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Metabolites predict cardiovascular disease events in persons living with HIV: a pilot case–control study

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Abstract

Introduction—Persons living with HIV (PLWH) are at higher risk for cardiovascular disease (CVD) events than uninfected persons. Current risk-stratification methods to define PLWH at highest risk for CVD events are lacking.

Methods—Using tandem flow injection mass spectrometry, we quantified plasma levels of 60 metabolites in 24 matched pairs of PLWH [1:1 with and without known coronary artery disease (CAD)]. Metabolite levels were reduced to interpretable factors using principal components analysis.

Results—Factors derived from short-chain dicarboxylacylcarnitines (SCDA) ($p = 0.08$) and glutamine/valine ($p = 0.003$) were elevated in CAD cases compared to controls.

Conclusion—SCDAs and glutamine/valine may be valuable markers of cardiovascular risk among persons living with HIV in the future, pending validation in larger cohorts.

Keywords

Cardiovascular disease; HIV; Metabolomics; Acute coronary syndrome; Myocardial infarction

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Conflict of interest The authors declare that there is no conflict of interest.

Compliance with ethical standards

Research involving human participants and/or animals Ethical approval for the use of patient clinical data and specimens were approved by the Duke University Institutional Review Board.

Informed consent Patient consent was obtained at the time of sample collection for the Duke HIV Biorepository with the understanding that their samples would be used for future research studies.

1 Introduction

Persons living with HIV (PLWH) are at higher risk for major cardiovascular disease (CVD) events than uninfected persons. The increase in CVD events in this population are not accounted for by traditional risk factors alone (Freiberg et al. 2013). Perhaps due to the complexities of atherosclerosis in chronic HIV infection, accurate estimation of CVD risk in PLWH has proven elusive (Hsue et al. 2009). Traditional CVD risk assessment tools have consistently underestimated risk in PLWH (Thompson-Paul et al. 2016). Attempts at constructing CVD risk calculators specifically for PLWH have fallen short (Friis-Moller et al. 2010). Technologic advances in molecular profiling present the opportunity to simultaneously identify biomarkers of disease risk as well as report on the biological mechanisms mediating risk (Kraus et al. 2015). The evolving field of metabolomics seeks to understand disease states by detecting alterations in normal physiology by detecting variations in levels of by-products of metabolism. Our group has previously utilized targeted metabolomic profiing techniques, with an emphasis on the quantification of acylcarnitines (metabolites of fatty acid oxidation) and amino acids, to identify circulating metabolites that predict CVD in uninfected persons. These cuttingedge techniques have never been utilized to understand the unique pathophysiology of CVD in persons living with HIV (Shah et al. 2012b; Kraus et al. 2015). We report a pilot case–control study of PLWH using a well-established profile of CVD-associated metabolites to identify potential biomarkers associated with CVD risk in this population (Shah et al. 2010, 2012a, b).

2 Methods

We conducted a retrospective, nested-case control study of PLWH cared for at the Duke Infectious Diseases Clinic between 2001 and 2012. Cases were defined as PLWH who were diagnosed with an acute coronary syndrome (ACS) at Duke. ACS diagnoses were verified by billing data for ACS events using International Classifications of Disease, Ninth Edition (ICD-9) codes (410.00–410.92, 411.x). Billing diagnoses were further adjudicated through medical chart review, creatine phosphokinase-MB isoenzyme fraction of over 8 ng/mL and/or troponin T of > 0.10 ng/ mL, and evidence of cardiac catheterization or coronary artery bypass grafting. For cases, the event date was the date of presentation to the hospital with ACS symptoms. Controls were chosen from HIV clinic patients without documentation of CAD, ACS, stroke or peripheral vascular disease in their medical record. Cases and controls were matched 1:1 by the following criteria (at the time of sample collection): age (± 2 years), sex, race, mean of last three outpatient systolic blood pressures (± 5 mmHg) and CD4 count. Patients with detectable viremia within 6 months of sample collection were excluded. Participants were linked to plasma samples in the Duke HIV Biorepository. For cases, the most recent outpatient blood sample collected prior to documented date of event was used for the analysis. For controls, the analysis specimen was the blood sample collected closest to the age at sample collection of the complementary matched “case” patient. Patient consent was obtained at the time of sample collection for the Duke HIV Biorepository with the understanding that their samples would be used for future research studies. Use of patient data and specimens were approved by the Duke University Institutional Review Board.

Levels of 45 acylcarnitines and 15 amino acids (Table 1) were quantified using tandem flow injection mass spectrometry (MS/MS) in the positive ionization mode (Ferrara et al. 2008; An et al. 2004). Briefly, plasma samples were spiked with a cocktail of heavy-isotope internal standards consisting of D_3-acetyl, D_3-propionyl, D_3-butyryl, D_9-isovaleryl, D_3-octanoyl, and D_3-palmitoyl carnitines; $^{15}N_1$,$^{13}C_1$-glycine, D_4-alanine, D_8-valine, D_7-proline, D_3-serine, D_3-leucine, D_3-methionine, D_5-phenylalanine, D_4-tyrosine, D_3-aspartate, D_3-glutamate, D_2-ornithine, D_2-citrulline, and D_5-arginine (Cambridge Isotope Laboratories, MA, USA; CDN Isotopes, Canada) and deproteinated with methanol. The methanol supernatants were dried and esterified by acidified methanol for the acycarnitine analysis and by acidified butanol for the amino acid analysis. Esterification was used to increase the ionization efficiency of the analytes of interest. Mass spectra for acylcarnitine and amino acid esters were obtained using precursor ion and neutral loss scanning methods, respectively. The data were acquired using a Waters TQ (triple quadrupole) detector equipped with Acquity™ UPLC system and a data system controlled by MassLynx 4.1 operating system (Waters, Milford, MA). Ion ratios of analyte to respective internal standard computed from centroided spectra were converted to concentrations using calibrators constructed from authentic aliphatic acylcarnitines and amino acids (Sigma, MO, USA; Larodan Sweden) and dialyzed Fetal Bovine Serum (Sigma, MO, USA). Standard non-MS based clinical chemistry assays were used for the detection of ketones (total and β-hydroxybutyrate) and free fatty acids (Wako Diagnostics, Richmond, VA). Measurements were performed using a Hitachi 911 clinical chemistry analyzer (Hitachi Chemical Diagnostics, Mountain View, CA).

Given the correlation between many of the measured metabolites (representing by-products of similar metabolic pathways), principal components analysis (PCA) was used to reduce the large number of correlated metabolites into uncorrelated (orthogonal) factors consisting of clusters of related metabolites, as previously described (Shah et al. 2012b). Given the data volume, we used factor scores from a larger study of CVD and applied the weights to calculate PCA-derived factors in this study (Shah et al. 2012b). PCA factors scores are unit-less and reflect the relative levels of the factors constituent metabolites. Reported values reflect the number of standard deviations the detected serum level of the factors are from the historical mean of all samples ever run at our facility (“0”). Metabolite factor scores were calculated from the metabolomic profiling data for each group (cases and controls) and group mean factor values were compared using paired *t* test. p-Values for the *t* tests were reported. For purposes of comparison, we also display historical means of uninfected persons from the CATHGEN cohort. The CATHGEN biorepository consists of subjects recruited sequentially through the cardiac catheterization laboratories at Duke University Medical Center as previously described (Shah et al. 2010). Given the exploratory nature of this study, metabolite factors with a nominal p-value < 0.10 were considered of potential significance. Statistical analysis was conducted with SAS Version 9.4 (Cary NC).

3 Results

Overall, 3749 PLWH were cared for at the Duke Adult ID Clinic between 2001 and 2012, of which 141 were diagnosed with an ACS event at Duke. For this study, 96 of these 141 had available blood samples; after careful matching, 24 suitable matched controls were identified

for a final analysis cohort of 48 patients. Cohort characteristics were as follows: median age 52 years (SD 8.2), 79% male, 50% Black (Table 2). Over half of the patients were current or prior cigarette users. The median systolic blood pressure for the cohort was 137 mmHg (SD 16 mmHg). The mean body mass index for the cohort was 26.7 kg/m^2 (SD 4.9 kg/m^2). The median proximal CD4 was 573 cells/ uL (IQR: 214–654 cells/uL) for cases and 476 cells/uL (IQR 285–762 cells/uL) for controls. Fifteen of 24 case (63%) patients were on a protease inhibitor (PI) at the time of sample collection compared to 10 of 24 control (42%) patients ($p = 0.15$). A similar number of case and control patients had any historical exposure to protease inhibitors (16 vs. 15, $p = 0.82$). Per inclusion criteria, all patients had HIV-1 RNA suppression of < 400 copies/mL (Table 2).

Among cases, 12 patients presented with unstable angina, six patients presented with non-ST elevation MI and six presented with ST-elevation MI. The median ejection fraction at the time of event was 48.5% (IQR 45–55%). On cardiac catheterization, thirteen patients had 1-vessel CAD, eight had 2-vessel disease and three had 3-vessel disease. Overall, eight cases underwent subsequent coronary artery bypass grafting, 12 received percutaneous coronary intervention (PCI) and four received medical management alone. The median time prior-to-event for the samples used for the analysis was 185 days (IQR 163, 251 days).

Fourteen PCA-derived metabolite factors were calculated for this analysis (Table 1), clustering in biologically relevant pathways (Shah et al. 2012b). Levels of three of the 14 metabolite factors were different between cases and controls: glutamine/valine ($p = 0.003$), short-chain dicarboxylacylcarnitines (SCDA, $p = 0.08$), and medium chain acylcarnitines ($p = 0.05$) (Table 3). Within the SCDA factor, metabolite levels that differed the most between the two groups was C5-DC acylcarnitine ($p = 0.06$). The significance of the glutamine/valine factor was entirely driven by glutamine ($p = 0.003$). Notably, levels of SCDA and glutamine/valine factors were higher in cases compared to controls. Conversely, levels of medium chain acylcarnitines were lower in HIV-infected cases compared to controls.

4 Discussion

Using a metabolomic profiling platform targeted at metabolites previously shown to be associated with atherosclerotic cardiovascular disease (ASCVD) in uninfected persons, we identified circulating metabolites of which abnormally elevated levels preceded ACS events in comprehensively phenotyped cohort of PLWH (Shah et al. 2010, 2012a, b). Specifically, we found elevated levels of glutamine and SCDA metabolites in cases compared to controls. We also found that circulating levels of medium chain acylcarnitines are significantly lower in HIVinfected case patients compared to infected controls. Our study is the first to demonstrate potential prognostic utility for these previously validated biomarkers of CVD among PLWH and consistent with findings in clinical studies conducted in uninfected persons (Shah et al. 2010, 2012a, b).

Circulating levels of valine/glutamine were elevated in HIV-infected CAD cases compared to controls ($p = 0.003$). Interestingly, elevated levels of both amino acids were also detected in our analysis of uninfected persons study cohorts: ($p < 0.0001$ for glutamine and $p = 0.05$ for valine) (Shah et al. 2012b). Glutamate/glutamine and valine have previously been

associated with type 2 diabetes and represent key metabolites in the branched chain amino acid (BCAA) catabolic pathway. Elevated levels of these metabolites in incident ACS cases may represent subclinical insulin resistance not reflected by a clinical diagnosis of diabetes (Newgard et al. 2009).

We also found elevated levels of SCDA metabolites in cases compared to controls. The potential of SCDAs as prognostic biomarkers was first established in a nested case–control study of two cohorts of uninfected patients from the Duke CATHGEN biorepository (initial: 174 case–control pairs, replication: 140 case–control pairs). In these cohorts, elevated SCDA levels were found to be associated with CVD events. (OR 1.52, 95% CI 1.08–2.14) (Shah et al. 2010). In a larger cohort (n = 2023) of patients presenting for cardiac catheterization, SCDAs were independently associated with MI (HR 1.11, 95% CI 1.01–1.23) (Shah et al. 2012b). Our study is the first to replicate these findings in PLWH. In fatty acid oxidation, shortening of long chain dicarboxylic acids via β-oxidation, results in the formation of SCDAs (Shah et al. 2012b). The significance of these metabolites remain unclear, however our recent work integrating genomics, epigenetics and metabolites suggests that circulating SCDA levels report on endoplasmic reticulum (ER) stress (Kraus et al. 2015).

Our analysis also reveals a significant difference in levels of medium chain acylcarnitines between HIV-infected cases and HIV-infected controls (Table 3). This difference in factor levels has also been demonstrated in previous studies in uninfected patients (Shah et al. 2009, 2010). In one study, medium chain acylcarnitines (MCA) levels were independently associated with all-cause mortality in the Measurement to Understand the Reclassification of Disease of Cabarrus and Kannapolis Cardiovascular Study (MURDOCK CV) clinical cohort (HR 1.12, 95% CI 1.04–1.21) (Shah et al. 2012b). Our report is the first to validate divergent values of circulating MCA between HIV-infected persons with CAD and those without.

In comparing samples from our HIV study cohort with historical CATHGEN controls, we are careful not to compare the value means between the two groups because of the large discrepancy in the sample sizes between the groups (48 vs. 3776). We believe that a direct comparison of values between groups must be interpreted with caution. However, the CATHGEN comparison values are valuable in instructing us on the directionality of the divergence of factor values within the groups. In comparing the directionality of the differences within groups, the directionality of the divergence (higher levels in cases vs. higher levels in controls) between HIV cases and controls mirrors that of uninfected CATHGEN samples in 5 of 6 factors with a paired *t* test p-value of 0.25 or less (Factors 1, 3, 10, 12 and 13). As expected for factors with a paired *t* test p-value of > 0.5, the concordance of the directionality of divergence was low (1 out of 5 factors). This consistency gives significant validation to our findings especially with the small sample size of our study cohort. It is also important to note that in this study we chose to explore a very restricted set of metabolites. We approached our analysis this way because we wanted to demonstrate utility of a set of metabolites, previously discovered and validated for the prediction of CVD events in uninfected persons, among PLWH (Shah et al. 2010, 2012). The above report is strictly a validation of previously identified metabolites with prognostic utility; however, future studies will employ a liquid chromatography/mass spectrometry based (LC–MS/MS)

non-targeted approach that will seek to cover of the metabolome in an effort to identify novel biomarkers indicative of CVD risk in PLWH.

Our study has limitations. Our sample size limits quantification of the discriminative capacity of the metabolites in a robust manner. However, we are currently performing metabolic profiling on biospecimens of PLWH with adjudicated ACS in a larger multi-center validation cohort. Our study was a lso not designed to determine how long prior to a major CVD event elevated levels of SCDA can be detected. Future studies will determine how SCDAs level change over time leading up to ACS events relative to expected baseline levels. Lastly, although we included only virologically-suppressed patients in the study, we did not account for the impact of ART on metabolite levels. This limitation will be addressed in our larger study.

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Table 1

Description of principal component analysis-derived metabolite factors

Factor	Description	MSI Level	Metabolites	Eigen value	Variance
1	Medium chain acylcarnitines	1	Octanoyl carnitine, decanoyl carnitine, tetradecanoyl carnitine, hexadecadienoyl carnitine, palmitoeloyl carnitine, tetradecadienoyl carnitine, dodecenoyl carnitine, decenoyl carnitine	14.06	0.23
2	Long chain dicarboxyl acylcarnitines	1	Octadecenedioyl carnitine, hexadecanedioyl carnitine, octadecanedioyl carnitine, 3-hydroxy-palmitoleoyl carnitine, 3-hydroxy-octadecanoyl carnitine, arachidoyl carnitine, 3-hydroxy-dodecanoyl carnitine, 3-hydroxy-tetradecanoyl carnitine	5.64	0.09
3	Short chain dicarboxyl acylcarnitines	1	Glutaryl carnitine, 3-hydroxy-*cis*-5-octenoyl carnitine, octenedioyl carnitine, adipoyl carnitine, methylmalonyl carnitine, 3-hydroxy-decanoyl carnitine, 3-hydroxy-dodecanoyl carnitine, Cit	4.86	0.08
4	Long chain acylcarnitines	1	Oleyl carnitine, 3-hydroxy-linoleyl carnitine, stearoyl carnitine, palmitoyl carnitine, arachidonoyl carnitine, 3-hydroxy-palmitoleoyl carnitine	3.8	0.06
5	Ketone related	1	KET, HBUT, hydroxy-butyryl carnitine, acetyl carnitine, (Ala)	2.52	0.04
6	Medium chain acylcarnitines	1	Decatrienoyl carnitine, octenoyl carnitine, decenoyl carni-tine	2.47	0.04
7	Branched chain amino acids	1	Phe, Tyr, Leu/Ile, Val, Met	2.32	0.04
8	Urea cycle amino acids		Gly, Met, Ser, Orn, Arg, C5:1, Pro	1.6	0.03
9	C3–C5's	1	Butyryl carnitine, propionyl carnitine, Isovaleryl carnitine	1.47	0.02
10	Miscellaneous	1	Asx	1.42	0.02
11	Miscellaneous	1	Tiglyl carnitine, His, 3-hydroxy-linoleyl carnitine, Arg	1.22	0.02
12	Glutamine/glutamic acid, valine	1	Glx, Val	1.12	0.02
13	NEFA	1	Ala, Pro, NEFA	1.07	0.02
14	C22	1	Behenoyl carnitine	1.01	0.02

Ala alanine, *Arg* arginine, *Asx* aspartate, *Cit* citrulline, *Glx* glutamine, *Hbut* hydroxybutyrate, *His* histamine, *Ile* isoleucine, *Ket* ketones, *Leu* leucine, *Met* methionine, *NEFA* non-esterified fatty acids, *Orn* ornithine, *Phe* phenylalanine, *Pro* proline, *Val* valine

Table 2

Baseline characteristics of study cohort

Variable	Cases (n = 24)	Controls (n = 24)
Age, years [mean (SD)]	52.2 (8.4)	52.6 (7.8)
Female, n (%)	(21)	(21)
Black race, n (%)	(50)	(50)
Tobacco use (current or former), n (%)	(75)	(55)
Diabetes, n (%)	(13)	(4)
Hypertension, n (%)	(54)	(54)
Systolic blood pressure, mmHg [mean (SD)]	137.6 (16.0)	137.4 (19.2)
Diastolic blood pressure, mmHg [mean (SD)]	81.6 (11.8)	83.8 (11.4)
Body mass index, kg/m^2 [mean(SD)]	26.5 (4.0)	26.8 (4.9)
Proximal CD4 count, cells/uL [median (IQR)]	(214,654)	(285,762)
ART regimen at time of specimen collection, n (%) PI-based	(63)	(42)
NNRTI-based	(33)	(54)
INSTI-based	0	0
Other	(4)	(4)
AZT, DDI or D4T backbone	(50)	(25)
ABC backbone	(21)	(17)
Any PI exposure	(67)	(63)

IQR interquartile range, *SD* standard deviation, *ABC* abacavir, *ART* antiretroviral therapy, *AZT* azidothymidine, *DDI* didanosine, *D4T* stavudine, *INSTI* integrase strand transfer inhibitor, *NNRTI* non-nucleoside reverse transcriptase inhibitor, *PI* protease inhibitor

Table 3

T-tests for comparison of metabolite factor levels between HIV cases and controls and historical uninfected CATHGEN means

Factor #	Factor description	MSI level	HIV cases (n = 24)	HIV controls (n = 24)	Historical CATH-GEN uninfected cases (n = 2185)	Historical CATHGEN uninfected controls (n = 1591)	Matched paired t-test p-values (HIV cases vs. controls)
1	Medium chain acylcarnitines	1	− 0.15 (− 0.5, 0.1)	− 0.08 (− 0.4, 0.2)	− 0.02	0.03	**0.048**
2	Long chain dicar-boxylacylcarni- tines	1	0.15 (0, 0.4)	0.01 (− 0.3, 0.3)	0.05	− 0.08	0.32
3	Short chain dicar-boxylacylcarniti- nes (SCDA)	1	− 0.07 (− 0.2, 0.1)	0.28 (− 0.5, 0.1)	0.04	− 0.05	**0.08**
4	Long chain acyl- carnitines	1	0.67 (− 0.5, 1.2)	0.1 (− 0.2, 0.4)	0.01	− 0.01	0.66
5	Ketone related	1	0.96 (− 1.3, 0.7)	0.95 (− 1.3, 0.6)	0.003	− 0.01	0.79
6	Medium chain acylcarnitines	1	− 0.16 (− 0.9, 0.4)	0.06 (− 0.5, 0.5)	0.06	− 0.07	0.23
7	Branched chain amino acids	1	− 0.07 (− 1, 0.8)	− 0.1 (− 0.5, 1.1)	0.05	− 0.07	0.34
8	Urea cycle amino acids	1	− 0.46 (− 1, 0.6)	− 0.26 (− 0.9, 0.4)	0.03	− 0.07	0.95
9	Short chain acyl- carnitines	1	− 0.1 (− 0.5, 0.7)	0.28 (− 0.6, 0.7)	0.12	− 0.17	0.65
10	Miscellaneous metabolites	1	− 0.12 (− 0.8, 0.4)	0.59 (− 0.9, 0.1)	0.05	− 0.04	0.22
11	Miscellaneous metabolites	1	− 0.62 (− 1.3, 0)	− 0.66 (− 1.1, - 0.2)	− 0.02	0.06	0.71
12	Glutamine/gluta- mate, valine	1	0.75 (− 0.2, 1.4)	− 0.16 (− 0.6, 0.1)	0.06	− 0.08	**0.003**
13	Non-esterified fatty acids	1	0.36 (− 0.1, 0.7)	− 0.13 (− 0.6, 0.4)	0.03	− 0.005	0.11
14	C22	1	0.21 (− 0.3, 0.6)	0.41 (− 0.3, 1)	0.01	− 0.01	0.40

Factors with p-value ≤ 0.1 are bold