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Proteomic Signature of Subclinical Coronary Artery Disease in People With HIV: Analysis of the REPRIEVE Mechanistic Substudy

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Background. People with HIV (PWH) have subclinical coronary artery disease (CAD) despite low traditional atherosclerotic cardiovascular disease (ASCVD) risk scores. Coronary plaque in PWH presents as a unique phenotype, but little is known about the contributions of specific inflammatory pathways to plaque phenotypes in PWH.

Methods. The REPRIEVE Mechanistic Substudy enrolled PWH on ART without known cardiovascular disease. We used a targeted discovery proteomics approach to evaluate 246 unique proteins representing cardiovascular, inflammatory, and immune pathways. Proteomic signatures were determined for presence of coronary artery calcium (CAC > 0) and presence of coronary plaque.

Results. Data were available for 662 participants (aged 51 [SD 6] years, ASCVD risk score 4.9% [SD 3.1%]). Among 12 proteins associated with both CAC and presence of coronary plaque, independent of ASCVD risk score, the odds ratios were highest for NRP1: 5.1 (95% confidence interval [CI], 2.3–11.4) for CAC and 2.9 (95% CI, 1.4–6.1) for presence of plaque. Proteins uniquely related to presence of plaque were CST3, LTBR, MEPE, PLC, SERPINA5, and TNFSF13B; in contrast, DCN, IL-6RA, OSMR, ST2, and VCAM1 were only related to CAC.

Conclusions. Distinct immune and inflammatory pathways are differentially associated with subclinical CAD phenotypes among PWH. This comprehensive set of targets should be further investigated to reduce atherosclerosis and ASCVD in PWH.

Clinical Trials Registration. NCT02344290.

Keywords. proteomics; HIV; coronary artery disease; plaque; CTA.

A significant proportion of antiretroviral therapy (ART) treated people with human immunodeficiency virus (PWH) have subclinical coronary artery disease (CAD) despite relatively low traditional atherosclerotic cardiovascular disease (ASCVD) risk scores and effective ART [1]. This population demonstrates a unique phenotype of subclinical atherosclerosis, with relatively high prevalence of plaque but more modest degrees of coronary artery calcium (CAC) [1]. Prior studies suggest that inflammation and immune activation are associated with cardiac events in PWH [2, 3], but a comprehensive assessment of these factors using a targeted discovery proteomic approach in relation to this plaque phenotype has not been performed.

The Randomized Trial to Prevent Vascular Events (REPRIEVE) is a global primary cardiovascular disease (CVD) prevention trial enrolling ART-treated PWH with low-to-moderate traditional cardiovascular risk and without known history of CVD [4]. Embedded within REPRIEVE is a mechanistic substudy including performance of coronary computed tomography angiography (CTA) [5]. Baseline data demonstrated the presence of CAD in almost 50% and CAC in 35% [1]. In analyses utilizing enzyme-linked immunosorbent assay (ELISA) assays, we demonstrated that markers of arterial inflammation and immune activation, including lipoprotein-associated phospholipase A2 (Lp-PLA2) and interleukin 6 (IL-6), were significantly associated with coronary plaque.

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To further our understanding of the potential mechanism of subclinical CAD in PWH with low-to-moderate traditional risk, we now perform a targeted discovery proteomic assessment among participants in the mechanistic substudy of REPRIEVE focusing on proteins with known associations with CVD, inflammation, cardiometabolism, and immunology with the following objectives: (1) identify an association between individual proteins and specific phenotypes of subclinical CAD, (2) assess for potential knowledge-based interactions between proteins to provide mechanistic insight into subclinical CAD in PWH, (3) evaluate whether the proteins are potential mediators of CAD independent of traditional ASCVD risk, and (4) determine the relationship of these proteins to standard measures of immune status.

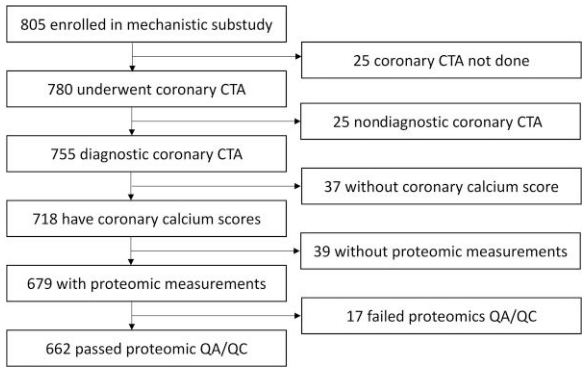

Figure 1. Study flow chart. Overall, 662 PWH were analyzed enrolling in the REPRIEVE mechanistic substudy. Abbreviations: CTA, computed tomography angiography; HIV, human immunodeficiency virus; PWH, people with HIV; QA/QC, quality assurance and quality control.

METHODS

Study Population

ART-treated PWH aged between 40 and 75 years with low-to-moderate cardiovascular risk and low-density lipoprotein levels, without known CVD or statin therapy, were recruited into REPRIEVE (NCT02344290) [4, 6]. We assessed ASCVD risk prospectively using the 2013 American College of Cardiology/American Heart Association pooled-cohort equation [6] including terms for age, sex, race, cholesterol (high-density lipoprotein and total), diabetes, blood pressure, and smoking. Individuals enrolled into the mechanistic substudy from 1 of 31 participating US REPRIEVE sites, mostly from the AIDS Clinical Trials Group (ACTG) Network. Detailed inclusion and exclusion criteria have been published [4, 5]. The Mass General Brigham Human Research Committee and local institutional review boards approved the study protocol, and participants provided written informed consent. Race, ethnicity, and natal sex were self-reported in accordance with ACTG guidelines. We analyzed data from individuals with diagnostic-quality CTA imaging and paired baseline proteomic measurements. Data from individuals with proteomic measurements not meeting quality assurance criteria were excluded (Figure 1). Immune parameters were obtained prior to the performance of CTA [1].

Coronary CTA Acquisition and Analysis

Detailed coronary CTA acquisition and analysis protocols have been previously described [1, 5]. In brief, electrocardiogram (ECG)-synchronized coronary CTA were performed on 64 or more slice computed tomography (CT) scanners at baseline using a standardized study protocol [1, 5]. CAC was evaluated using noncontrast CT images using a modified Agatston score [7]. Coronary arteries were evaluated on CTA images for the presence and composition of atherosclerotic plaque and the degree of stenosis. All CTA images were evaluated at a core laboratory facility in a standardized fashion [1, 5]. To assess proteomic signatures of subclinical CAD, CAC >0 on noncontrast CT and the presence of any coronary artery plaque (including both noncalcified and calcified plaques) were chosen as our primary outcomes given their relatively high prevalence in our population and the known relationship of CAC and plaque to major adverse cardiovascular events (MACE) in the general population [8]. An exploratory analysis was performed for the smaller subset with vulnerable plaques [9], defined as the presence of positive remodeling (dilatation of the coronary artery outer wall with remodeling index, >1.1), low-density plaque (CT attenuation <30 Hounsfield units), or napkin-ring sign (low central attenuation with ring-like peripheral high attenuation) [1].

Proteomic Measurements and Quality Control

For proteomic analysis, fasting plasma samples were drawn prior to study drug initiation, and stored at -80°C. Three commercially available multiplex immunoassays were used (Olink Target 96 Cardiovascular III, Immuno-oncology, and Cardiometabolic) based on prior studies in smaller cohorts [10] to quantify 275 unique proteins. Definitions, reproducibility, and validation information regarding the proteins can be found at: https://www.olink.com. Data for individuals in whom all measurements on 1 of the protein panels were flagged with warnings were excluded ($n=17$; Figure 1). Consistent with the evaluation methodology of other CVD cohorts with this proteomic technology [11], data for 29 proteins were excluded as $\geq$50% of study samples had values below the limit of detection; 246 proteins met quality control requirements and were analyzed using Normalized Protein eXpression values provided by Olink. A list of proteins included and excluded in the analysis can be found in Supplementary Tables 1 and 2.

Statistical Analysis

Continuous variables are presented as means and standard deviations, or medians and interquartile ranges, while categorical parameters are presented as counts and percentages. To assess associations between proteins and CAD phenotypes, logistic regression was used to estimate the odds ratios (OR) for CAD outcomes per a doubling of each protein. Multiple comparisons were adjusted for using the false discovery rate (FDR) method by Benjamini and Hochberg [12]. To assess whether these associations are independent of traditional risk, we ran multivariate logistic regression models adjusting for ASCVD risk score. Proteins showing an association with any CAD outcome at an FDR level <0.1 were further analyzed. We calculated the interpair Pearson correlation between these proteins and used the 1-correlation value as a distance metric for hierarchical clustering to assess whether there are any apparent protein clusters among significant proteins.

We used the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) knowledge-based protein-protein interaction database to further elucidate potential connections between the significant proteins [13]. Enrichment analysis was not done due to the lack of sufficient background proteins.

We conducted mediation analysis to determine whether key proteins mediated the direct association of traditional ASCVD risk to CAD outcomes. We simulated mediation using 1000 Monte Carlo draws for quasi-Bayesian approximation using the mediation R package (version 4.5.0) [14]. Analyses were done using the *ggstatsplot* (version 0.9.0) R package.

Protein expression levels were compared by CD4, nadir CD4, viral load, and ART regimen (Mann-Whitney or Kruskal-Wallis ANOVA as appropriate) with post hoc comparisons (Dunn-test with Holm's *P* value correction).

An FDR corrected *P* value of $<.1$ was considered significant in assessing the relationship of proteins to plaque outcomes in our primary analysis. We used a nominal *P* value threshold of $\leq.01$ in exploratory analyses. For comparisons between those with and without plaque or CAC, and for our mediation analysis, a 2-sided *P* value smaller than .05 was considered statistically significant. All statistical analyses were done using R (version 4.0.2) [15].

RESULTS

Patient Characteristics

Overall, full data were available and analyzed for 662 individuals (Figure 1) (aged 50.8 [SD 5.8] years; male sex, 83%, 547/662; ASCVD risk score, 4.9% [SD 3.1%]). Age and demographics were similar in the analysis cohort and the full cohort recruited into the substudy (Supplementary Table 3). All participants were taking ART (duration 11.6 [SD 6.5] years) and 88% had undetectable viral load. Among the 662 PWH, 321 (48%) had evidence of coronary plaque, of whom 231 (72%, 231/321) had CAC >0 and 145 (45%, 145/321) demonstrated vulnerable plaque. Of those with CAC, the majority, 163/231 had CAC values between 1 and 100. Smaller subsets, 56/231 and 12/231 demonstrated CAC between 101 and 400 and greater than 400, respectively. Most individuals with plaque had nonobstructive CAD, with only 22 individuals having stenoses greater than 50%. Demographic, risk factor, and HIV-associated parameter information can be found in Table 1, stratified by presence of any CAC and any plaque.

Associations Between Proteins and Plaque Phenotypes

Proteins significantly associated with CAD outcomes before adjustment for ASCVD are shown in Supplementary Figure 1, while results after adjustment are shown in Figure 2. The known biological function and relationship to ASCVD of individually significant proteins are shown in Table 2. Results for all proteins are presented in Supplementary Table 2.

Overall, 23 proteins were significant for at least 1 of our 2 CAD outcomes in models adjusting for ASCVD risk (Figure 3). Of these 23 proteins, 12 were associated with both CAC >0 and presence of plaque. Of these, the ORs were highest for NRP1: 5.1 (95% confidence interval [CI], 2.3–11.4) for CAC and 2.9 (95% CI, 1.4–6.1) for plaque. CST3, LTBR, MEPE, PLC, SERPINA5, and TNFSF13B were uniquely related to presence of plaque (see Supplementary Table 2 for protein functions and adjusted ORs for plaque phenotypes). In contrast, DCN, IL-6RA, OSMR, ST2, and VCAM1 were associated with the presence of CAC. Hierarchical clustering showed 1 or 2 clusters of moderately interrelated proteins among those significantly related to either plaque or CAC. For the 23 proteins, adjusted ORs and nominal *P* values for both outcomes can be found in Figure 3.

In exploratory analyses, NRP1, AOC3, MEPE, and PGF were most strongly related to the presence of vulnerable plaque adjusting for ASCVD risk, with NRP1 having the highest OR, 3.3 (95% CI, 1.4–7.9) (Supplementary Table 2 and Supplementary Figure 2).

Knowledge-Based Interactions Between Significant Proteins Using the STRING Database

To provide biologic insight, we annotated the 23 proteins described above using the STRING database and found multiple connections. The most common reactome pathways associated with our proteins were (pathway name [number of proteins involved]): immune system (n = 11), cytokine signaling in immune system (n = 9), signaling by interleukins (n = 6), TNFR2 noncanonical NF-κB pathway (n = 4), and TNFs binding to physiological receptors (n = 3). The resulting protein-protein interaction network is presented in Figure 4A. Patterns of proteins uniquely associated with either CAC, coronary plaque, or both are shown in Figure 4B. STRING

Table 1. Patient Characteristics

Variable	Overall (n = 662)	Presence of Coronary Calcium		Presence of Coronary Plaque	
		No (n = 431)	Yes (n = 231)	No (n = 341)	Yes (n = 321)
Demographic and behavioral characteristics					
Age, y	50.8 ± 5.8	49.8 ± 5.5	52.6 ± 5.8	49.6 ± 5.5	52.0 ± 5.8
Natal sex					
Female	115 (17)	88 (20)	27 (12)	78 (23)	37 (12)
Male	547 (83)	343 (80)	204 (88)	263 (77)	284 (88)
Race					
Asian	8 (1.2)	5 (1.2)	3 (1.3)	5 (1.5)	3 (0.9)
Black or African American	233 (35)	166 (39)	67 (29)	141 (41)	92 (29)
White	354 (53)	214 (50)	140 (61)	159 (47)	195 (61)
Other	67 (10)	46 (11)	21 (9.1)	36 (11)	31 (9.7)
Ethnicity (n = 653)					
Hispanic or Latino	163 (25)	117 (28)	46 (20)	85 (25)	78 (25)
Not Hispanic or Latino	490 (75)	307 (72)	183 (80)	251 (75)	239 (75)
Smoking status (n = 661)					
Current	158 (24)	100 (23)	58 (25)	81 (24)	77 (24)
Former	211 (32)	131 (30)	80 (35)	99 (29)	112 (35)
Never	292 (44)	200 (46)	92 (40)	161 (47)	131 (41)
Cardiovascular and metabolic characteristics					
ASCVD, %	4.9 ± 3.1	4.4 ± 2.9	5.8 ± 3.3	4.3 ± 2.9	5.6 ± 3.2
ASCVD risk group					
0–2.5	156 (24)	120 (28)	36 (16)	107 (31)	49 (15)
2.5–5	224 (34)	154 (36)	70 (30)	116 (34)	108 (34)
5–7.5	154 (23)	91 (21)	63 (27)	70 (21)	84 (26)
7.5–10	88 (13)	47 (11)	41 (18)	32 (9.4)	56 (17)
>10	40 (6.0)	19 (4.4)	21 (9.1)	16 (4.7)	24 (7.5)
Family history of premature CVD	145 (23)	90 (21)	55 (25)	65 (20)	80 (26)
Hypertension	213 (32)	127 (29)	86 (37)	100 (29)	113 (35)
Diabetes	3 (0.5)	1 (0.2)	2 (0.9)	0 (0)	3 (0.9)
BMI, kg/m^2	27.3 ± 4.3	27.4 ± 4.5	27.3 ± 4.1	27.4 ± 4.5	27.3 ± 4.2
Fasting glucose, mg/dL	92.9 ± 12.5	92.3 ± 12.1	94.0 ± 13.2	91.7 ± 11.0	94.1 ± 13.8
eGFR, mL/min/1.73m^2	88.4 ± 16.3	88.7 ± 16.5	87.6 ± 15.8	89.3 ± 16.5	87.3 ± 15.9
LDL-C, mg/mL	108.3 ± 30.5	106.8 ± 30.3	111.0 ± 30.8	104.1 ± 29.4	112.6 ± 31.1
HDL-C, mg/dL	50.9 ± 18.7	50.6 ± 18.5	51.3 ± 19.0	51.7 ± 19.3	49.9 ± 17.9
Triglycerides, mg/dL	133.6 ± 85.2	135.0 ± 86.0	131.2 ± 83.7	133.1 ± 88.9	134.2 ± 81.1
Prior statin use	53 (8.0)	28 (6.5)	25 (11)	17 (5.0)	36 (11)
Prior antihypertensive medication	133 (20)	76 (18)	57 (25)	61 (18)	72 (22)
HIV-related health history					
Total ART use, y	11.6 ± 6.5	11.1 ± 6.4	12.6 ± 6.7	11.0 ± 6.4	12.2 ± 6.6
Entry ART regimen					
NRTI + INSTI	293 (44)	187 (43)	106 (46)	147 (43)	146 (45)
NRTI + NNRTI	167 (25)	111 (26)	56 (24)	90 (26)	77 (24)
NRTI + PI	118 (18)	80 (19)	38 (16)	64 (19)	54 (17)
NRTI-sparing	20 (3.0)	11 (2.6)	9 (3.9)	9 (2.6)	11 (3.4)
Other NRTI-containing	64 (9.7)	42 (9.7)	22 (9.5)	31 (9.1)	33 (10)
HIV RNA, copies/mL, (n = 654)					
<LLQ	573 (88)	374 (88)	199 (88)	291 (86)	282 (89)
LLQ–400	65 (9.9)	39 (9.1)	26 (11)	33 (9.8)	32 (10)
400+	16 (2.4)	14 (3.3)	2 (0.9)	13 (3.9)	3 (0.9)
CD4 category, cells/mm^3	624.2 ± 275.1	642.4 ± 274.4	590.3 ± 273.8	635.2 ± 266.1	612.6 ± 284.3
Nadir CD4 category, cells/mm^3 (n = 646)					
<50	146 (23)	84 (20)	62 (27)	64 (19)	82 (26)
50–199	192 (30)	127 (30)	65 (29)	102 (31)	90 (28)
200–349	180 (28)	116 (28)	64 (28)	95 (29)	85 (27)
350+	128 (20)	92 (22)	36 (16)	69 (21)	59 (19)

Data are presented as mean ± standard deviations, or as frequencies (percentages). Percentages are presented considering the proportion of available data presented in parenthesis for parameters with missing values.

Abbreviations: ART, antiretroviral therapy; ASCVD, atherosclerotic cardiovascular disease; BMI, body mass index; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; HIV, human immunodeficiency virus; INSTI, integrase strand transfer inhibitor; LDL-C, low-density lipoprotein cholesterol; LLQ, lower limit of quantification; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

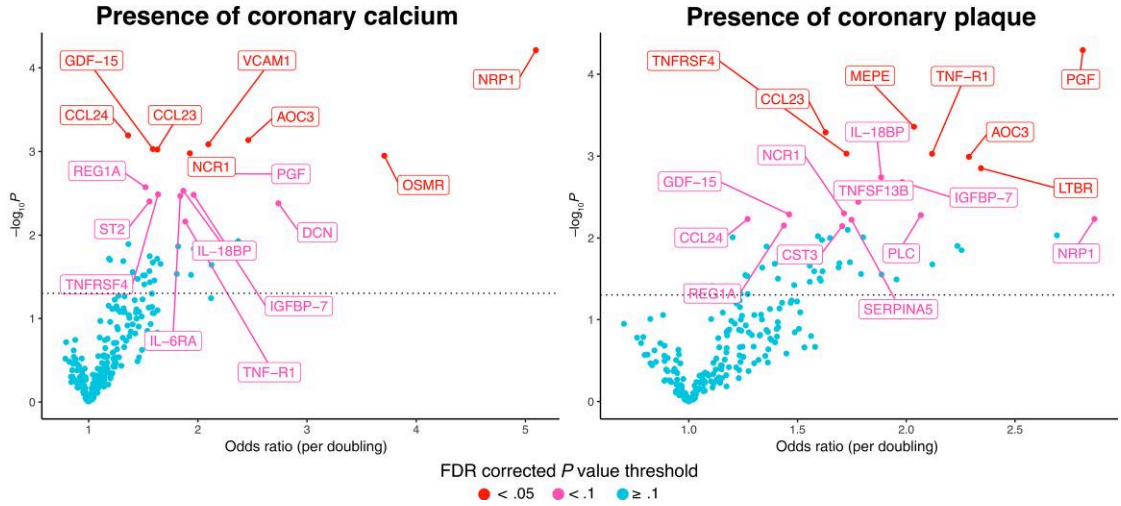

Figure 2. Volcano plots of ASCVD risk adjusted odds ratios between proteins and CAD outcomes. Each point represents a single protein. The position of each point represents the odds ratio and the *P* value for the association between the protein and the given CAD outcome corrected for ASCVD risk. The y axis shows the $-\log_{10}$ of the *P* values, therefore the higher it is the more significant the association. The dotted horizontal line indicates $P = .05$. The proteins are colored according to FDR corrected *P* values, where *P* values >.1 are blue, *P* values between .05 and .1 are pink, and $P < .05$ are red. All proteins with FDR corrected $P < .1$ are labeled on the plots. Abbreviations: ASCVD, atherosclerotic cardiovascular disease; CAD: coronary artery disease; FDR, false discovery rate; OR, odds ratio. Protein name abbreviations can be found in Supplementary Material.

characteristics on homology, coexpression, experimentally determined interaction, and text mining scores are shown in Supplementary Table 4.

Mediation Analysis on the Effects of ASCVD on CAD Outcomes

We further assessed whether these 23 proteins mediated the associations between ASCVD risk score and plaque phenotypes. ASCVD risk score was highly related to both CAD outcomes. Several proteins were shown to have a significant individual effect to mediate the association between ASCVD risk score and CAC, including IL-18BP, TNF-R1, IGFBP-7, PLC, NCR1, PGF, CST3, DCN, GDF-15, and ST2. However, the absolute value of the proportion of the effect of ASCVD mediated through these proteins was very low (range, 4.6%–12.6%; Table 3). Results in the mediation analysis for any plaque were generally similar, with the addition of MEPE as having a significant effect to mediate the association of ASCVD risk score. Again, however, the absolute proportion of the effect of ASCVD mediated through these proteins was very low (range, 3.9%–10.3%; Table 3).

Association With Immune Function Indices

Lower nadir CD4 counts were associated with higher GDF-15 levels and lower SERPINA5 levels. Lower CD4 counts were associated with higher CST3, IL-18BP, IL-6RA, NCR1, PLC, TNF-R1, and TNFRSF4 levels (Supplementary Table 5 and Supplementary Figure 3). Minimal differences were seen comparing by detectable viral load and by ART regimen, without a consistent pattern (Supplementary Figure 4).

DISCUSSION

In this study, we demonstrate a novel proteomic signature of subclinical CAD in PWH, with different sets of proteins relating to the presence of CAC and any plaque, after controlling for traditional ASCVD risk. Key findings include discovery of proteins representing inflammatory and immune pathways not previously associated with CAD in PWH, including NRP1, involved in cell migration and endothelial function [16]. Clear relationships of proteins to known pathways of NF-κB, cytokine response and TNF-related inflammation are seen. These findings inform us of novel biological pathways beyond traditional risk factors that may contribute to subclinical CAD and may be targets for specific immune modulation strategies.

We identified a rich set of proteins associated with presence of subclinical CAD, controlling for traditional ASCVD risk in PWH. Among the proteins most strongly associated were some that have been shown in general-population studies to be highly relevant to CAD including: TNF-R1, associated with CV mortality in those with chronic coronary heart disease (CHD) [17]; Il-18BP, increased in acute coronary patients [18]; IGFBP-7, a recently discovered marker of CAD [19]; PGF, thought to represent a chronic source of vascular inflammation, and a marker of overall mortality after non-ST-segment acute coronary syndrome [20]; CCL23, a chemokine that may contribute to vascular inflammation and a useful marker to detect atherosclerosis [21]; AOC3, associated with cardiovascular risk factors and related to preclinical atherosclerosis [22, 23]; TNFRSF4, postulated to influence atherosclerosis [24];

Table 2. Known Biological Function and Relationship to ASCVD in Significant Proteins

Protein	Biological Function, From STRING	Clinically Relevant Function, From the Literature	Related to CAC	Related to Presence of Plaque
AOC3	Amine oxidase, copper containing 3; membrane primary amine oxidase Cell adhesion protein that participates in lymphocyte extravasation and recirculation by mediating the binding of lymphocytes to peripheral lymph node vascular endothelial cells in an L-selectin-independent fashion Has semicarbazide-sensitive (SSAO) monoamine oxidase activity May play a role in adipogenesis	• Associated with cardiovascular risk factors	X	X
CCL23	C-C motif chemokine ligand 23	• May contribute to vascular inflammation • Marker to detect atherosclerosis • Has chemotactic properties for monocytes, resting T-lymphocytes, and neutrophils • Increased in HIV	X	X
CCL24	C-C motif chemokine 24 Chemotactic for resting T-lymphocytes, and eosinophils Has lower chemotactic activity for neutrophils but none for monocytes and activated lymphocytes Is a strong suppressor of colony formation by a multipotential hematopoietic progenitor cell line Binds to CCR3 Belongs to the intercrine β (chemokine CC) family	• Marker of cardiac ageing	X	X
CST3	Cystatin-C As an inhibitor of cysteine proteinases, this protein is thought to serve an important physiological role as a local regulator of this enzyme activity Belongs to the cystatin family	• Plays pleiotropic roles in human vascular pathophysiology • Strong association between circulating cystatin C and risk of future coronary heart disease, ischemic stroke, and heart failure • Cystatin C concentrations were associated with CVD risk after adjusting for age, sex, and traditional risk factors (however, this is not thought to be causal)		X
DCN	Decorin May affect the rate of fibrils formation Small leucine rich repeat proteoglycans	• Decorin has antifibrotic, anti-inflammation, antioxidant, and antiangiogenic properties	X	
GDF-15	Growth differentiation factor 15 Belongs to the TGF-β family	• GDF15 associated with incident ASCVD, incident heart failure, all-cause mortality, CVD death • While GDF-15 is weakly expressed in most tissues under physiological conditions, its expression may significantly increase in response to cardiovascular inflammation and tissue injury • Ischemia, mechanical stretch, neurohormones, and proinflammatory cytokines stimulate the expression of GDF-15 in cardiac myocytes • Its prominent antiapoptotic, antihypertrophic, and anti-inflammatory actions in cardiovascular disease models suggest that GDF-15 may play a counter-regulatory role in the context of cardiovascular injury	X	X
IGFBP-7	Insulin-like growth factor-binding protein 7 Binds IGF-I and IGF-II with a relatively low affinity Stimulates prostacyclin (PGI2) production Stimulates cell adhesion I-set domain containing	• Marker of CAD	X	X
IL-18BP	Interleukin-18-binding protein Isoform A binds to IL-18 and inhibits its activity Functions as an inhibitor of the early Th1 cytokine response Immunoglobulin-like domain containing	• Increased in acute coronary patients	X	X
IL-6RA	Interleukin-6 receptor subunit α Part of the receptor for interleukin 6 Binds to IL-6 with low affinity, but does not transduce a signal Signal activation necessitate an association with IL6ST Activation may lead to the regulation of the immune response, acute-phase reactions, and hematopoiesis CD molecules		X	

Table 2. Continued

Protein	Biological Function, From STRING	Clinically Relevant Function, From the Literature	Related to CAC	Related to Presence of Plaque
LTBR	Tumor necrosis factor receptor superfamily member 3 Receptor for the heterotrimeric lymphotoxin containing LTA and LTB, and for TNFS14/LIGHT Promotes apoptosis via TRAF3 and TRAF5 May play a role in the development of lymphoid organs Tumor necrosis factor receptor superfamily	• Involved in NF-kB signaling pathways		X
MEPE	Matrix extracellular phosphoglycoprotein Promotes renal phosphate excretion and modulates mineralization SIBLING family	• Associated with ischemic stroke		X
NCR1	Natural cytotoxicity triggering receptor 1 Cytotoxicity-activating receptor that may contribute to the increased efficiency of activated natural killer (NK) cells to mediate tumor cell lysis		X	X
NRP1	Neuropilin-1 The membrane-bound isoform 1 is a receptor involved in the development of the cardiovascular system, in angiogenesis, in the formation of certain neuronal circuits, and in organogenesis outside the nervous system It mediates the chemorepulsant activity of semaphorins It binds to semaphorin 3A, PLGF-2 isoform of PGF, VEGF165 isoform of VEGFA, and VEGFB Coexpression with KDR results in increased VEGF165 binding to KDR as well as increased chemotaxis Regulate VEGF-induced angiogenesis	• Role in signaling and angiogenesis	X	X
OSMR	Oncostatin-M-specific receptor subunit β Associates with IL31RA to form the IL-31 receptor Binds IL-31 to activate STAT3 and possibly STAT1 and STAT5 Capable of transducing OSM-specific signaling events Fibronectin type III domain containing	• Anti-inflammatory, though to decrease IL-8 mediated recruitment of neutrophils	X	
PGF	Placental growth factor; placenta growth factor Growth factor active in angiogenesis and endothelial cell growth, stimulating their proliferation and migration It binds to the receptor FLT1/VEGFR-1 Isoform PlGF-2 binds NRP1/neuropilin-1 and NRP2/neuropilin-2 in a heparin-dependent manner Also promotes cell tumor growth	• Chronic source of background vascular inflammation • Marker of overall mortality after non-ST segment acute coronary syndrome	X	X
PLC	Basement membrane-specific heparan sulfate proteoglycan core protein Integral component of basement membranes Component of the glomerular basement membrane, responsible for the fixed negative electrostatic membrane charge, and provides a barrier which is both size- and charge-selective It serves as an attachment substrate for cells Plays essential roles in vascularization Critical for normal heart development and for regulating the vascular response to injury Also required for avascular cartilage development I-set domain containing	• Regulates inflammation and angiogenesis		X
REG1A	Lithostathine-1-α Might act as an inhibitor of spontaneous calcium carbonate precipitation May be associated with neuronal sprouting in brain, and with brain and pancreas regeneration C-type lectin domain containing		X	X
SERPINA5	Plasma serine protease inhibitor Heparin-dependent serine protease inhibitor acting in body fluids and secretions Inactivates serine proteases by binding irreversibly to their serine activation site Involved in the regulation of intravascular and extravascular proteolytic activities Plays hemostatic roles in the blood plasma Acts as a procoagulant and proinflammatory factor by			X

Table 2. Continued

Protein	Biological Function, From STRING	Clinically Relevant Function, From the Literature	Related to CAC	Related to Presence of Plaque
	inhibiting the anticoagulant activated protein C factor as well as the generation of activated protein C factor by the thrombin/thrombomodulin complex			
ST2	Interleukin-1 receptor-like 1 Receptor for IL-33 Signaling requires association of the coreceptor IL1RAP Its stimulation recruits MYD88, IRAK1, IRAK4, and TRAF6, followed by phosphorylation of MAPK3/ERK1 and/or MAPK1/ERK2, MAPK14, and MAPK8 Possibly involved in helper T-cell function I-set domain containing	• Associated with death, heart failure, MACE (sST2)	X	
TNF-R1	Tumor necrosis factor receptor superfamily member 1A Receptor for TNFSF2/TNF-αand homotrimeric TNFSF1/ lymphotoxin-α The adapter molecule FADD recruits caspase-8 to the activated receptor The resulting death-inducing signaling complex (DISC) performs caspase-8 proteolytic activation which initiates the subsequent cascade of caspases (aspartate- specific cysteine proteases) mediating apoptosis Contributes to the induction of noncytocidal TNF effects including antiviral state and activation of the acid sphingomyelinase CD molecules	• Associated with cardiovascular mortality in patients with chronic coronary heart disease	X	X
TNFRSF4	Tumor necrosis factor receptor superfamily member 4 Receptor for TNFSF4/OX40L/GP34 A costimulatory molecule implicated in long-term T-cell immunity CD molecules	• Blocking of the TNFRSF4 pathway leads to reducedatherogenesis and a regression of atherosclerosis	X	X
TNFSF13B	Tumor necrosis factor ligand superfamily member 13B Cytokine that binds to TNFRSF13B/TACI and TNFRSF17/ BCMA TNFSF13/APRIL binds to the same 2 receptors Together, they form a 2 ligands-2 receptors pathway involved in the stimulation of B- and T-cell function and the regulation of humoral immunity A third B-cell specific BAFF-receptor (BAFFR/BR3) promotes the survival of mature B cells and the B-cell response CD molecules			X
VCAM1	Vascular cell adhesion protein 1 Important in cell-cell recognition Appears to function in leukocyte-endothelial cell adhesion Interacts with integrin α-4/β-1 (ITGA4/ITGB1) on leukocytes, and mediates both adhesion and signal transduction The VCAM1/ITGA4/ITGB1 interaction may play a pathophysiologic role both in immune responses and in leukocyte emigration to sites of inflammation C2-set domain containing	• Marker of endothelial dysfuntion, increased in rheumatoid arthritis, associated with plaque and carotid intima-media thickness (CIMT)	X	

Abbreviations: ASCVD, atherosclerotic cardiovascular disease; CAC, coronary artery calcium; CAD, coronary artery disease; CVD, cardiovascular disease; HIV, human immunodeficiency virus; IL, interleukin; MACE, major adverse cardiovascular events; NF-kB, nuclear factor-κB; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins.

MEPE, associated with ischemic stroke [25]; PLC, which regulates inflammation and angiogenesis [26]; CCL24, which acts on CCR3-bearing cells including T-helper cells type 2, found to be a strong marker of cardiac ageing [27]; and CST3, which encodes cystatin 3, a potent cysteine protease inhibitor, thought to play a role in human vascular pathophysiology, relating strongly to future CHD and ischemic stroke in humans [28]. Significant interest has been focused on GDF-15, which was recently associated with incident all-cause mortality and CVD death among a population of patients without HIV infection [29], as well as death and MACE in the Framingham Heart Study. GDF-15 expression is thought to increase in relation to cardiovascular inflammation and tissue injury, potentially in a counter-regulatory protective role [30].

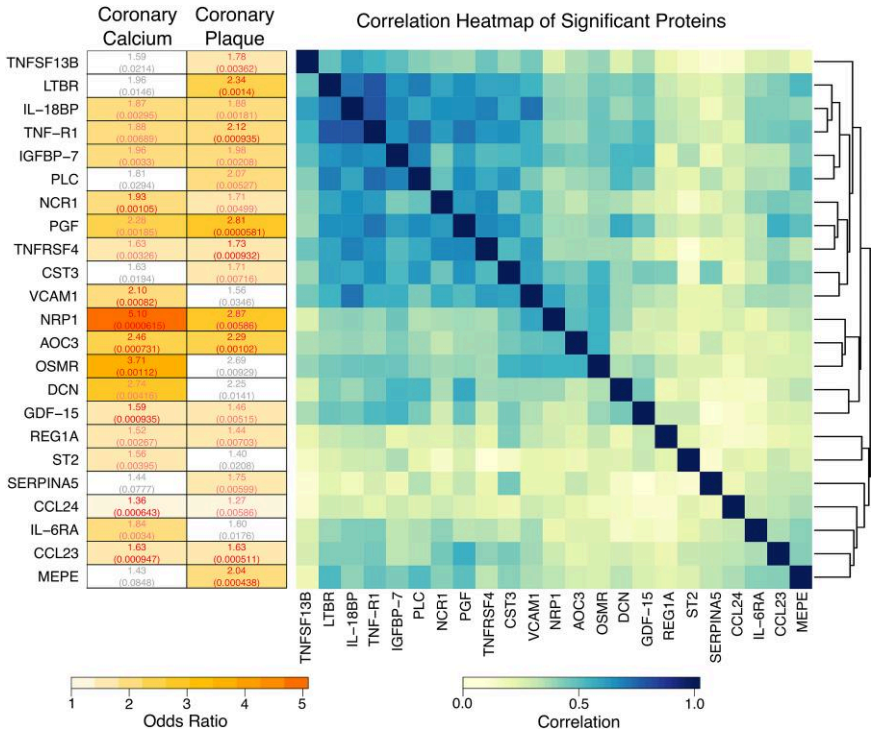

Figure 3. Odds ratios and correlation heatmap of significant proteins. Left, odds ratios and the corresponding nominal *P* values in parenthesis are shown for proteins that had a significant association with at least 1 of the CAD outcomes after correcting for ASCVD risk score. The numbers are colored according to the FDR corrected values, where grey is $P > .1$, pink is *P* between .05 and .1, and red indicates $P < .05$. The boxes are colored according to the magnitude of the odds ratio, where nonsignificant associations are white. Right, Pearson correlation heatmap of the proteins with the corresponding hierarchical clustering dendrogram is shown. Abbreviations: ASCVD, atherosclerotic cardiovascular disease; CAD, coronary artery disease; FDR, false discovery rate. Protein name abbreviations can be found in Supplementary Material.

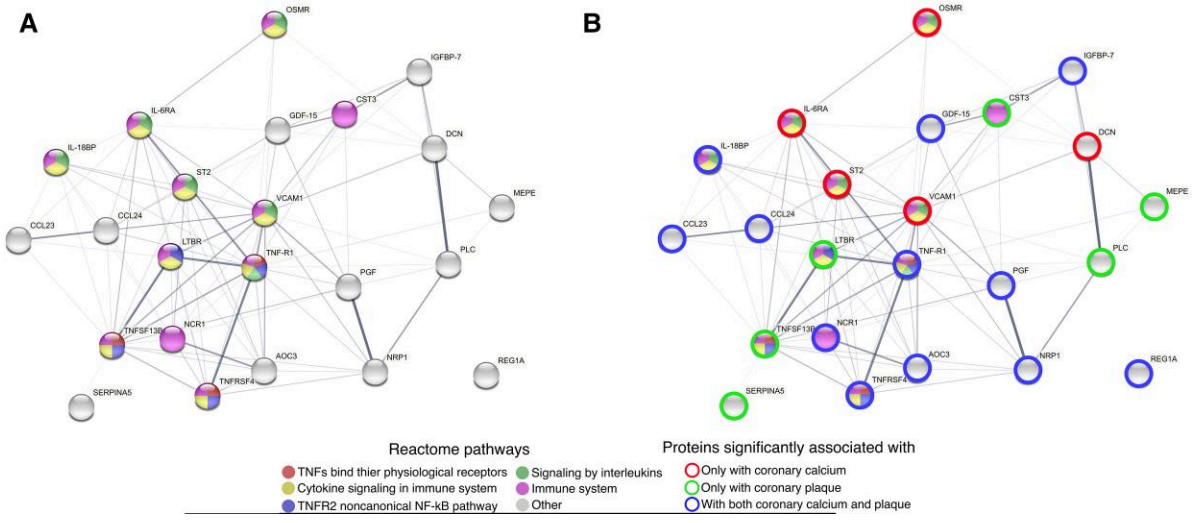

Figure 4. *A* and *B*, Protein-protein interaction network of significant proteins. Protein-protein interaction network of proteins that had a significant association with 1 of the CAD outcomes after correcting for ASCVD risk score. The edges between the protein nodes are proportional to the interaction score between the proteins from the STRING database considering all types of evidence. Only edges with interaction scores >0.15 are shown. The color of the node indicates the reactome pathway with which the protein is associated. The 5 most enriched reactome pathways are displayed. Summary of the function of the proteins and the interactions between them are provided in Supplementary Material. Image was generated using: https://string-db.org/. Abbreviations: ASCVD, atherosclerotic cardiovascular disease; CAD, coronary artery disease.

Table 3. Mediation Analysis Results Considering the Presence of Coronary Calcium and Coronary Plaque

	Direct Effect of ASCVD			Indirect Effect of ASCVD			
Proteins	OR	(95% CI)	*P*	OR	(95% CI)	*P*	Proportion Mediated, %
Presence of coronary calcium							
TNFSF13B	1.024	(1.017–1.030)	<.0001	1.001	(1.000–1.002)	.084	2.600
LTBR	1.024	(1.017–1.030)	<.0001	1.001	(1.000–1.002)	.088	2.740
IL-18BP	**1.024**	**(1.017–1.029)**	**<.0001**	**1.001**	**(1.000–1.003)**	**.016**	**4.650**
TNF-R1	**1.023**	**(1.016–1.029)**	**<.0001**	**1.001**	**(1.000–1.003)**	**.002**	**5.711**
IGFBP-7	**1.022**	**(1.015–1.028)**	**<.0001**	**1.002**	**(1.001–1.005)**	**.002**	**9.396**
PLC	**1.023**	**(1.017–1.029)**	**<.0001**	**1.001**	**(1.000–1.003)**	**.024**	**4.844**
NCR1	**1.026**	**(1.020–1.032)**	**<.0001**	**0.999**	**(.997–1.000)**	**.010**	**−5.476**
PGF	**1.023**	**(1.016–1.029)**	**<.0001**	**1.002**	**(1.001–1.004)**	**.004**	**8.225**
TNFRSF4	1.025	(1.018–1.030)	<.0001	1.000	(.999–1.001)	.694	−0.639
CST3	**1.023**	**(1.015–1.029)**	**<.0001**	**1.002**	**(1.000–1.004)**	**.012**	**7.501**
VCAM1	1.025	(1.018–1.031)	<.0001	1.000	(.998–1.001)	.642	−0.993
NRP1	1.024	(1.017–1.029)	<.0001	1.001	(1.000–1.003)	.128	4.381
AOC3	1.025	(1.018–1.031)	<.0001	0.999	(.998–1.001)	.376	−1.876
OSMR	1.025	(1.018–1.030)	<.0001	1.000	(.999–1.001)	.782	0.597
DCN	**1.023**	**(1.016–1.029)**	**<.0001**	**1.001**	**(1.000–1.003)**	**.016**	**5.527**
GDF-15	**1.021**	**(1.014–1.027)**	**<.0001**	**1.003**	**(1.001–1.006)**	**.002**	**12.578**
REG1A	1.024	(1.018–1.030)	<.0001	1.001	(.999–1.002)	.290	2.112
ST2	**1.023**	**(1.016–1.029)**	**<.0001**	**1.001**	**(1.000–1.003)**	**.018**	**5.224**
SERPINA5	1.024	(1.018–1.030)	<.0001	1.000	(1.000–1.001)	.358	1.102
CCL24	1.025	(1.019–1.030)	<.0001	1.000	(.998–1.001)	.778	−0.656
IL-6RA	1.026	(1.019–1.032)	<.0001	0.999	(.998–1.000)	.094	−3.559
CCL23	1.025	(1.018–1.031)	<.0001	1.000	(.999–1.001)	.968	−0.084
MEPE	1.025	(1.019–1.031)	<.0001	0.999	(.998–1.000)	.098	−2.786
Presence of coronary plaque							
TNFSF13B	1.031	(1.021–1.040)	<.0001	1.001	(1.000–1.003)	.064	3.305
LTBR	1.032	(1.021–1.041)	<.0001	1.001	(1.000–1.003)	.082	3.576
IL-18BP	**1.031**	**(1.021–1.040)**	**<.0001**	**1.002**	**(1.000–1.004)**	**.016**	**4.685**
TNF-R1	**1.030**	**(1.020–1.040)**	**<.0001**	**1.002**	**(1.001–1.004)**	**.002**	**6.763**
IGFBP-7	**1.029**	**(1.018–1.039)**	**<.0001**	**1.003**	**(1.001–1.006)**	**<.0001**	**9.519**
PLC	**1.031**	**(1.021–1.040)**	**<.0001**	**1.002**	**(1.001–1.004)**	**<.0001**	**5.944**
NCR1	**1.035**	**(1.024–1.043)**	**<.0001**	**0.998**	**(.997–1.000)**	**.010**	**−4.416**
PGF	**1.029**	**(1.019–1.038)**	**<.0001**	**1.003**	**(1.001–1.006)**	**<.0001**	**10.291**
TNFRSF4	1.033	(1.023–1.042)	<.0001	1.000	(.998–1.001)	.694	−0.724
CST3	**1.030**	**(1.019–1.039)**	**<.0001**	**1.003**	**(1.001–1.005)**	**<.0001**	**8.152**
VCAM1	1.033	(1.022–1.042)	<.0001	1.000	(.999–1.001)	.674	−0.512
NRP1	1.032	(1.021–1.041)	<.0001	1.001	(1.000–1.003)	.144	2.777
AOC3	1.033	(1.022–1.043)	<.0001	0.999	(.998–1.001)	.376	−1.704
OSMR	1.033	(1.022–1.042)	<.0001	1.000	(.999–1.001)	.786	0.370
DCN	**1.031**	**(1.021–1.040)**	**<.0001**	**1.002**	**(1.000–1.003)**	**.028**	**4.473**
GDF-15	**1.029**	**(1.018–1.038)**	**<.0001**	**1.003**	**(1.001–1.006)**	**.008**	**10.315**
REG1A	1.032	(1.022–1.041)	<.0001	1.001	(.999–1.002)	.296	1.777
ST2	**1.031**	**(1.020–1.041)**	**<.0001**	**1.001**	**(1.000–1.003)**	**.030**	**3.917**
SERPINA5	1.032	(1.022–1.041)	<.0001	1.001	(.999–1.002)	.314	1.896
CCL24	1.033	(1.023–1.041)	<.0001	1.000	(.998–1.001)	.782	−0.454
IL-6RA	1.034	(1.023–1.043)	<.0001	0.999	(.997–1.000)	.100	−2.679
CCL23	1.033	(1.023–1.042)	<.0001	1.000	(.998–1.002)	.968	−0.085
MEPE	**1.035**	**(1.025–1.043)**	**<.0001**	**0.998**	**(.996–1.000)**	**.014**	**−5.565**

Proteins that significantly mediate the effects of ASCVD risk are shown in bold.

Abbreviations: ASCVD, atherosclerotic cardiovascular disease; CI, confidence interval; OR, odds ratio.

GDF-15, CCL24, PGF, and DCN related to CAD in a prior proteomics study of patients in the general population with chest pain [31]. Activation of such pathways in an asymptomatic younger population with HIV and lower traditional ASCVD risk provides insight into the biology of the development of subclinical atherosclerotic changes in PWH. Other proteins found to be significant in the general population did not relate to CAD in our cohort of PWH, suggesting

unique pathways may also be associated with CAD among PWH [31].

PWH have been shown to have a CAD pattern enriched with noncalcified plaque [1, 32]. Indeed, we saw a relatively low percentage with CAC > 0 (35%) and a higher percentage with any plaque (48%) among PWH. In contrast, among an older, general population of European participants enrolled in the SCAPIS study without known CVD, plaque was seen in 42%, but the vast majority also had CAC > 0 [33]. While several proteins related to both CAC and plaque in our study, there are differences in the protein patterns, suggesting that different pathways may contribute more specifically to early calcification compared to a more mixed plaque pattern. Among the strongest related proteins to both CAC and presence of plaque in our study was NRP1, a neuropilin, that has roles in signaling and angiogenesis [34]. NRPs are thought to be involved in cell migration and promotion of endothelial cell proliferation, motility, and apoptosis [16]. Recent data in animal models suggests that increased NRP1 expression on T cells is atherogenic, results in increased IFN secretion, and facilitates T-cell migration to the aorta [35]. Increased expression of NRP1 may mark inflammatory T cells, which expand to facilitate atherosclerosis [35]. Moreover, NRP1 mediates vascular disease in the context of PDGF and TGFB signaling [36]. In contrast, unique proteins more associated with CAC in our study included VCAM1, a marker of endothelial function associated with plaque and carotid intima–media thickness (CIMT) [37]; OSMR, an anti-inflammatory protein thought to decrease IL-8–mediated recruitment of neutrophils [38]; DCN, decorin, with antifibrotic and anti-inflammatory properties [39]; and IL-6RA. Differential upregulation of pro- and anti-inflammatory pathways may contribute to the development of specific plaque phenotypes.

We have previously shown a relatively high proportion of asymptomatic PWH to have vulnerable plaque [1], which has been linked to increased MACE in the general population [40]. In exploratory analyses, we now demonstrate that NRP1, AOC3, MEPE, and PGF are most strongly associated with the presence of vulnerable plaque in our study population. Demonstration of overlapping proteins, including NRP1 and PGF, associated with multiple plaque phenotypes assessed in this analysis reinforces the potential role of these pathways in subclinical CAD among PWH. Indeed, NRP1 was the protein most strongly and consistently associated with all 3 plaque phenotypes examined in this study. Further work is needed to understand the unique pathways contributing to these subclinical plaque phenotypes, relating specific proteins to progression of disease and assessing the effects of modulating these pathways on CAD in PWH.

We demonstrated interrelationships among the proteins associated with CAD, after adjustment for ASCVD risk score. A critical pathway in atherogenesis involves activation of NF-κB, with subsequent activation of the NLRP3 inflammasome as well as IL-1β. Analysis of reactome pathways, showed 4 key proteins involved in the NF-κB signaling pathways: LTBR, TNF-R1, TNFSF13B, and TNFRSF4. Multiple studies have demonstrated that activation of the inflammasome is involved in atherosclerosis [41]. Recently, activation of caspase-1, a critical component of the inflammasome pathway, was associated with atherosclerosis among PWH [42]. Additional pathways associated with CAD in this study include proteins related to immune function as well as TNF, cytokine, and interleukin signaling. Among these proteins, TNFSF13B (survival of mature B-cells), LTBR (tumor necrosis factor superfamily), NCR1 (activates NK cells), and TNFRSF4 (involved in long-term T-cell immunity) have key immune regulatory functions which may link persistent immune activation to subclinical atherogenesis among PWH.

Among PWH, ART reduces viremia and increases CD4 count, but indices of inflammation and immune activation persist [2]. Drivers of this inflammatory milieu are thought to include persistent low-level viral replication, coinfections, and increased intestinal permeability. Prior work has demonstrated key proteins in these domains to be upregulated in PWH compared to individuals without HIV infection [10]. This study advances the field by now linking proteins previously found to be upregulated within these domains among PWH, including CST3, GDF-15, IGFBP-7, IL-18BP, REG1A, SERPINA5, TNFSF13B, and VCAM1 [10], to distinct subclinical CAD phenotypes in a well-defined low–traditional-risk population and further builds on work in smaller pilot studies initially linking protein pathways to CAD [43].

To further address potential relationships with HIV-specific factors, we investigated if these proteins were associated with current CD4 (determined at time of CT acquisition) and nadir CD4 count, as surrogates of immune function. We found some significant relationships, which were generally consistent in their directionality, eg higher inflammatory protein levels were associated with lower CD4 count and lower nadir CD4 count. We demonstrated a novel relationship between GDF-15 and nadir CD4 count. Nonetheless, differences in protein levels between CD4 groups, although statistically significant, were relatively small in absolute terms, suggesting that standard measures of immune function among those on long-term ART may not robustly predict inflammatory pathways contributing to subclinical CAD. Minimal differences were seen comparing by detectable viral load, although a majority of participants were on ART.

In prior studies, we have shown statin effects on LTBR and CCL24 in PWH [43]. Through the longitudinal assessment of statin effects in REPRIEVE, we will determine if effects on specific proteins mediate reductions in plaque volume. Our data suggest that strategies to modulate the NF-κB pathways with canakinumab, a monoclonal antibody targeting IL-1β, may be useful as seen in the CANTOS trial [44] and in a small pilot

study of PWH [45] to reduce arterial inflammation among PWH. However, IL-1β may increase infections, suggesting potential limitations for PWH [44]. Other strategies, including strategies to reduce IL-6 [46], may be useful, given our prior findings of IL-6 association with plaque in this cohort and the new finding of IL-6RA in the current analysis [1].

This study has strengths and limitations. The data are cross-sectional and represent participants from the United States only. We compared our data to a similar analysis in patients without HIV, but future studies will need to validate these findings in longitudinal cohorts, which we will do in relationship to events over time in REPRIEVE. Nonetheless, this study is the first comprehensive proteomic assessment of pathways associated with subclinical CAD among PWH. We adjusted for ASCVD risk and assessed relationships to different plaque phenotypes, further performing a mediation analysis to assess independent effects of these proteins. This study population is representative of the large number of PWH with only low-to-moderate traditional risk factors, on ART with restored CD4 count, but demonstrating CAD, and thus at risk for future events. We assessed CAD in a standardized fashion to ensure generalizability. Moreover, the proximity extension assay allows for sensitive and precise measurement of proteins and has been used to create predictive models of CAD in people without HIV [31]. Given that higher-risk features were less prevalent, we focused primarily on the proteomic signature of more common phenotypes of plaque presence and CAC, to assess potential mechanisms of subclinical disease but performed exploratory analyses for higher-risk phenotypes.

Our data extend a growing body of work identifying related patterns of elevated plasma biomarkers and cellular gene expression profiles related to CVD in PWH [47–49]. These data provide a road map for future investigation of the mechanisms of subclinical CAD in HIV, highlighting key interrelated immune and inflammatory pathways significantly associated with coronary phenotypes in PWH. These data support a construct in which upregulated inflammatory and immune pathways further contribute independently to CAD beyond traditional risk factors. Critically, the field needs to assess whether these abnormal protein signatures will identify PWH at greater risk for increased plaque and cardiovascular events over time, to optimize preventive care.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Data sharing. Data will be shared in accordance with National Institutes of Health policy.

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