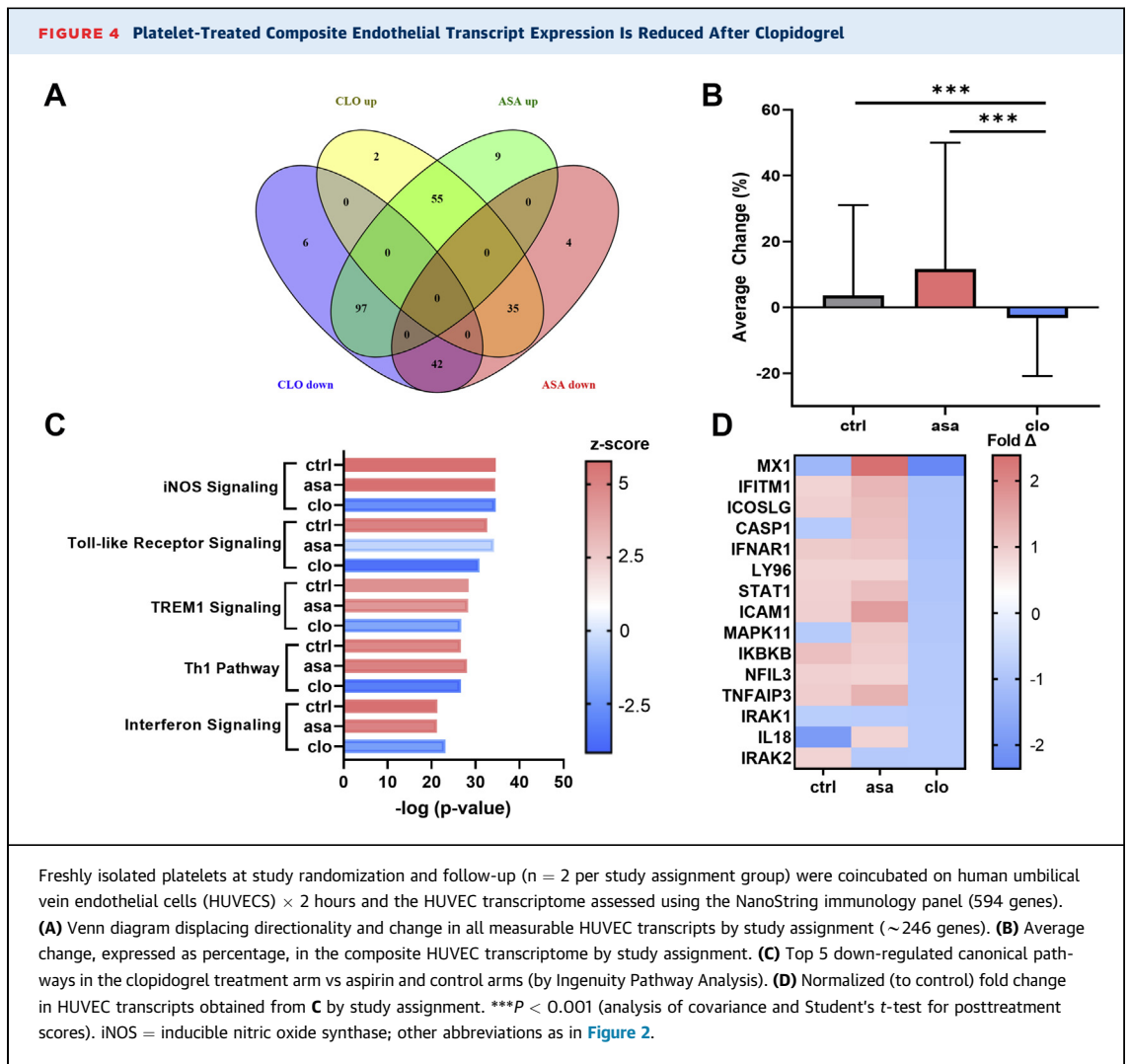
The underlying mechanisms in HIV driving enhanced CV risk may include the virus itself, complications from ART including enhanced platelet activation,[39,40] chronic inflammation,[41] immune dysregulation,[42] gut microbial disturbances, and endothelial dysfunction.[43] Although the crosstalk between HIV-induced inflammation and CVD has not been fully elucidated, an activated platelet phenotype is likely another prominent contributor.

Platelets play an important role in CVD both in the pathogenesis of atherosclerosis and in the development of acute thrombotic events.[27,44] Moreover, previous data show that PWH have an activated platelet phenotype as assessed by increased surface expression of P-selectin, activated glycoprotein αIIbβ3 (PAC-1), and increased aggregation in response to submaximal agonist simulation,[17,45] suggesting the importance of studying antiplatelet strategies in PWH. In prior studies of treated PWH, low-dose aspirin diminished, but did not abolish, the heightened platelet reactivity observed in HIV.[40] Certain antiviral therapies, including protease inhibitors[46] and abacavir,[40] increase platelet activation, whereas others, including ritonavir, inhibit bioactivation of

clopiogrel.[47] In line with this, in PWH with a recent acute coronary syndrome, dual antiplatelet therapy (aspirin plus clopidogrel) inhibited biomarkers of platelet activity less, when compared with the non-HIV patient population. These findings, and our own, highlight the importance of studying antiplatelet strategies in HIV, and potentially even monitoring for therapeutic efficacy.[46]

In our clinical trial, and similar to other clinical trials in non-HIV populations of aspirin on platelet function, we observed an uncoupling of the relationship in platelet aggregation and activation.[48] Despite a broader ability of aspirin to reduce platelet aggregation in response to arachidonic acid and ADP, we observed no decrease in platelet receptor expression with aspirin. By contrast, 2 weeks of clopidogrel decreased ADP-induced (but not arachidonic acid–induced) platelet aggregation and also reduced P-selectin and PAC-1 expression, suggesting that platelet activation markers are not solely dependent on COX-1 signaling. P-selectin is stored in alpha granules and secreted during stimulation.[49] P2Y$_{12}$ receptors amplify degranulation via signaling pathways, including thrombin mediated, and induce

FIGURE 4   Platelet-Treated Composite Endothelial Transcript Expression Is Reduced After Clopidogrel

Freshly isolated platelets at study randomization and follow-up (n = 2 per study assignment group) were coincubated on human umbilical vein endothelial cells (HUVECS) × 2 hours and the HUVEC transcriptome assessed using the NanoString immunology panel (594 genes). **(A)** Venn diagram displacing directionality and change in all measurable HUVEC transcripts by study assignment (~246 genes). **(B)** Average change, expressed as percentage, in the composite HUVEC transcriptome by study assignment. **(C)** Top 5 down-regulated canonical pathways in the clopidogrel treatment arm vs aspirin and control arms (by Ingenuity Pathway Analysis). **(D)** Normalized (to control) fold change in HUVEC transcripts obtained from **C** by study assignment. ***$P < 0.001$ (analysis of covariance and Student's *t*-test for posttreatment scores). iNOS = inducible nitric oxide synthase; other abbreviations as in **Figure 2**.

conformational changes in αIIbβ3 (PAC-1) receptors,[50] thus suggesting potential mechanisms of how a P2Y$_{12}$ inhibitor (clopidogrel), via ADP receptor antagonism, may inhibit platelet activation.

Clinically, a pilot study in 25 PWH and 44 healthy control subjects initially suggested that 1 week of low-dose 81-mg aspirin ameliorated platelet activation in ART-treated PWH and non-HIV participants via reduction of P-selectin levels, PAC-1 expression, and percentage of platelet-monocyte aggregates.[17] However, when compared with non-HIV, PWH after aspirin administration had a lessor reduction in thromboxane B$_2$ and higher aggregation after arachidonic acid stimulation, suggesting reduced efficacy in COX-1 mediated mechanisms of platelet inhibition in HIV. These studies, and the discordant results with the clinical trial of aspirin therapy presented here, highlight the importance of extending beyond pilot analyses and conducting adequately powered randomized clinical trials in PWH. Consistently, a randomized controlled trial of 121 PWH found that aspirin treatment for 12 weeks failed to reduce biomarkers of systemic inflammation and immune activation (ie, CD14), inflammation (ie, IL-6), coagulation (ie, D-dimer), or improve endothelial function.[18] A subsequent pilot clinical trial in PWH which included clopidogrel, also showed that despite no reduction of biomarkers of systemic inflammation, blood thrombogenicity was reduced.[24]

Although antiplatelet strategies are a cornerstone of CV care in patients with established CVD, use of antiplatelet therapies for primary prevention are far from certain, and underutilized, even when appropriate, in the HIV patient population.[51] More than 10 trials of aspirin for the prevention of a first CV event have yielded conflicting results.[52] In a high-risk group of patients with established CVD, clopidogrel confers a clinical benefit when compared with aspirin.[14]

When assessing the mechanistic impact of antiplatelet strategies, in non-HIV populations, clopidogrel, to a greater extent than aspirin, reduces platelet-activated glycoprotein αIIbβ3, P-selectin, and aggregation to various agonists and may be one reason why clopidogrel shows higher therapeutic benefit in high-CV-risk populations.[14,53] Consistently, a pilot randomized controlled trial of 14 subjects with PWH found that clopidogrel (and not aspirin) significantly reduced blood thrombogenecity after 24 weeks.[24]

To add to these prior preliminary data and further explore aspirin and clopidogrel impact on CVD development in ART-treated PWH, we investigated the effect of platelets on endothelial inflammation in vitro before and after various antiplatelet strategies. We discovered that clopidogrel reduced the platelets effector cell properties on endothelial inflammation, whereas aspirin did not. These findings may be attributed to reduced platelet P-selectin and activated glycoprotein αIIbβ3 expression (which were reduced in the clopidogrel-treated PWH arm) because these factors are shown to impact platelet-endothelial adhesion (via P-selectin–PSLG-1 interaction), which induces endothelial inflammation.[54] Pathway analyses of platelet-treated endothelial cells revealed down-regulation of interferon-regulated transcripts with clopidogrel as opposed to aspirin. Interferon pathways are dysregulated even in virologically controlled PWH, and altered interferon signaling in PWH is associated with clinical CVD.[55] In summary, the preferential impact on clopidogrel, both at the platelet inhibitory and endothelial inflammation reduction level, suggests potential efficacy of an ADP antagonist antiplatelet strategy to prevent atherothrombosis in PWH. However, further studies are required to explore the mechanistic underpinning of our clinical trial results and how this may modulate CV risk.

STUDY LIMITATIONS. The primary goal of this study was to investigate the impact of antiplatelet strategies in PWH, a high–CV-risk patient population with an activated platelet phenotype. Although we observed that clopidogrel reduces platelet activation and platelet effector function on the endothelium in vitro, we were unable to assess the clinical effect of clopidogrel in this high-risk group. Other limitations include our small sample size and open label design, and further studies are required to confirm these preliminary findings. Although we found an effect of antiplatelet therapy on platelet mediated endothelial cell activation in vitro, we did not measure circulating biomarkers nor the function of the vascular endothelium. Finally, although our findings may not be unique to the PWH population, our study provides exciting preliminary data on the potential therapeutic implications of clopidogrel in an understudied patient population at high CV risk.

CONCLUSIONS

We demonstrate that clopidogrel is more potent than aspirin to inhibit platelet activation as well as proinflammatory and atherogenic endothelial pathways in PWH. Clopidogrel could be a potential therapy to prevent atherothrombotic events in this high–CV-risk cohort. Placebo controlled studies with clinically important endpoints are needed to substantiate our findings in PWH and to determine the benefit to improve CV health vs the expected risk of bleeding.

ADDRESS FOR CORRESPONDENCE: Dr Jeffrey S. Berger, Center for the Prevention of Cardiovascular Disease, New York University School of Medicine, 435 East 30th Street, 7th Floor, New York, New York 10016, USA. E-mail: Jeffrey.berger@nyumc.org. Twitter: @plateletdoc.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: In persons with HIV, clopidogrel, as opposed to aspirin, reduces platelet activation and platelet-induced endothelial inflammation.

TRANSLATIONAL OUTLOOK: In persons with HIV, clopidogrel for the primary prevention of cardiovascular disease should be tested prospectively in more extensive clinical trials with hard clinical outcomes.

REFERENCES

1. Freiberg MS, Chang CC, Kuller LH, et al. HIV infection and the risk of acute myocardial infarction. *JAMA Intern Med*. 2013;173(8):614–622.

2. Rasmussen LD, May MT, Kronborg G, et al. Time trends for risk of severe age-related diseases in individuals with and without HIV infection in Denmark: a nationwide population-based cohort study. *Lancet HIV*. 2015;2(7):e288-e298.

3. Legarth RA, Ahlstrom MG, Kronborg G, et al. Long-term mortality in HIV-infected individuals 50 years or older: a nationwide, population-based cohort study. *J Acquir Immune Defic Syndr*. 2016;71(2):213–218.

4. Nkambule BB, Mxinwa V, Mkandla Z, et al. Platelet activation in adult HIV-infected patients on antiretroviral therapy: a systematic review and meta-analysis. *BMC Med*. 2020;18(1):357.

5. Morrell CN, Aggrey AA, Chapman LM, Modjeski KL. Emerging roles for platelets as immune and inflammatory cells. *Blood*. 2014;123(18):2759-2767.

6. Franco AT, Corken A, Ware J. Platelets at the interface of thrombosis, inflammation, and cancer. *Blood*. 2015;126(5):582-588.

7. Semple JW, Italiano JE Jr, Freedman J. Platelets and the immune continuum. *Nat Rev Immunol*. 2011;11(4):264–274.

8. Koupenova M, Clancy L, Corkrey HA, Freedman JE. Circulating platelets as mediators of immunity, inflammation, and thrombosis. *Circ Res*. 2018;122(2):337–351.

9. Rondina MT, Weyrich AS, Zimmerman GA. Platelets as cellular effectors of inflammation in vascular diseases. *Circ Res*. 2013;112(11):1506–1519.

10. Klinger MH, Jelkmann W. Role of blood platelets in infection and inflammation. *J Interferon Cytokine Res*. 2002;22(9):913–922.

11. Gawaz M, Langer H, May AE. Platelets in inflammation and atherogenesis. *J Clin Invest*. 2005;115(12):3378-3384.

12. Marcantoni E, Allen N, Cambria MR, et al. Platelet transcriptome profiling in HIV and ATP-binding cassette subfamily C member 4 (ABCC4) as a mediator of platelet activity. *J Am Coll Cardiol Basic Trans Science*. 2018;3(1):9–22.

13. Khawaja AA, Taylor KA, Lovell AO, et al. HIV antivirals affect endothelial activation and endothelial-platelet crosstalk. *Circ Res*. 2020;127(11):1365–1380.

14. Koo BK, Kang J, Park KW, et al. Aspirin versus clopidogrel for chronic maintenance monotherapy after percutaneous coronary intervention (HOST-EXAM): an investigator-initiated, prospective, randomised, open-label, multicentre trial. *Lancet*. 2021;397(10293):2487–2496.

15. CAPRIE Steering Committee. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). *Lancet*. 1996;348(9038):1329–1339.

16. Hsue PY. Mechanisms of cardiovascular disease in the setting of HIV infection. *Can J Cardiol*. 2019;35(3):238–248.

17. O'Brien M, Montenont E, Hu L, et al. Aspirin attenuates platelet activation and immune activation in HIV-1-infected subjects on antiretroviral therapy: a pilot study. *J Acquir Immune Defic Syndr*. 2013;63(3):280–288.

18. O'Brien MP, Hunt PW, Kitch DW, et al. A randomized placebo controlled trial of aspirin effects on immune activation in chronically human immunodeficiency virus-infected adults on virologically suppressive antiretroviral therapy. *Open Forum Infect Dis*. 2017;4(1):ofw278.

19. Ho WK, Hankey GJ, Eikelboom JW. Prevention of coronary heart disease with aspirin and clopidogrel: efficacy, safety, costs and cost-effectiveness. *Expert Opin Pharmacother*. 2004;5(3):493–503.

20. Donadini MP, Bellesini M, Squizzato A. Aspirin plus clopidogrel vs aspirin alone for preventing cardiovascular events among patients at high risk for cardiovascular events. *JAMA*. 2018;320(6):593–594.

21. Berger JS, Bhatt DL, Cannon CP, et al. The relative efficacy and safety of clopidogrel in women and men a sex-specific collaborative meta-analysis. *J Am Coll Cardiol*. 2009;54(21):1935–1945.

22. Berger JS, Abramson BL, Lopes RD, et al. Ticagrelor versus clopidogrel in patients with symptomatic peripheral artery disease and prior coronary artery disease: insights from the EUCLID trial. *Vasc Med*. 2018, 1358863X18775594.

23. Berger JS. Aspirin, clopidogrel, and ticagrelor in acute coronary syndromes. *Am J Cardiol*. 2013;112(5):737–745.

24. O'Brien MP, Zafar MU, Rodriguez JC, et al. Targeting thrombogenicity and inflammation in chronic HIV infection. *Sci Adv*. 2019;5(6):eaav5463.

25. Totani L, Evangelista V. Platelet-leukocyte interactions in cardiovascular disease and beyond. *Arterioscler Thromb Vasc Biol*. 2010;30(12):2357-2361.

26. Blann AD, Nadar SK, Lip GY. The adhesion molecule P-selectin and cardiovascular disease. *Eur Heart J*. 2003;24(24):2166–2179.

27. Barrett TJ, Schlegel M, Zhou F, et al. Platelet regulation of myeloid suppressor of cytokine signaling 3 accelerates atherosclerosis. *Sci Transl Med*. 2019;11(517):eaax0481.

28. Yee DL, Sun CW, Bergeron AL, Dong JF, Bray PF. Aggregometry detects platelet hyperreactivity in healthy individuals. *Blood*. 2005;106(8):2723-2729.

29. Nhek S, Clancy R, Lee KA, et al. Activated platelets induce endothelial cell activation via an interleukin-1beta pathway in systemic lupus erythematosus. *Arterioscler Thromb Vasc Biol*. 2017;37(4):707–716.

30. Newman JD, Echagarruga CT, Ogando YM, et al. Hyperglycemia enhances arsenic-induced platelet and megakaryocyte activation. *J Transl Med*. 2017;15(1):55.

31. Johnson-Tidey RR, McGregor JL, Taylor PR, Poston RN. Increase in the adhesion molecule P-selectin in endothelium overlying atherosclerotic plaques. Coexpression with intercellular adhesion molecule-1. *Am J Pathol*. 1994;144(5):952-961.

32. Guo L, Sun G, Wang G, Ning W, Zhao K. Soluble P-selectin promotes acute myocardial infarction onset but not severity. *Mol Med Rep*. 2015;11(3):2027–2033.

33. Alfonso F, Angiolillo DJ. Targeting p-selectin during coronary interventions: the elusive link between inflammation and platelets to prevent myocardial damage. *J Am Coll Cardiol*. 2013;61(20):2056–2059.

34. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation*. 2002;105(9):1135-1143.

35. Huo Y, Xia L. P-selectin glycoprotein ligand-1 plays a crucial role in the selective recruitment of leukocytes into the atherosclerotic arterial wall. *Trends Cardiovasc Med*. 2009;19(4):140–145.

36. Angiolillo DJ, Ueno M, Goto S. Basic principles of platelet biology and clinical implications. *Circ J*. 2010;74(4):597–607.

37. Marcus JL, Leyden WA, Chao CR, et al. HIV infection and incidence of ischemic stroke. *AIDS*. 2014;28(13):1911–1919.

38. Butt AA, Chang CC, Kuller L, et al. Risk of heart failure with human immunodeficiency virus in the absence of prior diagnosis of coronary heart disease. *Arch Intern Med*. 2011;171(8):737-743.

39. Freiberg MS, So-Armah K. HIV and cardiovascular disease: we need a mechanism, and we need a plan. *J Am Heart Assoc*. 2016;4(3):e003411.

40. Falcinelli E, Francisci D, Schiaroli E, et al. Effect of aspirin treatment on abacavir-associated platelet hyperreactivity in HIV-infected patients. *Int J Cardiol*. 2018;263:118–124.

41. Nou E, Lo J, Grinspoon SK. Inflammation, immune activation, and cardiovascular disease in HIV. *AIDS*. 2016;30(10):1495–1509.

42. Lichtenstein KA, Armon C, Buchacz K, et al. Low CD4+ T cell count is a risk factor for cardiovascular disease events in the HIV outpatient study. *Clin Infect Dis*. 2010;51(4):435–447.

43. Torriani FJ, Komarow L, Parker RA, et al. Endothelial function in human immunodeficiency virus-infected antiretroviral-naive subjects before and after starting potent antiretroviral therapy: the ACTG (AIDS Clinical Trials Group) study 5152s. *J Am Coll Cardiol*. 2008;52(7):569–576.

44. Davi G, Patrono C. Platelet activation and atherothrombosis. *N Engl J Med*. 2007;357(24):2482–2494.

45. Holme PA, Muller F, Solum NO, Brosstad F, Froland SS, Aukrust P. Enhanced activation of platelets with abnormal release of RANTES in human immunodeficiency virus type 1 infection. *FASEB J*. 1998;12(1):79–89.

46. Hauguel-Moreau M, Boccara F, Boyd A, et al. Platelet reactivity in human immunodeficiency virus infected patients on dual antiplatelet therapy for an acute coronary syndrome: the EVERE2ST-HIV study. *Eur Heart J*. 2017;38(21):1676–1686.

47. Itkonen MK, Tornio A, Lapatto-Reiniluoto O, et al. Clopidogrel increases dasabuvir exposure with or without ritonavir, and ritonavir inhibits the bioactivation of clopidogrel. *Clin Pharmacol Ther*. 2019;105(1):219–228.

48. Gurbel PA, Bliden KP, DiChiara J, et al. Evaluation of dose-related effects of aspirin on platelet function: results from the Aspirin-Induced Platelet Effect (ASPECT) study. *Circulation*. 2007;115(25):3156–3164.

49. Blair P, Flaumenhaft R. Platelet alpha-granules: basic biology and clinical correlates. *Blood Rev*. 2009;23(4):177–189.

50. Wijeyeratne YD, Heptinstall S. Anti-platelet therapy: ADP receptor antagonists. *Br J Clin Pharmacol*. 2011;72(4):647–657.

51. Park TE, Yusuff J, Sharma R. Use of aspirin and statins for the primary prevention of myocardial infarction and stroke in patients with human immunodeficiency virus infection. *Int J STD AIDS*. 2016;27(6):447–452.

52. Arnett DK, Blumenthal RS, Albert MA, et al. 2019 ACC/AHA guideline on the primary prevention of cardiovascular disease: executive summary: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *J Am Coll Cardiol*. 2019;74(10):1376–1414.

53. Moshfegh K, Redondo M, Julmy F, et al. Antiplatelet effects of clopidogrel compared with aspirin after myocardial infarction: enhanced inhibitory effects of combination therapy. *J Am Coll Cardiol*. 2000;36(3):699–705.

54. Dole VS, Bergmeier W, Patten IS, Hirahashi J, Mayadas TN, Wagner DD. PSGL-1 regulates platelet P-selectin-mediated endothelial activation and shedding of P-selectin from activated platelets. *Thromb Haemost*. 2007;98(4):806–812.

55. Santinelli L, De Girolamo G, Borrazzo C, et al. Alteration of type I interferon response is associated with subclinical atherosclerosis in virologically suppressed HIV-1-infected male patients. *J Med Virol*. 2021;93(8):4930–4938.

KEY WORDS aspirin, clopidogrel, endothelium, HIV, platelets

APPENDIX For supplemental figures and tables, please see the online version of this paper.