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Circulating Levels of Tissue Factor Microparticle Procoagulant Activity are Reduced with Antiretroviral Therapy and are Associated with Persistent Inflammation and Coagulation Activation among HIV Positive Patients

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Abstract

Activation of coagulation pathways may contribute to risk for non-AIDS related conditions among HIV positive patients. We measured tissue factor-dependent procoagulant activity on circulating microparticles (MP-TF) in the plasma of 163 HIV positive participants, both untreated and treated, with viral suppression. MP-TF activity was 39% lower among treated versus untreated participants ($p<0.001$), which persisted in adjusted models (−36%; $p=0.03$). Among treated participants, MP-TF activity correlated modestly with D-dimer ($r=0.24$; $p=0.01$), vWF ($r=0.36$; $p<0.001$), and IL-6 ($r=0.20$; $p=0.04$) levels. Future research should focus on mechanisms driving residual functional TF activity and whether these alterations have clinical consequences for non-AIDS defining complications.

INTRODUCTION

The spectrum of clinical disease among contemporary HIV positive patients receiving effective treatment with antiretroviral therapy (ART) now more commonly consists of non-AIDS defining chronic conditions such as cardiovascular disease.[12] Plasma D-dimer, a marker of coagulation activity, strongly predicts risk for all-cause mortality (largely non-AIDS related) as well as specific conditions like CVD and thromboembolic events among HIV patients.[3–7] In addition, D-dimer levels are correlated with HIV viral replication after starting or stopping ART, but remain elevated among treated patients when compared to uninfected controls.[689] Currently, mechanisms underlying persistent HIV-related

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abnormalities in coagulation biology, and the potential consequences for non-AIDS related clinical risk, remain poorly understood.

Tissue factor (TF) is a transmembrane protein that when complexed with factor VII(a), is responsible for initiating coagulation through activation of the extrinsic pathway. Circulating TF exists on cell surfaces (e.g., activated monocytes), as soluble cell-free TF in plasma (where it is largely inactive in coagulation), and on cell-derived microparticles (MP). MPs are membrane-encapsulated vesicles released from activated or apoptotic cells that contain and express surface proteins derived from the parent cell.[10] Though TF positive MPs (MP-TF) constitute only a small fraction of circulating TF, they likely represent a functionally active form of TF given their origin from activated cells (e.g., released form monocytes in response to endotoxemia).[1011] In this report we study the effect of ART treatment on MP-TF procoagulant activity, and explore associations between MP-TF activity with biomarkers of coagulation and systemic inflammation that in turn predict clinical risk among HIV positive patients.

METHODS

Study Population

Participants with HIV infection were recruited from 2007 to 2011 as part of ongoing research protocols at Hennepin County Medical Center (HCMC) HIV clinic in Minneapolis, MN. Plasma biomarkers of inflammation and coagulation had previously been measured among most of these participants. MP-TF activity was assessed for this study using stored specimens from participants who were either: a) naïve to treatment or off ART for ≥1 year (i.e., 'untreated'), or b) receiving ART with HIV viral load <200 copies/mL for ≥6 months (i.e., 'treated'). Patients were excluded for presence of a current bacterial infection, pregnancy, or known CVD. Study measures were obtained at a single study visit. Framingham Risk Score (FRS) for 10 year coronary heart disease risk was estimated using published algorithms.[12] All study protocols, including use of stored specimens for future research, were approved by the HCMC human subject research committee, and participants underwent written informed consent prior to enrollment.

Laboratory Measures

Plasma specimens were collected using EDTA (ethylenediaminetetraacetic acid) tubes and processed within 30 minutes of collection and frozen at −70°C until analysis. Plasma was isolated after a 2500g spin for 15 minutes in a refrigerated centrifuged at 4°C. Measures obtained from fresh blood at HCMC clinical laboratory included: HIV RNA level, serologies for hepatitis B and C, total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglycerides. All samples were handled in a blinded fashion.

D-dimer levels, interleukin-6 (IL-6), and high sensitivity CRP (hsCRP) were measured using methods previously described,[13] and von Willebrand factor (vWF) levels were measured on Stago STA-R analyzer (Stago Diagnostics, Parsipanny NJ), at the Laboratory for Clinical Biochemistry Research at the University of Vermont. These markers were chosen to assess coagulation activity and inflammation, and because they have been associated with clinical risk for CVD and all-cause mortality in HIV infected and uninfected populations.[6714–16]

MP-TF activity was measured as previously reported,[17] on MPs isolated from stored EDTA plasma by performing another centrifugation at 20,000g for 30 minutes at 4oC. The MP pellet was re-suspended in HBSA (20mM HEPES, 120mM NaCl, 1mg/ml) *via* mild sonication. A previously described two stage chromogenic assay was employed with the following modifications: 1) MPs were incubated for 2 hours with 2.5mM $CaCl_2$, 1nM FVIIa

and 150nM FX in the presence and absence of a TF blocking antibody, 2) Absorbance measurements were made every 30 seconds for 30 minutes after the addition of EDTA and FXa chromogenic substrate (Pefachrome 8595, Centerchem, Norwalk, CT).[18] MP-TF activity was calculated in relation to an Innovin™ tissue factor standard.

Statistical Methods

Descriptive statistics are reported as means with standard deviation (SD) and medians with inter-quartile range (IQR). Wilcoxon rank tests and the chi square test for categorical variables were used to compare characteristics of the untreated and ART-treated groups. For comparisons between groups, the relative percent difference (with 95% confidence interval) was obtained by exponentiating the mean difference on the natural log scale using generalized linear models. Fully adjusted models included the following covariates: age, gender, race/ethnicity, smoking status, co-infection with hepatitis B/C, prior AIDS, current CD4 count, total-to-HDL-C ratio and lipid lowering therapy. Hepatitis B/C co-infection was chosen over injection drug use (IDU) in covariate models as hepatitis B/C co-infection is more likely to influence the degree of current systemic inflammation, and findings were similar with and without inclusion of IDU in multivariate models. Total-to-HDL-C was included over other lipid parameters given the strong association with clinical event risk,[19,20] and that these 2 lipid measures differed the most between groups. Determinants of residual MP-TF activity were then explored among treated participants. Correlations were assessed using non-parametric rank tests due non-normal distribution of data. The level of significance was defined as $p<0.05$, and all analyses were conducted with SAS (version 9.2) and R statistical software (Version 2.10.1; http://www.cran.r-project.org).

RESULTS

Study Sample

Among the 163 HIV positive participants, 54 were untreated and 109 were receiving ART with a suppressed viral load. Demographic and clinical characteristics are presented in Table 1. Compared with untreated participants, those receiving ART were older, had a higher CD4 count, a higher proportion with a prior AIDS event, and a lower proportion with a history of IDU or who currently smoked cigarettes. Overall, traditional cardiovascular disease risk factors were more abnormal for treated versus untreated participants as indicated by 10-year FRS, use of lipid lowering drugs and cholesterol levels. Among treated participants, 51% were taking tenofovir disoproxil fumarate (DF), 50% were taking abacavir, 27% were taking a non-nucleoside reverse transcriptase inhibitor (efavirenz for 23%), 12% were taking raltegravir, and 49% were taking a protease inhibitor (26% atazanavir, 8% lopinavir, and 6% darunavir).

Comparison Between Untreated and ART-Treated Participants

Median values for MP-TF activity and other plasma biomarkers, including differences by ART use from unadjusted comparisons, are presented in Table 1. When compared to untreated participants, treated participants have 39% lower MP-TF activity, 52% lower D-dimer, 41% lower vWF, and 36% lower IL-6, but hsCRP levels were not significantly different. In fully adjusted models, differences were similar for MP-TF activity (36% lower; $p=0.03$), D-dimer (61% lower; $p<0.001$), wWF (37% lower; $p<0.001$), IL-6 (49% lower; $p<0.001$), and hsCRP (47% lower; $p=0.02$).

MP-TF Activity, Co-morbid Disease, Inflammation, and Coagulation Activity

Among untreated participants, MP-TF activity was higher for those with hepatitis B or C co-infection (74%; $p=0.03$), and was correlated with age ($r=0.30$; $p=0.03$), 10-year FRS

(r=0.34; p=0.01), and current CD4 count (r=0.43; p=0.001). In contrast, among treated participants, MP-TF activity was not correlated with age, 10-year FRS or current CD4 count, but was 28% lower (p=0.09) among those taking lipid-lowering medication versus not.

Finally, we then explored associations between MP-TF activity among treated patients with plasma biomarker levels to see if TF activity corresponded to residual abnormalities in biomarkers reflecting ongoing inflammation and coagulation. Scatter plots with correlations between MP-TF activity and inflammation and coagulation biomarkers are presented in Figure 1. In linear regression models, the change in log-e MP-TF activity for each 1 log-e increase in biomarker level was 0.19 for D-dimer (95% CI −0.02, 0.41; p=0.08), 0.84 for vWF (95% CI 0.44, 1.23; p<0.001), 0.26 for IL-6 (95% CI 0.02, 0.50; p=0.04), and 0.09 for hsCRP (95% CI −0.06, 0.24; p=0.22). Corresponding associations tended to be stronger in fully adjusted models at 0.31 for D-dimer (p=0.01), 0.86 for vWF (p<0.001), 0.32 for IL-6 (p=0.02), and 0.21 for hsCRP (p=0.01).

DISCUSSION

Activation of coagulation pathways has been identified as a potential mechanism contributing to risk for non-AIDS-defining complications among HIV positive patients.[4–6] We assessed the functional procoagulant activity of MP-TF, an active form of circulating cell-free TF, and determined that ART treatment with viral suppression was associated with lower TF pro-coagulant activity. Coagulation activity, as assessed by D-dimer levels, is persistently elevated among HIV-infected persons, despite viral suppression, though questions remain about the mechanisms up-regulating coagulation pathways.[9] Our findings that residual MP-TF activity among treated patients is associated with biomarkers of inflammation and coagulation is consistent with the hypothesis that residual inflammation may be contributing to HIV-related coagulation abnormalities through increased circulating TF activity.

In a sentinel study by Funderburg et. al., monocyte expression of TF was increased between HIV infected versus uninfected persons and also correlated directly with HIV RNA and D-dimer levels.[21] The authors subsequently reported that HIV increases the frequency of ‘activated’ monocyte phenotypes (CD14+/-CD16+), which express TF at high levels.[2122] Monocyte and tissue macrophage activation is an important mediator of coagulation and disease risk (e.g., premature atherosclerosis),[2324] and sCD14, a plasma marker of monocyte activation, is an independent predictor of mortality (largely non-AIDS related) among HIV positive patients.[25] Circulating MP-TF typically originate from monocytes that express TF, and our findings describing associations between MP-TF procoagulant activity with D-dimer and vWF levels are consistent with the notion that activation of TF-pathways is an important component of HIV-related coagulation abnormalities.

The clinical significance of MP-TF activity among HIV positive patients is unknown, but is potentially important given IL-6 and D-dimer associations with clinical risk. Epidemiologic data show that these plasma biomarkers are elevated among HIV infected persons, even after viral suppression, and elevated levels predict increased risk for myocardial infarction and all-cause mortality.[4–79] Specifically, in the SMART (Strategic Timing of AntiRetroviral Therapy) study, mortality risk associations with IL-6 and D-dimer were robust, present for those on and off ART, present for events in short and longer (>2 years) term, and were more extreme than what has been reported in uninfected populations.[62627] The hypothesis that persistent up-regulation of TF pathways in HIV patients explains, at least in part, D-dimer clinical risk prediction requires validation with additional measures of TF activity in larger studies.

The vast majority of TF in circulating blood is contained within mononuclear cells,[1011] and the inability to assess this fraction of peripheral blood was a limitation of our study. Newer methods that assess TF activity in whole blood (i.e., reflecting both cell-free and cell-associated activity) may now be applied to study epidemiologic associations with clinical event risk.[10] Similarly, we were not able to determine whether monocyte activation is driving TF activity, or accounts for the associations with inflammatory biomarkers. Additional limitations of our study include the cross-sectional design and lack of an HIV uninfected control group. Although median MP-TF activity for both treated and untreated participants are higher that what we've found in healthy controls while developing the assay, comparisons of TF activity between treated HIV positive patients and uninfected persons need to account for additional confounding factors and this will be important to clarify in future studies.

In summary, ART treatment is associated with reduced MP-TF pro-coagulant activity, though residual activity among virally suppressed patients is related to ongoing inflammation and may be an important contributor to coagulation abnormalities. Future research should focus on the underlying mechanisms driving TF activity and whether these alterations contribute to clinical event risk.

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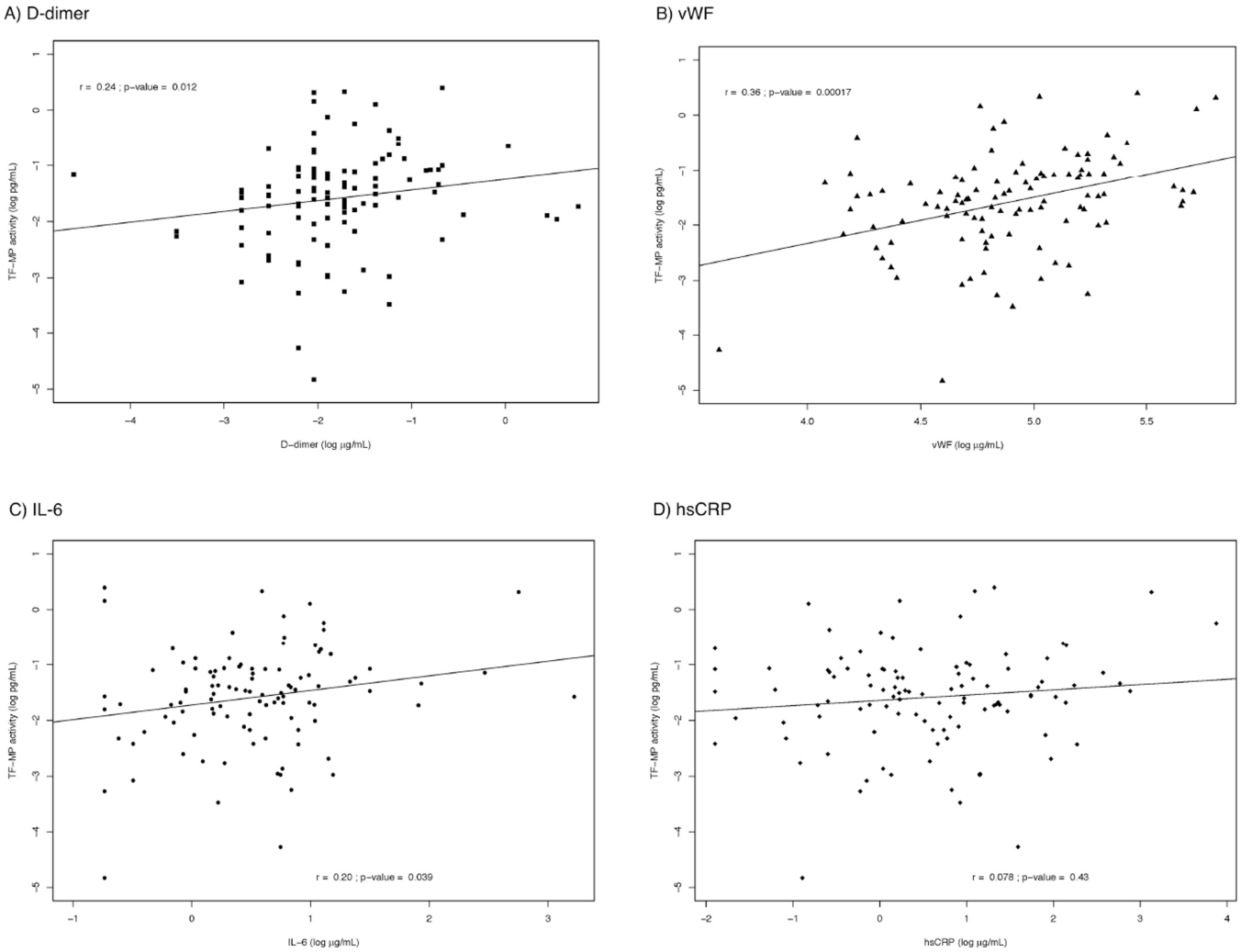

Figure 1. Correlations Between MP-TF Activity and Plasma Biomarker Levels

Legend: Scatter plot with trend line describes the correlation between MP-TF activity and D-dimer (a; square), vWF (b; triangle), IL-6 (c; circle) and hsCRP (d; diamond) levels. Spearman's rank correlation coefficient and p-value are reported.

TABLE 1

Clinical Characteristics and Laboratory Measures

	Untreated	ART-Treated	*p*-value*
Number	**54**	**109**	
Characteristic			
Age, median years (IQR)	41 (31, 45)	47 (43, 54)	<0.001
Male Gender, # (%)	45 (83)	98 (90)	0.23
Race/Ethnicity White, # (%) African American, # (%) Other, # (%)	23 (43) 24 (44) 7 (13)	63 (58) 36 (33) 10 (9)	--
Current Smoker, # (%)	35 (65)	48 (44)	0.01
Prior IDU, # (%)	17 (35)	8 (7)	<0.001
Hepatitis B or C, # (%)	14 (26)	15 (14)	0.12
Prior AIDS, # (%)	6 (12)	48 (47)	<0.001
BMI, median kg/m^2 (IQR)	26 (23, 29)	27 (24, 29)	0.65
Diabetes, # (%)	3(6)	9(8)	0.81
Blood pressure medication, # (%)	11 (20)	17 (16)	0.44
Lipid lowering medication, # (%)	1 (3)	30 (28)	0.001
10-year FRS, median % (IQR)	5.14 (1.23, 9.15)	7.13 (4.51, 10.44)	0.008
ART regimen Tenofovir, # (%) NNRTI, # (%) PI, # (%)	-- -- --	56 (51) 29 (27) 53 (49)	-- -- --
Laboratory Measure			
CD4+ count, median cells/mm^3 (IQR)	342 (259, 446)	522 (377, 745)	<0.001
HIV RNA, median $\log_{10}$ copies/mL (IQR)	4.2 (3.9, 4.7)	--	--
Total Chol./HDL-C, median (IQR)	4.0 (3.3, 5.5)	4.0 (3.4, 5.2)	0.25
Total Cholesterol, median mg/dL (IQR)	162 (129, 185)	185 (167, 212)	<0.001
LDL-C, median mg/dL (IQR)	88 (71, 112)	103 (85, 123)	0.02
HDL-C, median mg/dL (IQR)	37 (30, 48)	46 (36, 54)	<0.001
Triglycerides, median mg/dL (IQR)	118 (78, 155)	142 (99, 226)	0.002
MP-TF activity, median pg/mL (IQR)	0.35 (0.24, 0.54)	0.22 (0.14, 0.34)	<0.001
D-dimer, median μg/mL (IQR)	0.35 (0.19, 0.57)	0.15 (0.11, 0.25)	<0.001
vWF, median (IQR)	224.0 (178.5, 303.0)	131.5 (107.0, 181.0)	<0.001
IL-6, median pg/mL (IQR)	2.35 (1.53, 4.64)	1.67 (1.17, 2.35)	<0.001
hsCRP, median μg/mL (IQR)	1.99 (0.80, 5.18)	1.66 (0.83, 3.74)	0.02

* p-value for comparison by ART use; IDU=injection drug use; BMI=body mass index; FRS=Framingham risk score; NNRTI=non-nucleoside reverse transcriptase inhibitor; PI=protease inhibitor; LDL-C=low-density lipoprotein cholesterol; HDL-C=high-density lipoprotein cholesterol; MP-TF= microparticles expressing active tissue factor; IL-6=interleukin-6; hsCRP=high sensitivity C-reactive protein; sICAM-1=soluble intercellular adhesion molecule; vWF=von Willebrand Factor