Plaque burden in HIV-infected patients is associated with serum intestinal microbiota-generated trimethylamine

Suman Srinivasa[a], Kathleen V. Fitch[a], Janet Lo[a], Hanane Kadar[b,c], Rachel Knight[d], Kimberly Wong[a], Suhny Abbara[e], Dominique Gauguier[b,c], Jacqueline Capeau[c,f], Franck Boccara[g] and Steven K. Grinspoon[a]

Objective: Some intestinal microbiota-generated metabolites of phosphatidylcholine are recognized to be proatherogenic. As the HIV population is vulnerable to cardiovascular disease and can develop intestinal dysbiosis associated with systemic inflammation, we investigated the novel relationship between microbiota-derived metabolites of phosphatidylcholine and coronary atherosclerosis in HIV.

Design/Methods: One hundred and fifty-five HIV-infected and 67 non-HIV-infected individuals without known history of cardiovascular disease were previously recruited to assess coronary plaque by computed tomography angiography. In the current study, we evaluate whether serum choline, trimethylamine (TMA), or trimethylamine-*N*-oxide (TMAO) levels are associated with plaque features.

Results: Young, asymptomatic HIV-infected patients (age 47 ± 7 years) demonstrated significantly higher prevalence of plaque (53 vs. 35%, $P = 0.01$) and number of total plaque segments (1.8 ± 2.5 vs. 1.2 ± 2.2, $P = 0.03$) when compared with well matched noninfected individuals with similar comorbidities. TMA was significantly associated with calcium score ($r = 0.22$, $P = 0.006$), number of total ($r = 0.20$, $P = 0.02$) and calcified ($r = 0.18$, $P = 0.03$) plaque segments, and calcium plaque volume ($r = 0.19$, $P = 0.02$) and mass ($r = 0.22$, $P = 0.009$) in the HIV cohort only. In multivariate modeling among HIV-infected patients, TMA remained significantly associated with calcium score ($P = 0.008$), number of total ($P = 0.005$) and calcified ($P = 0.02$) plaque segments, and calcium plaque volume ($P = 0.01$) and mass ($P = 0.007$), independent of Framingham risk score. In contrast, there was no association of TMAO to coronary plaque features in either cohort.

Conclusion: A link between TMA and atherosclerosis has not previously been established. The current study suggests that TMA may be a nontraditional risk factor related to the number of plaque segments and severity of calcified plaque burden in HIV.

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[a]Program in Nutritional Metabolism, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, USA, [b]INSERM UMRS1138, Cordeliers Research Centre, Paris, [c]Institute of Cardiometabolism and Nutrition, University Pierre & Marie Curie, Hospital Pitié Salpetrière, Paris, France, [d]University of Rochester School of Medicine, Rochester, New York, New York, [e]Cardiothoracic Imaging Division, Department of Radiology, UTSW Medical Center, Dallas, Texas, USA, [f]Biochimie et Hormonologie, Hôpital Tenon AP-HP Paris, CDR Saint-Antoine, Inserm UMR_S938, Université Paris 6-UPMC, Institute of Cardiometabolism and Nutrition, Paris, and [g]Department of Cardiology, Saint Antoine Hospital, Inserm U938, Université Paris 6-UPMC, Faculté de Médecine Pierre et Marie Curie, Paris, France.

Correspondence to Steven K. Grinspoon, MD, Director, Program in Nutritional Metabolism, Massachusetts General Hospital, 55 Fruit Street, LON207, Boston, MA 02114, USA.

Tel: +1 617 726 3890; fax: +1 617 724 8998; e-mail: sgrinspoon@mgh.harvard.edu
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Introduction

HIV-infected patients demonstrate increased risk of cardiovascular disease (CVD) beyond that predicted by traditional risk factors, such as age, sex, and tobacco use, when compared with controls [1–3]. Prior studies have suggested that unique, nontraditional mechanisms involving inflammation may potentiate the risk for atherosclerotic plaque in HIV [4–6], but the exact mechanism is yet to be determined. Emerging evidence suggests that alterations in intestinal microbial composition may contribute to CVD [7–9]. Indeed, HIV itself has been associated with changes in the gut microbiome [10] and disruption in intestinal wall integrity [11], which may in part contribute to a proatherogenic disease state in this population. In addition, recent studies demonstrate that altered gut microbiota is associated with increased inflammatory and immune activation markers among HIV-infected patients with good virologic control on antiretroviral therapy (ART) [12–14], thus further suggesting a relationship between gut microbiota and CVD in these patients.

Recent interest has focused on the metabolism of phosphatidylcholine in relationship to the gut as a potentially important pathway contributing to CVD. Dietary phosphatidylcholine is a major source of choline, which is further metabolized by intestinal flora to trimethylamine (TMA). In the liver, TMA is oxidized to trimethylamine-*N*-oxide (TMAO). Alternatively, dietary L-carnitine [15] and betaine [16] can also be metabolized to TMAO, but contribute less to TMAO production. Wang *et al.* [17] proposed that metabolites of dietary phosphatidylcholine, although essential to certain biologic roles, also carry atherogenic potential. In this regard, circulating levels of TMAO are associated with CVD among non-HIV-infected patients [15,17,18]. However, no prior studies have investigated whether enhanced metabolism of phosphatidylcholine contributes to increased CVD in the HIV population.

The aim of this study was to compare serum phosphatidylcholine metabolites among HIV-infected patients and healthy controls in relation to subclinical atherosclerotic disease. Whereas prior studies have determined the association of TMAO to cardiovascular events in the non-HIV-infected population, this study investigated for the first time the relationship of choline-related metabolites to coronary plaque burden in HIV, assessed by cardiac computed tomography angiography (CTA). This study, therefore, investigates a new potential mechanistic factor linking gut metabolism to CVD in HIV-infected patients. We hypothesized that HIV-infected patients would demonstrate unfavorable coronary plaque characteristics in association with increased serum levels of TMAO.

Methods

Study participants

One hundred and fifty-five HIV-infected patients and 67 non-HIV-infected controls were previously recruited. HIV-infected patients were recruited from Boston area HIV clinics, community healthcare centers, and newspaper advertisements. Healthy controls were recruited from the same communities to ensure similar demographic characteristics. Data on coronary plaque characteristics have been previously reported in this cohort [1,19,20], but data on choline-related metabolites have not previously been assessed.

Inclusion and exclusion factors were identical for both groups with the exception of HIV serostatus and are detailed elsewhere [1,20]. In brief, patients aged 18–60 years were recruited based on no known cardiac disease, or symptoms of heart disease [including current or prior angina, arrhythmias, valvular heart disease, pericarditis, congestive heart failure, or any prior treatment for coronary artery disease (CAD) or any heart disease]. Patients with known renal disease, creatinine levels more than 1.5 mg/dl or creatinine clearance less than 70 ml/min were excluded to minimize risk of contrast nephropathy. HIV-infected patients were receiving stable ART for more than 3 months.

All participants provided informed consent to participate. This study was approved by the institutional review board of Massachusetts General Hospital.

Study procedures and assessment of cardiovascular risk factors

Detailed interviews were performed to obtain sociodemographic information, medical and family history, cardiovascular risk factors, and tobacco, recreational intravenous drug and medication use. For HIV-infected patients, duration of diagnosis and detailed history of past and present ART use were also obtained.

Cardiac computed tomography angiography

Cardiac CTA imaging was performed using a 64-slice computed tomography scanner (Sensation 64; Siemens Medical Solutions, Forchheim, Germany) according to a standardized protocol [1]. Assessment of coronary plaque type, calcified or noncalcified, and other plaque characteristics are detailed elsewhere [1]. A consensus reading of coronary atherosclerotic plaque segments was established between a cardiologist and radiologist with significant experience in cardiac computed tomography interpretation who were blinded to patients' medical history, including HIV serostatus. Agatston calcium score was quantified using noncontrast computed tomography imaging [21].

Metabolic, biochemical, inflammatory, and body composition parameters

Creatinine and glucose and lipid parameters were determined using standard techniques. Plasma high-sensitivity interleukin 6 (R&D Systems, Minneapolis, Minnesota, USA) and high-sensitivity C-reactive protein (Labcorp, Cranford, New Jersey, USA) were quantified by ELISA. The endpoint limulus amebocyte lysate assay (Associates of Cape Cod, East Falmouth, Massachusetts, USA) was used to evaluate lipopolysaccharide (LPS) levels [20]. All patients fasted prior to blood draws. A cross-sectional computed tomography scan at the level of the L4 pedicle was performed to assess abdominal visceral and subcutaneous adipose tissue (visceral adipose tissue and subcutaneous adipose tissue, respectively) area [22].

Dietary assessment of choline and betaine levels

Dietary intake was collected through a 4-day food record and analyzed using Nutrition Data System for Research software [Nutrition Coordinating Center (NCC), University of Minnesota, Minneapolis, Minnesota, USA). Dietary choline and betaine were assessed.

Measurements of choline-related serum metabolites

Serum samples were obtained after a 12 h fast and aliquoted into 2 ml Sarstedt microtubes and stored at -80°C. A mass spectrometry-based method was specifically designed to assay choline, betaine, L-carnitine, TMA, and TMAO in serum samples. Serum aliquots were spiked with internal standards, mixed with acetonitrile and injected on a UPLC chromatographic system (Waters Corp, Saint-Quentin en Yvelines, France) coupled to a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Illkirch, France). The system was fitted with an Acquity BEH HILIC column and a corresponding guard column (Waters Corp). Data were acquired in full scan positive ion mode with a resolution of 70 000 FWHM at a scan range of m/z 50–400. Target analyte signals were extracted with an accuracy of 5 ppm. Additional studies were performed to assess correlations between plasma and serum and metabolite assessment in different storage conditions (see Supplemental Methods, http://links.lww.com/QAD/A627).

Statistical analysis

Normality of distribution was determined using the Shapiro–Wilk test. Data are presented as mean ± standard deviation or median (interquartile range), depending on normality of the distribution; all categorical variables are reported as proportions. Comparisons were made between HIV-infected and non-HIV-infected patients using the Student's t test for normally distributed continuous variables, the Wilcoxon rank-sum test for nonnormally distributed data, and the χ^2 test for categorical variables. Nonnormally distributed variables were log-transformed to establish a normal distribution. Linear regression was performed using Pearson's correlation coefficient within the HIV and non-HIV-infected cohorts, separately. Multivariate regression analysis was performed to assess the effects of individual choline-related serum metabolites on coronary plaque features among HIV-infected patients controlling for Framingham risk score (FRS), a composite score of traditional risk factors known to affect plaque. The focus of these analyses was TMA, as this metabolite was most strongly associated with coronary plaque features in univariate analyses. Sensitivity analyses including LPS in the models were also performed, as LPS was the systemic inflammatory marker most significantly associated with TMA. In addition, sensitivity analyses controlling for high-density lipoprotein (HDL), hepatitis C virus (HCV) status, and protease inhibitor, nonnucleoside reverse transcriptase inhibitors (NNRTI), atanazavir (ATV) and efavirenz (EFV) use were performed. Statistical significance was defined as $P < 0.05$. All statistical analysis was performed using SAS JMP (version 9.0; Cary, North Carolina, USA).

Results

Demographics and traditional cardiovascular disease risk factors

HIV-infected and noninfected patients were well matched with respect to age, sex, and race. Similar proportions of HIV patients and non-HIV-infected controls were identified to have dyslipidemia, hypertension, and diabetes mellitus. The HIV cohort presented with a long duration of HIV for 14 ± 6 years, and 99% of this population had exposure to ART for 8 ± 5 years. Current use of protease inhibitor, nucleoside/nucleotide reverse transcriptase inhibitors, and NNRTI treatments in the HIV-infected patients was 55, 95, and 37%, respectively. The majority of the HIV patients had evidence of good immunological control with $CD4^+$ cell count 552 ± 290 cell/μl. Undetectable viral load was demonstrated among 86% of the cohort. There was a higher prevalence of HCV in the HIV patients. With respect to metabolic parameters, HIV-infected patients demonstrated significantly increased fasting triglycerides compared with the noninfected patients. There was a trend toward lower SAT and in the HIV cohort. The prevalence of smoking was similar between the groups. Other lipid parameters, including total cholesterol, low-density lipoprotein, and HDL, were not significantly different between populations. Furthermore, FRS did not differ between the HIV and control populations. The HIV cohort demonstrated elevated liver transaminases when compared with healthy patients. In addition, LPS was increased in the HIV-infected patients compared with the non-HIV-infected patients (Table 1).

Dietary intake and serum levels of choline-related metabolites

Dietary intake of choline and betaine and measured serum levels of choline, betaine, L-carnitine, TMA, and

TMAO did not differ between HIV-infected and noninfected patients (Table 2). Dietary choline was related to serum TMA in both HIV-infected ($r = 0.19$, $P = 0.05$) and control ($r = 0.33$, $P = 0.05$) groups. There were no other significant associations between dietary intake and serum measures of choline-related metabolites. Serum TMA did not differ by current protease inhibitor use status vs. not [0.4(0.3, 1.4) vs. 0.4 (0.3, 1.5), $P = 0.30$), but was higher in those on NNRTI-containing regimens vs. not [0.4(0.3, 1.6) vs. 0.3(0.3, 0.4), $P = 0.001$]. Specific correlations to ART demonstrated that serum TMA levels were significantly higher in HIV-infected patients on EFV-containing regimens vs. non-EFV containing regimens [0.4(0.3, 1.6) vs. 0.3(0.3, 0.4), $P = 0.03$]. TMA did not differ by ATV-containing regimens vs. non-ATV containing regimens [0.3(0.3, 0.4) vs. 0.3(0.3, 1.5), $P = 0.38$].

Coronary plaque characteristics

HIV-infected patients demonstrated a significantly higher prevalence of plaque (53 vs. 35%, $P = 0.01$) and number of total plaque segments (1.8 ± 2.5 vs. 1.2 ± 2.2, $P = 0.03$) when compared with controls. Both cohorts had a similar number of calcified plaque segments in addition to other parameters related to calcified plaque, including calcium score and calcium plaque volume and

Table 1. Characteristics of control and HIV-infected patients.

	Controls ($n = 67$)	HIV-infected patients ($n = 155$)	P value
Demographics			
Age years	46 ± 7	47 ± 7	0.13
Sex (%)			
Male	58	61	0.67
Race (%)			
White	49	53	0.37
FRS	9 (5–11)	10 (7–12)	0.09
Family history of premature CHD by NCEP (%)	24	21	0.71
Dyslipidemia by NCEP (%)	24	34	0.13
Hypertension (%)	15	24	0.15
Diabetes mellitus (%)	7	10	0.50
Current smoker (%)	42	44	0.74
HCV (%)	9	27	0.002
History of IVDU (%)	13	23	0.12
HIV disease-related parameters			
Duration since HIV diagnosis (years)	N/A	14 ± 6	N/A
Ever on ART (%)	N/A	99	N/A
Duration of antiretroviral therapy (years)	N/A	8 ± 5	N/A
Current protease inhibitor treatment (%)	N/A	55	N/A
Current NRTI treatment (%)	N/A	95	N/A
Current NNRTI treatment (%)	N/A	37	N/A
$CD4^+$ T-lymphocytes (cells/μl)	N/A	552 ± 290	N/A
Log HIV RNA viral load (copies/ml)	N/A	1.8 ± 0.5	N/A
Undetectable HIV RNA < 50 copies/ml (%)	N/A	86	N/A
Anthropometric parameters			
BMI (kg/m^2)	27 ± 5.0	27 ± 5.0	0.39
Waist circumference (iliac crest) (cm)	93.9 (84.4–105.1)	96.2 (85.1–108.4)	0.60
VAT area (cm^2)	100 (52–172)	109 (61–210)	0.28
SAT area (cm^2)	238 (146–343)	199 (126–288)	0.07
Metabolic indices			
SBP (mmHg)	117 ± 15	119 ± 14	0.22
DBP (mmHg)	76 ± 9	76 ± 9	0.70
Fasting glucose (mg/dl)	86 (80–93)	88 (80–95)	0.47
Hemoglobin A1c (%)	5.5 (5.4–5.8)	5.5 (5.2–5.8)	0.08
Total cholesterol (mg/dl)	176 (156–200)	178 (155–206)	0.71
HDL cholesterol (mg/dl)	49 (42–66)	50 (41–62)	0.71
LDL cholesterol (mg/dl)	105 ± 30	102 ± 35	0.60
Triglycerides (mg/dl)	83 (62–119)	97 (77–172)	0.001
Creatinine (mg/dl)	0.94 (0.77–1.13)	0.93 (0.81–1.10)	0.81
ALT (U/dl)	24 ± 15	35 ± 28	0.0001
AST (U/dl)	28 ± 15	39 ± 30	0.0008
Inflammatory indices			
CRP (mg/dl)	0.13 (0.05–0.36)	0.14 (0.05–0.39)	0.76
hsIL-6 (pg/ml)	0.96 (0.61–1.83)	1.06 (0.74–1.86)	0.38
LPS (ng/ml)	0.07 (0.02–0.09)	0.09 (0.04–0.12)	0.003

Data reported as mean ± standard deviation, percentage, or median (interquartile range). ALT, alanine aminotransferase; ART, antiretroviral therapy; AST, aspartate aminotransferase; CHD, coronary heart disease; CRP, C-reactive protein; DBP, diastolic blood pressure; FRS, Framingham risk score; HCV, hepatitis C virus; HDL, high-density lipoprotein; hsIL-6, high-sensitivity interleukin-6; IVDU, intravenous drug use; LDL, low-density lipoprotein; LPS, lipopolysaccharide; N/A, not applicable; NCEP, National Cholesterol Education Program; NNRTI, nonnucleoside reverse transcriptase inhibitors; NRTI, nucleoside/nucleotide reverse transcriptase inhibitors; SAT, subcutaneous adipose tissue; SBP, systolic blood pressure; VAT, visceral adipose tissue.

Table 2. Dietary and serum choline-related metabolites among controls and HIV-infected patients.

	Controls ($n=67$)	HIV-infected patients ($n=155$)	*P* value
Dietary intake			
Betaine (mg)	155.9 (90.3–208.9)	128.9 (94.2–182.3)	0.43
Choline (mg)	349.2 (251.5–464.8)	330.7 (218.9–429.4)	0.60
Serum metabolites			
Betaine (μmol/l)	6.1 (4.7–7.7)	5.8 (5.1–7.0)	0.32
L-Carnitine (μmol/l)	5.9 (4.9–6.7)	5.8 (4.5–6.9)	0.58
Choline (μmol/l)	2.0 (1.8–2.2)	1.9 (1.7–2.3)	0.88
TMA (μmol/l)	0.3 (0.3–0.4)	0.3 (0.3–1.5)	0.50
TMAO (μmol/l)	0.7 (0.5–1.0)	0.7 (0.5–1.1)	0.79

Data reported as median (interquartile range). Food record data were available for 36 controls and 100 HIV-infected patients. TMA, trimethylamine; TMAO, trimethylamine-*N*-oxide.

mass, but the HIV cohort was observed to have more noncalcified segments compared with healthy controls (Table 3).

Relationship of serum metabolites and coronary plaque features among HIV-infected and non-HIV-infected patients

Among the HIV-infected patients, serum TMA was significantly and positively associated with calcium score ($r=0.22$, $P=0.006$), number of total plaque segments ($r=0.20$, $P=0.02$), number of calcified plaque segments ($r=0.18$, $P=0.03$), calcium plaque volume ($r=0.19$, $P=0.02$) and calcium plaque mass ($r=0.22$, $P=0.009$) (Table 4a). Serum betaine was not consistently related to plaque parameters, and no relationships were observed between serum choline, L-carnitine, or TMAO and plaque characteristics (Table 4a). In addition, dietary intake of choline and betaine did not relate to plaque parameters (data not shown). Serum TMA levels inversely correlated with HDL ($r=-0.17$, $P=0.03$) among HIV-infected patients.

Among the non-HIV-infected controls, no significant relationships with serum TMA or other metabolites were seen (Table 4b).

Relationship of serum trimethylamine and systemic inflammatory markers among HIV-infected and non-HIV-infected patients

Among the HIV-infected patients, serum TMA was positively correlated with LPS ($r=0.19$, $P=0.03$), whereas there was no significant association with high-sensitivity interleukin 6 and high-sensitivity C-reactive protein (Table 4a). Serum TMA did not differ based on HCV status in the HIV cohort (0.3[0.3, 1.5] vs. 0.3[0.3, 0.4], $P=0.38$, no HCV vs. presence of HCV).

Among the non-HIV-infected controls, neither TMA nor other choline-related metabolites were associated to systemic inflammatory markers (Table 4b).

Multivariate regression modeling among HIV-infected patients to assess effects of serum trimethylamine on coronary plaque features

As the relationship to serum TMA and coronary plaque features was significant in the HIV cohort, we performed multivariate modeling assessing the relationship of TMA to coronary plaque features simultaneously controlling for the effects of other common risk factors for calcified coronary plaque, using the composite Framingham risk score. In separate models for specific coronary plaque features, serum TMA remained significantly associated with calcium score ($\beta=25.7192$, $P=0.008$), number of total plaque segments ($\beta=0.9643$, $P=0.005$), number of calcified plaque segments ($\beta=0.1895$, $P=0.02$), calcium plaque volume ($\beta=21.0937$, $P=0.01$), and calcium plaque mass ($\beta=5.7989$, $P=0.007$), independent of FRS (Table 5a). In a model including HDL, rather than the aggregate total FRS, TMA remained significantly associated to these coronary plaque parameters independent of HDL (Supplemental Table 1, http://links.lww.com/QAD/A627**)**. Similarly, TMA remained related to

Table 3. Coronary plaque characteristics by computed tomography angiography of control and HIV-infected patients.

	Controls ($n=67$)	HIV-infected patients ($n=155$)	*P* value
Coronary plaque characteristics			
Log calcium score	1.5 ± 2.7	1.6 ± 2.5	0.81
Presence of plaques (%)	35	53	0.01
Total plaque segments (#)	1.2 ± 2.2	1.8 ± 2.5	0.03
Noncalcified segments (#)	0.5 ± 1.2	1.0 ± 1.6	0.003
Calcified segments (#)	0.3 ± 0.8	0.2 ± 0.6	0.41
Total calcium volume of plaque (mm^3)	19.9 ± 49.2	21.3 ± 59.7	0.35
Total calcium mass of plaque (mg)	4.2 ± 10.5	4.9 ± 14.9	0.35

Data reported as mean ± SD or percentage. For segments and plaque characteristics, results are reported as mean ± SD to provide more information because of the low number of lesions. *P* value comparison by Wilcoxon rank-sum test where appropriate.

Table 4. Univariate associations with choline-related metabolites.

Parameter	Log serum betaine		Log serum L-carnitine		Log serum choline		Log serum TMA		Log serum TMAO	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
(a) Among HIV-infected patients										
Coronary plaque characteristics										
Log calcium score	−0.02	0.83	−0.006	0.94	0.10	0.22	0.22	0.006	0.06	0.47
Total plaque segments (#)	−0.001	0.99	−0.07	0.43	0.03	0.71	0.20	0.02	0.007	0.93
Noncalcified plaque segments (#)	0.03	0.76	−0.13	0.13	0.02	0.82	0.11	0.18	0.007	0.93
Calcified plaque segments (#)	−0.19	0.03	0.10	0.23	−0.03	0.70	0.18	0.03	0.02	0.85
Total calcium volume of plaque (mm^3)	−0.07	0.42	0.07	0.39	0.09	0.26	0.19	0.02	0.04	0.67
Total calcium mass of plaque (mg)	−0.07	0.42	0.08	0.38	0.14	0.10	0.22	0.009	0.04	0.66
Inflammatory indices										
Log CRP (mg/dl)	0.10	0.22	0.12	0.15	−0.14	0.08	0.11	0.18	0.06	0.47
Log hsIL-6 (pg/ml)	0.05	0.58	−0.14	0.11	0.006	0.94	−0.10	0.25	0.15	0.08
Log LPS (ng/ml)	0.05	0.59	0.02	0.81	0.03	0.75	0.19	0.03	0.14	0.12
(b) Among control patients										
Coronary plaque characteristics										
Log calcium score	0.02	0.86	0.08	0.52	−0.08	0.54	0.09	0.47	0.16	0.21
Total plaque segments (#)	0.02	0.87	0.14	0.27	−0.05	0.72	0.14	0.29	0.02	0.90
Noncalcified plaque segments (#)	−0.03	0.80	0.18	0.17	−0.07	0.61	0.19	0.15	−0.14	0.27
Calcified plaque segments (#)	0.06	0.65	0.07	0.58	0.06	0.63	−0.17	0.17	0.18	0.15
Total calcium volume of plaque (mm^3)	0.22	0.08	0.24	0.06	−0.06	0.64	0.0003	1.00	0.12	0.34
Total calcium mass of plaque (mg)	0.21	0.09	0.24	0.06	−0.07	0.61	−0.02	0.89	0.13	0.29
Inflammatory indices										
Log CRP (mg/dl)	−0.0002	1.00	0.14	0.25	−0.10	0.44	0.16	0.20	−0.03	0.80
Log hsIL-6 (pg/ml)	−0.21	0.13	−0.10	0.48	−0.05	0.71	0.04	0.79	0.09	0.54
Log LPS (ng/ml)	0.15	0.28	0.15	0.28	0.07	0.64	−0.01	0.92	−0.08	0.56

r represents Pearson's correlation coefficient. CRP, C-reactive protein; hsIL-6, high-sensitivity interleukin-6; LPS, lipopolysaccharide; TMA, trimethylamine; TMAO, trimethylamine-*N*-oxide.

these plaque indices in multivariate regression models controlling for protease inhibitor, NNRTI, ATV and EFV use and HCV status (Supplemental Table 1, http://links.lww.com/QAD/A627). Moreover, TMA remained a significant and independent predictor of calcium score, number of total plaque segments, and calcium plaque mass and tended to be independently associated with number of calcified plaque segments and calcium plaque volume after further controlling for both FRS and LPS (Table 5b).

Discussion

This is the first study to evaluate the association of phosphatidylcholine metabolites to detailed plaque phenotype and burden of calcified plaque both in healthy controls and the HIV population. Our data demonstrate that serum TMA, but not serum TMAO, is associated with the presence of coronary plaque, and more specifically calcified plaque in HIV-infected patients. Furthermore, other parameters of calcified plaque, including calcium score and calcified plaque mass and volume were similarly related to serum TMA. The significant association of TMA to these detailed calcified plaque indices was demonstrated to be independent of traditional CVD risk factors, as represented by the FRS and HDL. Therefore, our data suggest that TMA may be a nontraditional CVD risk factor with relevance to HIV-infected patients. Prior studies have related CVD event rates and degree of coronary artery stenosis to circulating TMAO in non-HIV-infected individuals, but have not yet evaluated the relationship of plaque phenotype to this metabolite or to TMA and other metabolites in HIV.

Adequate amounts of dietary choline, commonly found in eggs, beef, and animal liver, are essential to biological roles in cholinergic neurotransmission, hepatic very low-density lipoprotein secretion, and synthesis of integral phospholipids [23]. Moreover, through its intermediate oxidation to betaine, choline is an effective methyl donor in the conversion of homocysteine to methionine. Because of its ability to decrease plasma homocysteine, dietary supplementation of choline may be cardioprotective [24]. However, contrary evidence suggests that choline may, in fact, serve as a substrate for atherosclerotic disease through metabolism by intestinal microbiota to TMA and subsequent production of TMAO [17,18]. It is possible that these contrasting data in the choline literature may be explained by a U-shaped curve, such that either deficiency or excess of choline could be detrimental to biologic function.

In our study, both HIV-infected and non-HIV-infected cohorts had similar dietary intake of choline and betaine, as well as measured serum levels of all substrates betaine, choline, L-carnitine, TMA, and TMAO. Choline can be synthesized endogenously or obtained through the diet. The dietary intake for choline in both cohorts was well

Table 5. Models to assess effects of serum TMA on coronary plaque parameters in HIV-infected patients.

	Calcium score ($n=146$) ($R^2=0.08$; $P=0.002$)		Total plaque segments (#) ($n=139$) ($R^2=0.16$; $P=<0.0001$)		Calcified plaque segments (#) ($n=139$) ($R^2=0.08$; $P=0.004$)		Calcium volume of plaque (mm^3) ($n=138$) ($R^2=0.08$; $P=0.003$)		Calcium mass of plaque (mg) ($n=138$) ($R^2=0.08$; $P=0.004$)	
	β estimate	*P*	β estimate	*P*	β estimate	*P*	β estimate	*P*	β estimate	*P*
(a)										
Variables										
Serum TMA	25.7192	0.008	0.9643	0.005	0.1895	0.02	21.0937	0.01	5.7989	0.007
FRS	2.8206	0.01	0.1718	<0.0001	0.0266	0.009	2.6245	0.01	0.5439	0.04
	Calcium score ($n=123$) ($R^2=0.08$; $P=0.02$)		Total plaque segments (#) ($n=118$) ($R^2=0.15$; $P=0.0003$)		Calcified plaque segments (#) ($n=119$) ($R^2=0.08$; $P=0.02$)		Calcium volume of plaque (mm^3) ($n=117$) ($R^2=0.09$; $P=0.02$)		Calcium mass of plaque (mg) ($n=117$) ($R^2=0.08$; $P=0.03$)	
	β estimate	*P*	β estimate	*P*	β estimate	*P*	β estimate	*P*	β estimate	*P*
(b)										
Variables										
Serum TMA	22.9497	0.04	0.8232	0.03	0.1720	0.07	18.2025	0.06	5.2065	0.03
FRS	3.2799	0.02	0.1605	0.0008	0.0293	0.02	3.1836	0.01	0.6456	0.04
LPS	36.0131	0.76	4.9143	0.21	0.6241	0.53	8.0591	0.94	6.7358	0.80

R^2 represents the coefficient of determination and the proportion of variance explained by the model. *P* value represents significance by the whole model analysis of variance test. FRS, Framingham risk score; LPS, lipopolysaccharide; TMA, trimethylamine.

below the proposed daily recommendation for adequate intake, measured to be 425 mg for women and 550 mg for men [25]. Food records may underreport the total amount of nutrients. Thus, serum metabolites under fasting conditions were obtained for more accurate comparisons.

Potential mechanisms for the presumed role of TMAO in atherosclerosis include inhibition of reverse cholesterol transport and altered cholesterol metabolism [15]. Moreover, TMAO may enhance formation of foam cells from macrophages [17]. The gut microbiome appears to be an essential component in the production of TMAO. After administration of antibiotics, TMAO production is attenuated, but production rebounds upon recolonization of the gut flora [17]. Indeed, Tang *et al.* [26] showed that in human studies, TMAO is correlated with an increased risk of major cardiovascular events, independent of traditional cardiovascular risk factors. In contrast, circulating levels of choline and betaine have been shown to be less predictive of major adverse cardiac events when compared with TMAO [16]. Furthermore, the relationship of dietary choline and betaine to CVD has been equivocal in prior studies [23,27,28]. Some prior studies that have cited a link between TMAO and CVD have incorporated a choline challenge. Tang *et al.* [26] asked participants to digest hard boiled eggs and radiolabelled phosphatidylcholine. In contrast, our individuals were observed after routine diet and under fasting conditions. Prior studies have not assessed serum measures of TMA and TMAO, but serum and plasma have given similar results for these analytes in our laboratory.

Limited details are available as to the function of TMA and its direct impact on atherosclerosis, apart from its central role as the serum intestinal microbiota-generated precursor to TMAO, and prior studies have not investigated the relationship between TMA and atherosclerotic disease. In this study, we investigated TMAO and additionally its precursor TMA in relationship to detailed indices of plaque morphology. There is site-specific enzymatic conversion of TMA to TMAO in the liver predominantly by flavin-containing monooxygenase 3 (FMO3). Our data demonstrate a novel relationship between calcified plaque features and TMA, but not TMAO, in this first study of HIV-infected patients. Whereas FMO3 expression may reportedly differ based on sex and dietary bile acid [18], other mechanisms may account for these findings.

First, our HIV cohort demonstrated significantly higher transaminases, suggestive of underlying hepatic inflammation. Because FMO3 is unique to the liver, chronic inflammation may promote downregulation of this enzyme and in turn affect the conversion of TMA to TMAO. Trimethylaminuria, a clinical model of disease in which there is a FMO3 loss of function mutation and concomitant elevated TMA levels, exists in varying degrees of severity, but has not yet been associated with increased CVD. Bennett *et al.* [18] created a murine model of FMO3 deficiency, which resulted in a 90% reduction of FMO3 mRNA in the liver, a 47% reduction in plasma TMAO, and more than doubling of plasma TMA levels when compared with control mice. In two murine models of inflammation, induced by introduction of *Citrobacter rodentium* and LPS, mRNA levels of FMO3 were significantly decreased [29]. In addition, more than half of HIV-infected patients in this study received a protease inhibitor-based regimen, which could inhibit

the activity of liver drug metabolizing enzymes. In addition, ATV can affect bile metabolism. In our HIV cohort, we did not see an association with serum TMA and protease inhibitor use, but we did see an association of serum TMA to NNRTI use. We performed additional analyses to investigate the contribution of EFV, a NNRTI known to affect liver-specific enzymes. There was a significant correlation between EFV use and TMA; however, the relationship of TMA to coronary plaque characteristics persisted regardless of EFV use. The strong independent relationship between TMA and plaque features in the current human study suggests that TMA may itself have atherogenic potential. Lastly, TMA may also be a marker for other processes, including microbial translocation, which may contribute to CVD.

Specific gut microbiota compositions have been associated with either circulating levels of TMA or TMAO, respectively [15]. During HIV infection, there is a relative reduction in a $CD4^+$ subset of Th17 cells that regulate the intestinal flora [30]. Moreover, taxa have been demonstrated to discriminate between HIV-infected and noninfected individuals [10]. Dysbiosis is present even in those HIV-infected patients well controlled on suppressive ART. Relative enrichment of *Enterobacteriaceae* and depletion of *Bacteriodes* have been profiled in the gut microbiome in HIV-infected patients when compared to non-HIV-infected patients [12,14]. In addition, specific microbiota has been identified that relate to increases in markers of microbial translocation and systemic inflammation in HIV-infected patients on ART-suppressive regimens [12–14]. Alterations in gut-associated T-cell regulation in HIV may impair the mucosal barrier and enhance bacterial translocation in the gut with subsequent elevation in systemic bacterial endotoxins, including LPS. Indeed, increased levels of LPS have been associated with atherosclerotic disease in HIV-infected patients [31,32]. Prior studies have demonstrated that alterations in gut flora may be linked to cardiometabolic disease, inflammation, and atherosclerosis. In our HIV cohort, LPS levels were increased and positively associated with serum TMA, but the relationship of TMA to plaque features remained independent of LPS. Assessment of specific gut flora was beyond the scope of the current data, but it will be interesting to compare gut flora with serum TMA in future studies of choline metabolism in HIV and non-HIV-infected patients.

In this study, HIV-infected and noninfected individuals had similar number of calcified plaque segments, but HIV-infected patients had a significantly higher number of noncalcified plaque segments. Our group has previously shown that HIV-infected patients demonstrate a higher prevalence of noncalcified plaque—a 'vulnerable' plaque feature more prone to rupture and increased atherosclerotic events [1,20]. However, in this study, we demonstrate that serum TMA was most consistently related to calcified plaque characteristics. As calcified and noncalcified plaque phenotypes have divergent pathophysiologies, factors other than choline-related metabolites, including sex, inflammation, and chronic immune activation, may play a more considerable role in noncalcified plaque formation in the HIV population [20]. In addition, longstanding effects of choline metabolism on nascent noncalcified lesions may over time contribute to calcified plaque formation.

This study has limitations. The study design was cross-sectional, so causality cannot be inferred. In addition, the role of TMA in CVD, independent of TMAO, needs further clarification. A choline challenge may also be warranted to more clearly accentuate pathophysiologic differences in phosphatidylcholine metabolism in HIV-infected patients compared with controls. Studies in the HIV population should evaluate expression of FMO3 and composition of the intestinal microbiome to investigate these relationships to TMA production. In addition, the effect of ART use and liver inflammation on FMO3 expression should be investigated in HIV.

Novel data from the current study suggest a potential contribution of choline metabolism to CVD in HIV-infected patients, assessed in relationship to plaque burden using a sensitive CT angiographic technique. This relationship may derive from altered gut flora or microbial translocation unique to HIV-infected patients, and further studies are required to answer these important questions, particularly as to the role of the gut microbiome and related metabolism in the HIV population.

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Conflicts of interest

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