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ORIGINAL RESEARCH

N8-Acetylspermidine: A Polyamine Biomarker in Ischemic Cardiomyopathy With Reduced Ejection Fraction

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BACKGROUND: Patients with ischemic cardiomyopathy (ICM) have worse outcomes than those with coronary artery disease alone and those with non-ICM. N8-acetylspermidine (N8AS) is a polyamine that regulates ischemic cardiac apoptosis and resultant cardiac dysfunction. We hypothesized that N8AS is a mechanistic biomarker of adverse outcomes in patients with ICM.

METHODS AND RESULTS: High-resolution plasma metabolomics profiling and mass spectrometry were used to quantitate N8AS levels in a discovery cohort of 474 patients with coronary artery disease (age: 68±11 years, 12% black, 26% women): 154 with ICM, and 320 without ICM; and in an external validation cohort of 85 patients with ICM (age: 60±12 years, 37% black, 19% women). Patients without heart failure (HF) at baseline were followed for incident HF. The association between N8AS (log_2-transformed, standardized) and outcomes of all-cause mortality and incident HF were examined using Cox regression. N8AS was higher (10.39 [interquartile range, 7.21–17.75] versus 8.29 nmol/L [interquartile range, 5.91–11.42]; $P<0.001$) in patients with ICM compared with patients who had coronary artery disease without ICM. Higher N8AS levels were associated with higher mortality in patients with ICM (hazard ratio [HR], 1.48; 95% CI, 1.19–1.85 per SD increase [$P=0.001$]), independent of B-type natriuretic peptide, high-sensitivity troponin I, and high-sensitivity C-reactive protein. Findings were validated in the independent cohort. Moreover, higher N8AS level was associated with incident HF in patients without HF at baseline (HR, 4.16; 95% CI, 1.41–12.25 per SD increase [$P=0.01$]).

CONCLUSIONS: Independent of traditional HF measures, higher N8AS levels are associated with higher mortality in patients with ICM and incident HF in those who have coronary artery disease without HF. N8AS is a novel mechanistic biomarker in ICM.

Key Words: biomarker ■ ischemic cardiomyopathy ■ risk prediction

While mortality associated with coronary artery disease (CAD) has declined over the past 2 decades, the incidence of heart failure (HF) attributable to CAD (ischemic cardiomyopathy [ICM]) has increased.[1] ICM is the leading cause of HF with reduced ejection fraction (EF), accounting for approximately two thirds of all cases,[2] and is associated with a worse prognosis compared with either CAD alone or with non-ICM (NICM).[1]

Several prognostic biomarkers associated with HF have been discovered. Most of these are reflective of either HF-associated myocyte stretch (natriuretic peptides), myocardial injury or ischemia (high-sensitivity troponin), or of the final-common pathophysiology of progressive HF, regardless of the mechanism of HF development.[3] In this context, a mechanistic biomarker that is upstream to the final HF phenotype may not only refine prognostication but also be a

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CLINICAL PERSPECTIVE

What Is New?

- N8-Acetylspermidine (N8AS), a polyamine that is involved in the regulation of cardiomyocyte cell death and resultant cardiac dysfunction during ischemic injury, is a mechanistic biomarker that is upstream to the final-common heart failure (HF) phenotype.
- Patients with ischemic cardiomyopathy (ICM) have higher N8AS levels than those with coronary artery disease without HF, or non-ICM.
- Independent of traditional biomarkers, higher circulating N8AS levels are associated with higher mortality in patients with ICM in 2 independent cohorts and also with greater risk of incident HF in patients who have coronary artery disease without HF.

What Are the Clinical Implications?

- A mechanistic biomarker that is upstream to the final HF phenotype may not only help refine prognostication but may also be a potential therapeutic target. In this context, N8AS is a novel mechanistic biomarker of ischemic cardiomyocyte death and resultant cardiac dysfunction.
- Independent of B-type natriuretic peptide, high-sensitivity troponin I, and high-sensitivity C-reactive protein, higher circulating N8AS is associated with higher mortality in patients with ICM and greater risk of incident HF in patients who have coronary artery disease without HF.
- Future studies should examine whether N8AS is a "modifiable" risk factor in ICM by examining the effect of modulation of polyamine levels by diet and pharmacological therapy on disease progression.

Nonstandard Abbreviations and acronym

ACEI	angiotensin-converting enzyme inhibitor
ARB	angiotensin receptor blocker
BNP	B-type natriuretic peptide
CAD	coronary artery disease
EF	ejection fraction
HF	heart failure
HMDB	Human Metabolome Database
HR	hazard ratio
hsCRP	high-sensitivity C-reactive protein
hsTnI	high-sensitivity troponin I
ICM	ischemic cardiomyopathy
KEGG	Kyoto Encyclopedia of Genes and Genomes
m/z	mass-to-charge ratio
MS/MS	tandem mass spectrometry
N8AS	N8-acetylspermidine
NICM	nonischemic cardiomyopathy
SAM	S-adenosyl methionine
SAT 1/2	spermidine/spermine acetyltransferase 1/2

potential therapeutic target.[4] Cardiac dysfunction in CAD is a complex process characterized by myocyte apoptosis and autophagy caused by both ischemia and ischemia/reperfusion injury.[5] Polyamines, including spermidine and its derivatives, are aliphatic molecules that modulate the cardiac stress response to ischemia by regulating cardiomyocyte apoptosis[5,6] and autophagy.[7,8] Intracellular spermidine is catabolized by the enzyme spermidine N8-acetyltransferase to N8-acetylspermidine (N8AS).[9,10] N8AS is then excreted from the cell, and is therefore a plasma indicator of intracellular polyamine activity.[9,10] We have previously demonstrated reliable quantification of plasma N8AS concentrations using high-resolution metabolomics profiling followed by reference standardization.[11] Prior preclinical studies have established a key regulatory role for polyamines in cardiomyocyte cell death during the ischemic cascade,[5–8] as well as HF development. Pharmacological polyamine depletion has been shown to protect cardiomyocytes from ischemia-induced apoptosis.[8,12,13] However, there is a paucity of data on the translation of these findings to the use of polyamines as prognostic biomarkers in this population.[7]

The purpose of our study was to investigate the association between plasma N8AS levels and progression of ICM. Our hypothesis was that: (1) N8AS levels will be higher in patients with ICM compared with those who have CAD without ICM and those with NICM; and (2) higher levels of N8AS, reflective of increased polyamine turnover and resultant cardiomyocyte death during ischemia will predict (a) adverse outcomes in ICM and (b) incident HF in those without HF. Prediction of adverse events was explored in a discovery cohort and replicated in an independent validation cohort. We further investigated whether the predictive capacity of N8AS was independent of BNP (B-type natriuretic peptide), high-sensitivity troponin I (hsTnI), and hsCRP (high-sensitivity C-reactive protein). Finally, using targeted and global metabolomics analyses, we investigated metabolic pathways that are associated with N8AS.

METHODS

Study Population

Discovery Cohort

We studied patients with CAD (defined as a history of myocardial infarction, percutaneous coronary intervention, coronary artery bypass grafting, or an index left heart catheterization showing ≥50% stenosis in at least 1 major epicardial vessel) undergoing cardiac catheterization who were enrolled in the Emory Cardiovascular Biobank at 3 Emory Healthcare sites between 2003 and 2008.[14] Participants were interviewed to collect information about demographic characteristics, medical history, and behavioral habits as previously described.[14] CAD severity was evaluated using the modified Duke CAD Index with ≥50% stenosis classified as clinically significant. Echocardiographic left ventricular EF was abstracted after reviewing medical records. Medical records and *International Classification of Diseases, Ninth Revision* (*ICD-9*) diagnostic codes were reviewed to confirm self-reported medical history.

Patients who had HF with preserved EF (defined as a history of HF at enrollment *and* EF ≥50%) were excluded. Prevalent ICM was defined as the presence of physician diagnosis of HF or *ICD-9* discharge diagnosis of HF *and* EF <50%, or EF <50% at the time of study enrollment.

Validation Cohort

Patients from the Atlanta Cardiomyopathy Consortium, a prospective cohort study that enrolled outpatients who had HF with reduced EF (EF <50%) from 3 Emory University–affiliated hospitals in the greater metropolitan Atlanta area from 2007 to 2011, constituted the external validation cohort.[15] Findings in the discovery cohort were validated in patients with ICM from this cohort. As a secondary analysis, we compared patients who had ICM with those who had NICM in the validation cohort.

All participants provided written informed consent at the time of enrollment, and both studies were approved by the institutional review board at Emory University, Atlanta, GA. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Follow-Up and Outcomes

Study participants were prospectively followed for the primary outcome of all-cause mortality and the secondary end point of HF hospitalization. Outcome censoring was performed on October 15, 2018. Follow-up data were obtained by annual phone contact, electronic medical record review, data from the social security death index, and state records.[14,15] Incident HF was defined as the absence of a documented history of HF *and* EF >50% at study enrollment, *and* HF hospitalization during follow-up.

High-Resolution Metabolomics for Metabolic Profiling

High-resolution metabolomics was performed using established methods.[11,16,17] All patients underwent an overnight fast before blood collection. Plasma specimens were collected before catheterization and stored at −80°C. Samples were extracted and analyzed as previously described.[11,17] Briefly, extractions were performed with acetonitrile containing a mixture of internal standards and maintained in an autosampler at 4°C until injection. Each sample was analyzed using a Thermo LTQ Velos Orbitrap high-resolution (60 000 mass resolution) mass spectrometer (Thermo Fisher Scientific) and C18 column chromatography.[11,17] For the validation cohort, samples were analyzed using liquid chromatography–Fourier transform mass spectrometry (Accela-LTQ Velos Orbitrap; mass-to-charge ratio [*m/z*] range from 85 to 850 ppm) with 10-uL injection volume using a dual chromatography setup (anion exchange and HILIC C18) and a formic acid/acetonitrile gradient. Electrospray ionization was used in the positive ion mode. Data were extracted using apLCMS[18] with modifications by xMSanalyzer as *m/z* features,[16] where an *m/z* feature is defined by *m/z*, retention time, and ion intensity (integrated ion intensity for the chromatographic peak). Metabolite annotation was performed using xMSannotator v1.3.2 using Human Metabolome Database (HMDB) v3.6.[19] Identity of N8AS was previously confirmed via tandem mass spectrometry (MS/MS) and matching fragmentation pattern and retention time with the authentic standard.[11]

Quantification of N8AS

In the discovery cohort, quantification of N8AS was accomplished using a reference standardization protocol using NIST SRM 1950 and a pooled reference plasma analyzed with each batch ("Q standard") as previously described.[11] Based on triplicate analysis of the Q standard, the concentration of N8AS within the Q standard was determined to be 5.62±1.92 nmol/L. In the validation cohort, N8AS was reported as feature intensities.

Biomarker Measurements

Levels of BNP (ARCHITECT BNP chemiluminescent microparticle immunoassay, Abbott Laboratories, reported in pg/mL), hsTnI (ARCHITECT STAT High Sensitive Troponin-I chemiluminescent microparticle immunoassay, Abbott Laboratories, reported in pg/mL), and hsCRP (MULTIGENT CRP Vario latex

immunoassay, Abbott Laboratories, reported in mg/L) were measured as previously described.[20,21]

Metabolome-Wide Association Study of N8AS

All metabolite features were measured in triplicates, and median intensities were taken from the nonzero readings. Features with >20% zero readings were excluded. Metabolome-wide association study of N8AS was then performed using Spearman rank–based correlation to determine metabolites that significantly correlated with N8AS using false discovery rate <0.01 (Benjamini and Hochberg method, denominator: 5719 detectable features).[22] These metabolites were selected for pathway enrichment analysis in Mummichog (version 2.0.6).[23] Identities of many of the *m/z* features are known from previous research using ion dissociation patterns by MS/MS, coelution with authentic standards, and cross-platform validation.[11,17,19] Possible identities of other *m/z* features were obtained using the METLIN Mass Spectrometry Database,[24] HMDB,[25] and Kyoto Encyclopedia of Genes and Genomes (KEGG).[26] Metabolite identification levels[27] were assigned by comparison with the in-house library of confirmed metabolites, which includes metabolites confirmed using MS/MS and retention time with authentic standards (level 1), comparison of MS/MS with experimental spectra in online databases or in silico predicted spectra (level 2), annotation at the metabolite class level (level 3), medium or high confidence databases matches from xMSannotator (level 4), and accurate mass match (level 5).

Targeted Network and Pathway Analysis

KEGG[26] Mapper was used to target polyamine metabolism. Matches to adducts for metabolites in pathways associated with polyamine metabolism were selected using HMDB[25] and KEGG.[26] Correlation of metabolites with N8AS was determined as previously described.

Statistical Analysis

Data are presented as mean±SD, median (interquartile range), or number (percentage) of patients. Baseline characteristics were compared between groups using Student *t* test or ANOVA for normally distributed continuous variables, Mann–Whitney *U* tor Kruskal–Wallis test for non-normally distributed continuous variables, and chi-square test for categorical variables.

N8AS levels were $\log_2$-transformed and standardized (expressed per 1 SD; *z* score) for all analyses. Cox proportional hazards models were used to determine the association of N8AS with outcomes, adjusted for age, sex, race, creatinine, body mass index, smoking history, diagnosis of diabetes mellitus, hypertension, and hyperlipidemia; with and without inclusion of other biomarkers (BNP, hsTnI, and hsCRP) in the models. We used Schoenfeld residuals to check the proportional hazards assumption and found no evidence of violation. Less than 10% of patients had missing biomarker data, and available case analysis was performed. $P<0.05$ was considered statistically significant.

Data were analyzed using SAS statistical software version 9.4 (SAS Institute Inc) and R statistical software (version 3.5.1, R Foundation for Statistical Computing).

RESULTS

Baseline Characteristics: Discovery Cohort

The discovery cohort consisted of 474 patients with CAD (mean age: 67.7±11.0 years; 25.9% women, 11.8% black), 320 without ICM, and 154 with ICM (Table 1). Risk factor profile was similar in those with and without ICM, but levels of BNP, hsTnI, and hsCRP were higher in patients with ICM. Patients with ICM had higher levels of N8AS than those without ICM (ICM: 10.39 [7.21–17.75] nmol/L; no ICM: 8.29 [5.91–11.42] nmol/L [$P<0.001$]). There was a similar variation in $\log_2$-transformed, standardized N8AS levels (ICM: 0.23±0.56; no ICM: −0.11±1.14 [$P<0.001$]). A modest correlation was observed between N8AS levels and BNP ($r=0.40$, $P<0.001$) and hsCRP ($r=0.26$, $P<0.001$), but not with hsTnI levels ($r=0.07$, $P=0.14$).

Elevated N8AS levels (per 1-SD increase in $\log_2$-transformed, standardized N8AS) were independently associated with higher risk of ICM (adjusted odds ratio, 2.97; 95% CI, 1.94–4.54 [$P<0.001$]).

N8AS Levels and Cardiovascular Outcomes in ICM: Discovery Cohort

During a median follow-up of 3.4 (interquartile range, 1.6–6.3) years, there were 109 (70.1%) all-cause death events and 62 (40.2%) HF hospitalizations in patients with ICM. Elevated N8AS levels (per 1-SD increase in $\log_2$-transformed, standardized N8AS) were independently associated with greater mortality (adjusted hazard ratio [HR], 1.48; 95% CI, 1.19–1.85 [$P=0.001$]) and higher risk for HF hospitalization (adjusted HR, 1.72; 95% CI, 1.27–2.33 [$P<0.001$]), independent of BNP, hsTnI, and hsCRP (Table 2, Figure 1).

Incremental Prognostic Value to BNP

In a multivariate model including all aforementioned covariates, together with BNP and N8AS, both were

Table 1. Baseline Characteristics of Participants in the Discovery Cohort

	CAD/No ICM (n=320)	Prevalent ICM (n=154)	*P* Value
Demographic data			
Age, y	67.44±10.77	68.32±10.50	0.40
Male sex	234 (73.1)	117 (76.0)	0.51
Black race	35 (10.9)	21 (13.6)	0.39
Clinical data			
Diabetes mellitus	124 (38.8)	72 (46.8)	0.10
Hypertension	230 (71.9)	105 (68.2)	0.41
Hyperlipidemia	225 (70.3)	100 (64.9)	0.24
Smoking history	216 (67.5)	107 (69.5)	0.67
Body mass index, kg/m^2	28.78±5.08	28.24±4.82	0.27
Serum creatinine, mg/dL	1.30±1.27	1.35±0.68	0.60
Ejection fraction, %	56.31±6.24	30.31±9.92	<0.001
Modified Duke CAD Index*	53.07±26.35	57.15±28.24	0.16
Medication history			
ACEI/ARB	215 (67.2)	116 (75.3)	0.07
β-Blocker	245 (76.6)	125 (81.2)	0.26
Biomarkers			
BNP, pg/mL	98.10 [47.08–206.78]	325.40 [125.60–739.38]	<0.001
hsTnI, pg/mL	6.90 [3.60–17.60]	20.75 [9.10–72.98]	<0.001
hsCRP, mg/L	3.10 [1.20–7.45]	5.35 [2.03–10.00]	0.001

Data are expressed as mean±SD, median [interquartile range], or number (percentage). ACEI indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BNP, B-type natriuretic peptide; CAD, coronary artery disease; hsCRP, high-sensitivity C-reactive protein; hsTnI, high-sensitivity troponin I; and ICM, ischemic cardiomyopathy.

*Data missing for 15.2% of patients.

independent predictors of events. When stratified by BNP (high BNP: >100 pg/mL) and N8AS (high N8AS: >median) levels, patients with high levels of both biomarkers had the highest risk, patients with low levels of both biomarkers had the lowest risk, and patients with elevation of only 1 biomarker had intermediate risk of all-cause mortality and HF hospitalizations (Table S1, Figure 2).

N8AS Levels and Cardiovascular Outcomes in ICM: Validation Cohort

The external validation cohort consisted of 85 patients with ICM (mean age: 60.3±11.6 years; 18.8% women, 36.5% black) (Table S2). Patients in the validation cohort were younger, more likely to be black and receive β-blockers, less likely to smoke, and had a lower left ventricular EF than patients with ICM in the discovery cohort. All other baseline characteristics were comparable.

During a median follow-up of 6.2 years (interquartile range, 3.3–9.3 years), there were 34 (40.0%) all-cause deaths. Similar to the discovery cohort, elevated N8AS levels (per 1-SD increase in $\log_2$-transformed, standardized N8AS) were associated with higher mortality in the validation cohort (adjusted HR, 1.97; 95% CI, 1.08–3.60 [*P*=0.03]). The association between N8AS and mortality was independent of BNP levels (adjusted HR, 2.30; 95% CI, 1.19–4.44 [*P*=0.01]).

N8AS Levels and Incident HF

Of the discovery cohort of patients with CAD without HF at the time of enrollment (n=320), 22 (6.9%) developed incident HF during a median follow-up of 6.1 years (interquartile range, 2.8–9.3 years). Patients with incident HF had a similar risk factor profile compared with those who did not, but levels of BNP were higher in patients who developed incident HF (Table S3). Elevated N8AS levels (per 1-SD increase in $\log_2$-transformed, standardized N8AS) were independently associated with higher risk of incident HF (adjusted

Table 2. HR Estimates for the Association Between N8AS Levels ($\log_2$-Transformed, Standardized), All-Cause Mortality, and HF Hospitalizations in Patients With ICM

Discovery Cohort	All-Cause Mortality*		HF Hospitalization*	
N8AS (HR per SD Increase)	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Unadjusted (n=154)	1.54 (1.26–1.87)	<0.001	1.54 (1.19–2.01)	0.001
Model 1 (n=154)†	1.48 (1.19–1.85)	0.001	1.72 (1.27–2.33)	<0.001
Model 1+BNP (n=144)	1.39 (1.11–1.75)	0.005	1.62 (1.17–2.24)	0.004
Model 1+hsTnI (n=152)	1.45 (1.16–1.81)	0.001	1.71 (1.26–2.32)	0.001
Model 1+hsCRP (n=152)	1.43 (1.14–1.79)	0.002	1.66 (1.22–2.25)	0.001
Model 1+BNP+hsTnI+hsCRP (n=142)	1.34 (1.06–1.69)	0.01	1.56 (1.13–2.16)	0.008

BNP indicates B-type natriuretic peptide (in pg/mL); HF, heart failure; hsCRP, high-sensitivity C-reactive protein (in mg/L); hsTnI, high-sensitivity troponin I (in pg/mL); ICM, ischemic cardiomyopathy; and N8AS, N8-acetylspermidine.

*Hazard ratios (HRs) were calculated with the use of Cox regression models incorporating covariates listed in the table.

†Model 1 adjusted for age, sex, race, creatinine, presence of diabetes mellitus, hypertension, hyperlipidemia, body mass index, and smoking history.

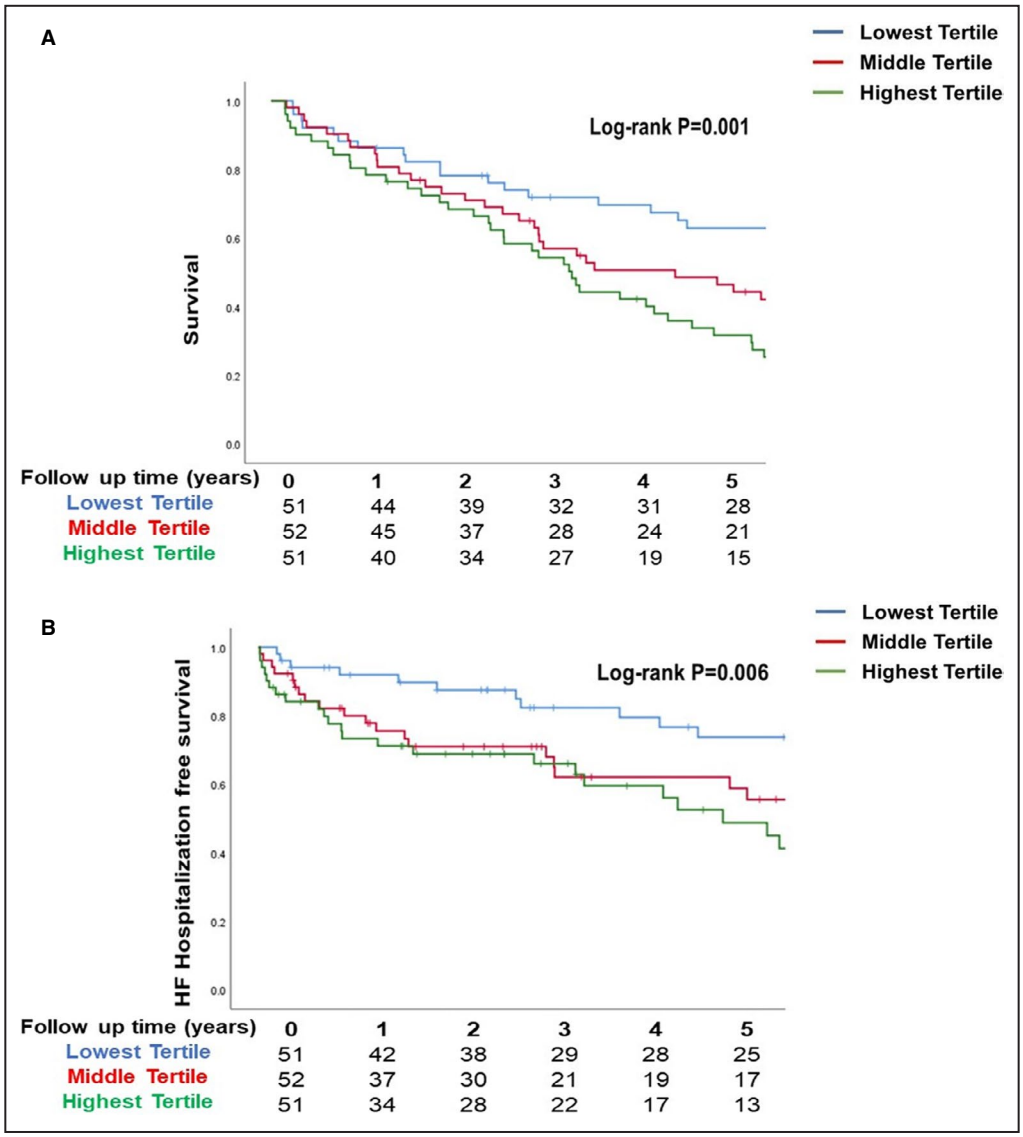

Figure 1. Kaplan–Meier curves for outcomes stratified by N8-acetylspermidine (N8AS) tertiles.
A, All-cause mortality by N8AS tertiles, and (**B**) heart failure hospitalization–free survival by N8AS tertiles.

HR, 4.16; 95% CI, 1.41–12.25 [*P*=0.01]), independent of BNP, hsTnI, and hsCRP (Table 3).

N8AS Levels and NICM

To explore the effect of Etiology on N8AS levels, we performed a secondary analysis comparing patients who had NICM (n=131) with those who had ICM (n=85) in the validation cohort. Patients with ICM had higher N8AS (log_2-transformed, standardized) levels (ICM: 0.18±0.81; NICM: −0.12±1.09 [*P*=0.02]) but similar levels of BNP (*P*=0.10) (Table S4). There was a significant Etiology*N8AS interaction in the prediction of all-cause mortality (*P*=0.02, ratio of HR in ICM:NICM per 1-SD increase in log_2-transformed, standardized N8AS: 1.97). Unlike in ICM, N8AS levels (per 1-SD increase in log_2-transformed, standardized N8AS) were not significantly associated with mortality in the NICM cohort (adjusted HR, 1.06; 95% CI, 0.77–1.46 [*P*=0.72]).

Metabolic Pathways Associated With N8AS

A total of 5719 detected features in the 474 patients in the discovery cohort entered global metabolomics analysis to determine metabolic pathways associated with N8AS. At a false discovery rate q threshold <0.01, there were 203 features that correlated with N8AS (Table S5). Pathway enrichment analysis demonstrated that these features mapped to 2 metabolic pathways: the carnitine shuttle (*P*=0.007) and the saturated fatty acids β-oxidation pathway (*P*=0.04) (Table 4).

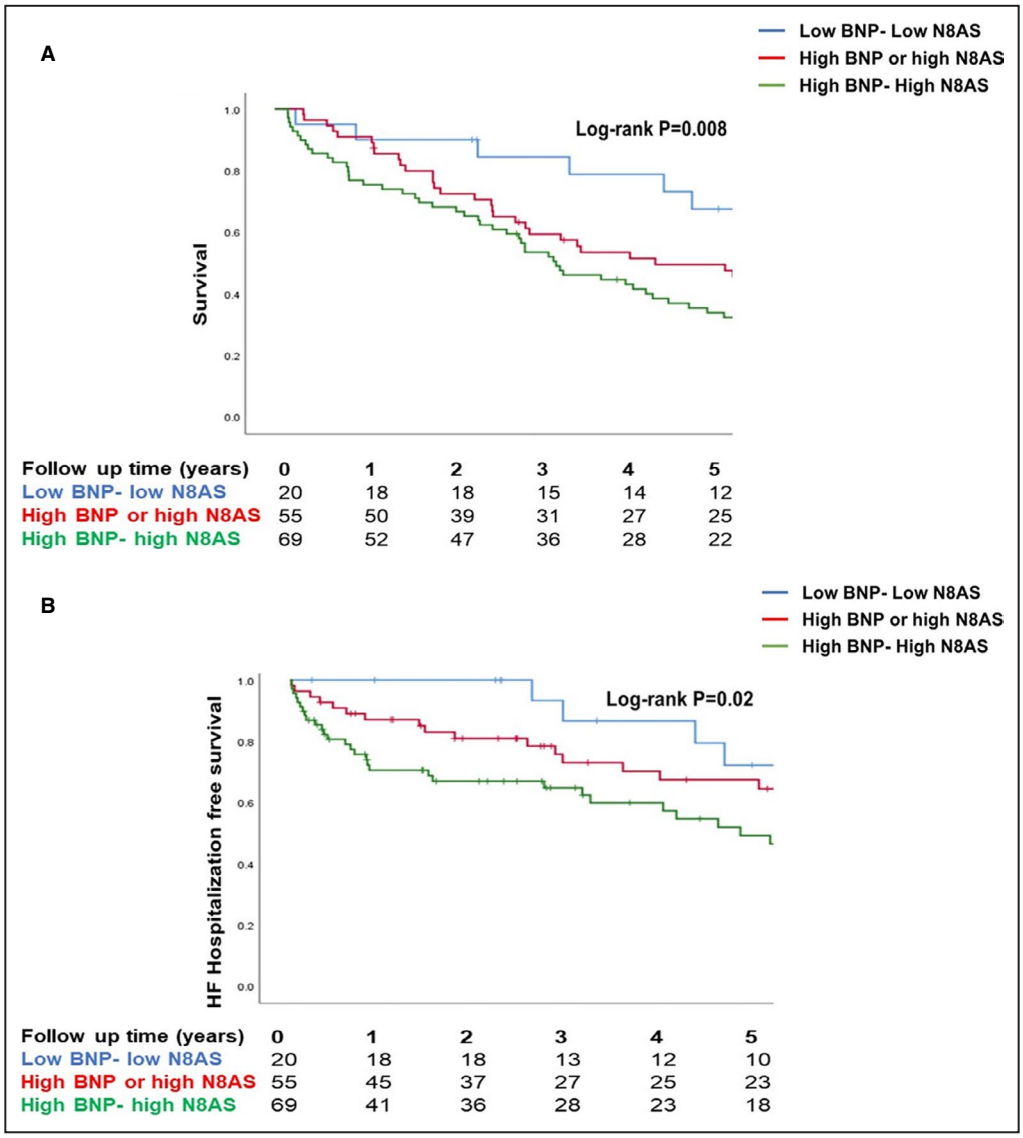

Figure 2. Kaplan–Meier curves for outcomes by BNP (B-type natriuretic peptide)–N8-acetylspermidine (N8AS) strata (high BNP: >100 pg/mL, high N8AS: >median).
A, All-cause mortality by BNP-N8AS strata, and (**B**) heart failure (HF) hospitalization–free survival BNP-N8AS strata.

Targeted correlation analysis of metabolites in 3 central pathways of N8AS-associated metabolism (polyamine metabolism, methionine metabolism, and urea cycle) (Figure 3) revealed 16 significant correlations at a false discovery rate q<0.2 (Table 4). Most urea cycle metabolites were negatively correlated, while those involved in methionine metabolism were positively correlated with N8AS.

DISCUSSION

The major findings of this study are: (1) N8AS levels are higher in patients with ICM compared with patients who have CAD without ICM and NICM; (2) higher circulating N8AS levels are associated with higher mortality in patients with ICM, independent of BNP; and (3) higher N8AS levels in patients who have CAD without HF are associated with greater risk of incident HF (Figure 4). N8AS levels correlate with metabolites in the carnitine shuttle and the saturated fatty acid β-oxidation pathway, as well as known pathways of N8AS-associated metabolism.

HF with reduced EF affects over 2.5 million Americans, with >50% mortality within 5 years of diagnosis.[28] The incorporation of natriuretic peptides into clinical practice has refined prognostication in patients with HF; however, the residual risk of adverse outcomes remains high.[3] Consequently,

Table 3. HR Estimates for the Association Between N8AS Levels (Log_2-Transformed, Standardized) and Incident HF in Patients Who Had CAD Without HF at Baseline

Discovery Cohort	Incident HF*	
N8AS (HR per SD Increase)	HR (95% CI)	*P* Value
Unadjusted (n=320)	5.19 (1.83–14.74)	0.002
Model 1 (n=320)†	4.16 (1.41–12.25)	0.01
Model 1+BNP (n=293)	4.35 (1.46–12.96)	0.008
Model 1+hsTnI (n=316)	3.94 (1.33–11.68)	0.01
Model 1+hsCRP (n=306)	4.14 (1.39–12.36)	0.01
Model 1+BNP+hsTnI+hsCRP (n=284)	4.14 (1.35–12.69)	0.01

BNP indicates B-type natriuretic peptide (in pg/mL); CAD, coronary artery disease; HF, heart failure; hsCRP, high-sensitivity C-reactive protein (in mg/L); hsTnI, high-sensitivity troponin I (in pg/mL); and N8AS, N8-acetylspermidine.

*Hazard ratios (HRs) were calculated with the use of Cox regression models incorporating covariates listed in the table.

†Model 1 adjusted for age, sex, race, creatinine, presence of diabetes mellitus, hypertension, hyperlipidemia, body mass index, and smoking history.

with the technological evolution of high-throughput -omics platforms over the past decade, there has been a surge in the identification of new biomarkers reflective of the final-common pathophysiology of progressive HF[29]: inflammation (CRP), extracellular-matrix remodeling (galectin-3), myocyte injury (troponins I and T), and myocyte stress (BNP, soluble ST2, growth differentiation factor 15).[3] However, a biomarker upstream to the final-common pathway for HF, and specific to the mechanism of HF development, would likely provide additional prognostic value.[4] Ischemic HF is a result of cardiomyocyte damage from calcium overload, oxidative stress, and activation of cellular apoptosis and autophagy occurring both during ischemia and reperfusion injury,[30] mechanisms unique to an ischemic pathogenesis for HF.[31] Moreover, since no currently available therapies successfully target ischemia/reperfusion injury, which accounts for nearly 50% of the total ischemic damage to the heart,[32] a biomarker specific to the ischemic cascade may also elucidate novel therapeutic targets.

Polyamines, including spermidine, spermine, and their derivatives, including N8AS, are ubiquitous aliphatic molecules with a well-recognized role in cardiac cell growth, differentiation, and protein synthesis.[13] Specifically, they are key mediators of the ischemic cascade,[33] by regulating apoptosis or programmed cell death,[5,6] and autophagy,[34] the principal cellular mechanisms for ischemia and reperfusion injury and subsequent cardiac failure.[35,36] In preclinical studies, acute ischemia activates the myocardial polyamine stress response, resulting in polyamine accumulation and consequent cardiomyocyte death.[8,37] On the other hand, polyamine degradation via acetylation is enhanced during ischemia/reperfusion injury,[37] a reaction that also mediates ischemic apoptosis and autophagy caused by the generation of toxic metabolic products and oxidative stress.[33] Additionally, polyamines regulate nitric oxide/cGMP pathway–mediated signaling[38] and calcium homeostasis[39] during ischemia/reperfusion injury. Inhibition of the rate-limiting enzyme of polyamine biosynthesis: ornithine decarboxylase, by α-difluoromethylornithine protects cardiomyocytes from ischemia-induced apoptosis via polyamine depletion.[8,12,13] In this study, we show that higher levels of plasma N8AS, an excretory product of intracellular spermidine that is reflective of increased polyamine turnover,[9,10] is reproducibly associated with worse clinical outcomes in 2 independent cohorts with ICM. Moreover, we confirmed the associations between N8AS and other known polyamine-associated pathway metabolites (Figure 4). Interestingly, while the regulation of cardiomyocyte death is the principal pathophysiological role for polyamines during the ischemic cascade,[5,6,34] we did not find a correlation between N8AS and hsTnI levels in our study. However, troponin is also released from intact cells by cleavage and membrane permeability during increased cardiac metabolic demand, potentially explaining our findings.

Other clinical studies have also helped to elucidate the role of polyamines in the pathogenesis of cardiovascular disease. In a prospective analysis of 658 patients from the Bruneck study,[7] higher dietary intake of spermidine, assessed by food questionnaires, was associated with a decreased risk of HF, as well as a composite outcome of acute coronary syndrome, stroke, and death from vascular disease. In an exploratory analysis, the authors showed inverse associations between spermidine intake and chitinase-3-like protein 1, implicated in atherosclerotic plaque inflammation and rupture, and with growth differentiation factor 15 levels, a known marker of HF progression.[7] Two previous metabolomics studies found that higher spermidine was part of a metabolite panel that predicted the presence of HF, as well as adverse outcomes in HF, independent of BNP and galectin-3.[40,41] However, since spermidine was part of a panel that also included essential amino acids, butyrylcarnitine and dimethylarginine/arginine ratio, the relative contribution of spermidine to the risk profile was not clearly elucidated. Additionally, these studies did not explore the effect of HF etiology on prognostication. We have shown that N8AS predicts outcomes in the ICM but not in the NICM population. Another novel finding of our study is that higher N8AS levels predict incident HF in patients with CAD. There is a paucity of data on

Table 4. Metabolites From Global and Targeted Network and Pathway Analysis That Significantly Correlated With N8AS

Name	*m/z*_Retention Time, s	Spearman Correlation Coefficient	*P* Value	False Discovery Rate Q Value	HMDB (Identification Level*)	Adduct
Global metabolic pathway and network analysis						
Carnitine shuttle						
α-Linolenyl carnitine	mz422.3266_t368	0.17	1.53E-04	5.83E-03	HMDB06319 (level 4)	M+H
Tetradecanoyl carnitine	mz372.3102_t366	0.22	1.09E-06	1.45E-04	HMDB05066 (level 2)	M+H
L-palmitoylcarnitin	mz400.3414_t398	0.16	3.54E-04	9.92E-03	HMDB00222 (level 1)	M+H
Trans-hexadec-2enoyl carnitin	mz398.3258_t376	0.20	7.18E-06	6.62E-04	HMDB06317 (level 4)	M+H
Linoelaidyl carnitine	mz424.3414_t384	0.20	8.17E-06	7.05E-04	HMDB06461 (level 4)	M+H
Timnodonyl carnitine	mz468.3082_t411	−0.17	1.72E-04	6.29E-03	NA (level 4)	M+Na
Saturated fatty acids β oxidation						
L-palmitoylcarnitine	mz400.3414_t398	0.16	3.54E-04	9.92E-03	HMDB00222 (level 1)	M+H
Targeted metabolic pathway and network analysis						
Polyamine metabolism						
Isoputreanine	mz161.129_t38	0.17	1.39E-04	5.55E-03	HMDB06009 (level 4)	M+H
Methionine/cysteine metabolism						
L-methionine	mz172.0403_t39	0.21	2.47E-06	2.83E-04	HMDB00696 (level 4)	M+Na
Cystathionine ketimine	mz204.0328_t142	0.14	1.89E-03	0.03	HMDB02015 (level 4)	M+H
Cystathionine sulfoxide	mz239.0712_t32	0.11	0.02	0.12	HMDB02399 (level 4)	M+H
N-ornithyl-L-taurine	mz240.0994_t438	−0.13	3.55E-03	0.04	HMDB33519 (level 4)	M+H
4-Hydroxy-17β-estradiol-2-S-glutathione	mz594.249_t421	−0.17	2.26E-04	7.58E-03	HMDB60139 (level 4)	M+H
Serine	mz106.0495_t42	0.11	0.01	0.09	HMDB00187; HMDB03406 (level 1)	M+H
Cysteinyl-cysteine	mz225.0347_t38	0.10	0.03	0.15	HMDB28772 (level 4)	M+H
Methylmalonate	mz141.0145_t33	0.11	0.01	0.10	HMDB00202 (level 4)	M+Na
γ-L-glutamyl-L-cysteine	mz251.0701_t374	0.15	1.07E-03	0.02	HMDB01049 (level 4)	M+H
Urea cycle metabolism						
Argininosuccinic acid; N2-(3-hydroxysuccinoyl)arginine; N2-(3-carboxy-2-hydroxy-1-oxopropyl) arginine	mz291.13_t416	−0.11	0.01	0.10	HMDB00052; HMDB32765; HMDB39408 (level 4)	M+H
Hippurate	mz180.0656_t68	0.19	4.55E-05	2.52E-03	HMDB00714 (level 2)	M+H
4-Acetamidobutanoate	mz146.081_t47	−0.12	8.93E-03	0.07	HMDB03681 (level 2)	M+H
Peptide 2-[3-carboxy-3-(methylammonio)propyl]-L-histidine	mz294.13_t420	−0.12	8.76E-03	0.07	NA	M+Na
Asparagine	mz133.0603_t47	−0.12	9.54E-03	0.07	HMDB00168 (level 1)	M+H
Asymmetric dimethylarginine; symmetric dimethylarginine	mz203.1503_t37	−0.10	0.03	0.15	HMDB01539; HMDB03334 (level 4)	M+H

M+Na indicates sodium adduct; *m/z*, mass-to-charge ratio; NA, not annotated in Human Metabolome Database (HMDB); and N8AS, N8-acetylspermidine.

1. Metabolite identification levels are adapted from the criteria proposed by Schymanski et al:
   a. Level 1 confirmed by tandem mass spectrometry (MS/MS) and co-elution with authentic standards;
   b. Level 2 confirmed by MS/MS and matches with online databases or in silico predicted spectra
   c. Level 3 confirmed by MS/MS at the chemical class level, but no evidence for a specific metabolite
   d. Level 4 computationally assigned annotation using xMSannotator (medium or high confidence)
   e. Level 5 accurate mass match
2. For metabolites with multiple adduct matches, only the hydrogen adduct (M+H) adduct is reported here.

the use of polyamines as biomarkers for incident HF prediction. Whether increased N8AS levels reflect a decrease in intracellular spermidine bioavailability, or increased spermidine production and degradation in response to ischemic stress, requires further exploration.

STUDY STRENGTHS AND LIMITATIONS

The major strengths of our study include the robust confirmation and quantification of N8AS levels, as well as the validation of our findings in an independent validation cohort that utilized different mass spectrometry

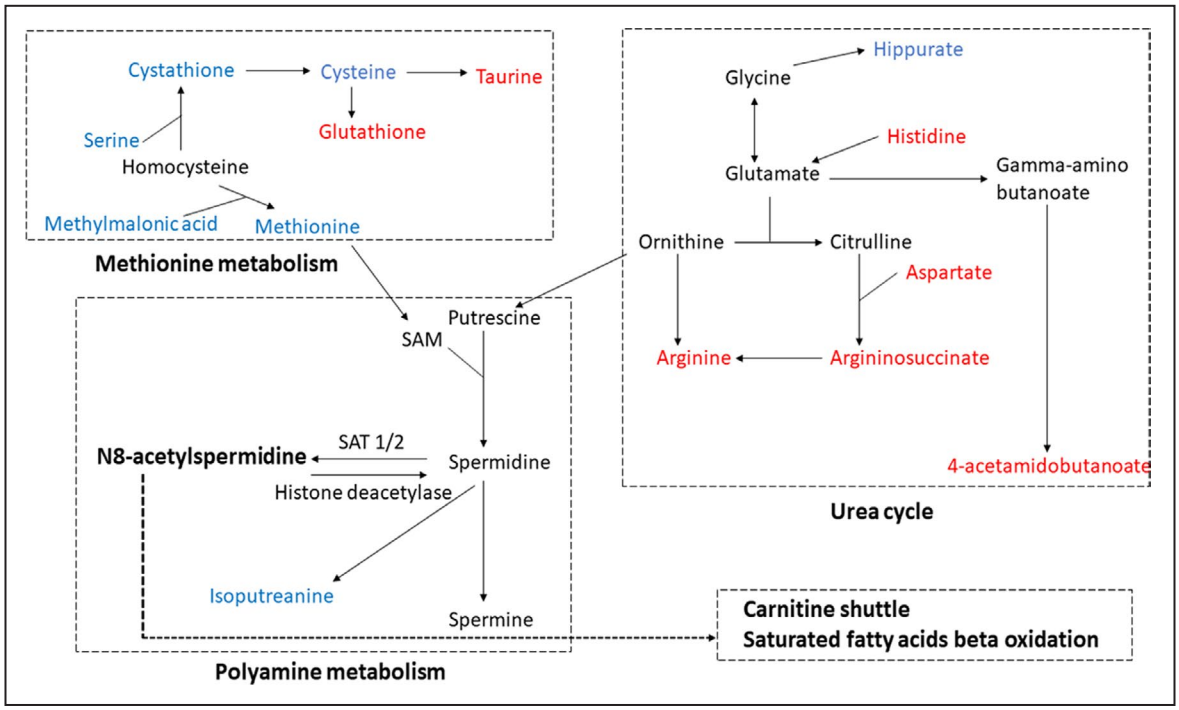

Figure 3. Correlated metabolites by targeted network and pathway analysis of N8-acetylspermidine (N8AS)-related pathways.
In red: Negatively correlated with N8AS. In blue: Positively correlated with N8AS. SAM indicates S-adenosyl methionine; and SAT 1/2, spermidine/spermine acetyltransferase 1/2.

and chromatography techniques, overcoming concerns regarding reproducibility of metabolomics studies. Limitations include the small validation cohort sample size and a lack of serial N8AS measurements. This precluded estimations of N8AS variability with acute decompensated HF.

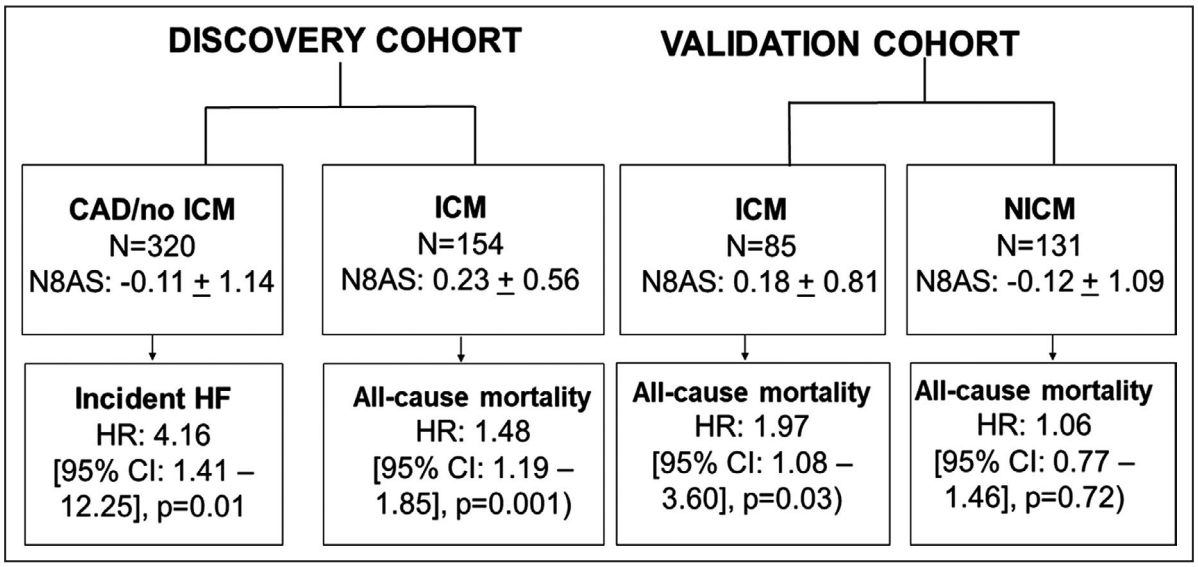

Figure 4. Summary of study findings.
N8-Acetylspermidine (N8AS) levels are higher in patients with ischemic cardiomyopathy (ICM) compared with patients who had coronary artery disease (CAD) without ICM ($P<0.001$) and non-ICM (NICM) ($P=0.02$). Higher circulating N8AS levels are associated with higher mortality in patients with ICM but not in patients with NICM. Higher N8AS levels in patients who have CAD without heart failure (HF) are associated with greater risk of incident HF. N8AS levels are $\log_2$-transformed and standardized (expressed per 1 SD). All analyses were adjusted for age, sex, race, creatinine, presence of diabetes mellitus, hypertension, hyperlipidemia, body mass index, and smoking history.

CONCLUSIONS

Circulating N8AS levels are higher in patients with ICM compared with those who have CAD without ICM and NICM. Higher levels are predictive of mortality and HF hospitalizations in patients with ICM, and with incident HF in those without HF, independent of BNP, hsTnI, and CRP levels. Whether N8AS is a risk factor for ICM and whether the modulation of polyamine metabolism and levels via diet[32] and pharmacological therapy, such as α-difluoromethylornithine,[8,12,13] affect disease progression needs further investigation.

ARTICLE INFORMATION

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Disclosures

None.

Supplementary Materials

Tables S1–S5

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SUPPLEMENTAL MATERIAL

Table S1. **Hazard ratio estimates for the association between BNP-N8AS strata, all-cause mortality and HF hospitalization in ICM.**

	All-cause mortality*		Heart failure hospitalization*	
BNP-N8AS strata †	**HR (95% CI)**	**P-value**	**HR (95% CI)**	**P-value**
Low-BNP Low-N8AS (n=20)	REF	REF	REF	REF
High-BNP or high-N8AS (n=55)	1.53 (0.74 – 3.16)	0.25	1.51 (0.54 – 4.18)	0.43
High-BNP and High-N8AS (n=69)	2.14 (1.05 - 4.28)	0.04	3.44 (1.23 – 9.62)	0.02

*Hazard ratios were calculated with the use of Cox regression models, adjusting for age, sex, race, creatinine, presence of diabetes, hypertension, hyperlipidemia, BMI and smoking history

†High BNP: >100 pg/mL, High N8AS: >median

Table S2. **Comparison of the baseline characteristics of ICM patients in the Discovery and Validation cohorts.**

	Discovery (n=154)	Validation (n=85)	P-value
Demographic data			
Age, years	68.32 ± 10.50	60.31 ± 11.64	<0.001
Male sex	117 (76.0)	69 (81.2)	0.35
Black race	21 (13.6)	31 (36.5)	<0.001
Clinical data			
Diabetes	72 (46.8)	34 (40.0)	0.31
Hypertension	105 (68.2)	63 (74.1)	0.34
Hyperlipidemia	100 (64.9)	58 (68.2)	0.61
Smoking history	107 (69.5)	10 (11.8)	<0.001
BMI, kg/m^2	28.24 ± 4.82	29.89 ± 6.77	0.05
Serum creatinine, md/dL	1.35 ± 0.68	1.33 ± 0.44	0.79
Ejection Fraction, %	30.31 ± 9.91	22.69 ± 9.19	<0.001
Medication history			
ACEi/ARB	116 (75.3)	70 (82.4)	0.21
BB	125 (81.2)	82 (96.5)	0.001
Biomarkers			

BNP, pg/mL	325.40 [125.60, 739.38]	247.00 [112.25, 786.25]	0.52

Data are mean ± standard deviation, median (interquartile range), or N (%). ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; BB, Beta blocker; BMI, body mass index; BNP, B-type natriuretic peptide in picograms per milliliter.

Table S3. Baseline characteristics of CAD patients with and without incident HF during follow-up in the Discovery cohort.

	CAD/No HF (n=298)	Incident HF (n=22)	P-value
Demographic data			
Age, years	67.15 ± 10.63	71.31 ± 12.10	0.08
Male sex	218 (73.2%)	16 (72.7%)	0.96
Black race	34 (11.4%)	1 (4.5%)	0.32
Clinical data			
Diabetes	115 (38.6%)	9 (40.9%)	0.83
Hypertension	212 (71.1%)	18 (81.8%)	0.28
Hyperlipidemia	209 (70.1%)	16 (72.7%)	0.78
Smoking history	198 (66.4%)	18 (81.8%)	0.14
BMI, kg/m^2	28.74 ± 5.12	29.30 ± 4.58	0.62
Serum creatinine, mg/dL	1.29 ± 1.31	1.39 ± 0.36	0.71
Ejection Fraction, %	56.27 ± 6.28	56.95 ± 5.88	0.62
Modified Duke CAD Index*	53.05 ± 26.60	53.37 ± 23.33	0.96
Medication history			
ACE/ARB	201 (67.4%)	14 (63.6%)	0.71

BB	228 (76.5%)	17 (77.3%)	0.93

Established biomarkers			
BNP, pg/mL	96.30 [45.85, 189.85]	183.20 [68.75, 414.75]	0.01
hsTnI, pg/mL	6.70 [3.50, 17.30]	9.60 [5.98, 24.85]	0.08
hsCRP, mg/L	3.10 [1.20, 7.50]	2.25 [1.15, 7.65]	0.77

*Data missing for 12.8% of subjects

Data are mean ± standard deviation, median (interquartile range), or N (%). ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; BB, Beta blocker; BMI, body mass index; BNP, B-type natriuretic peptide in picograms per milliliter; CAD, Coronary Artery Disease; HF, Heart Failure; hsCRP, High-sensitivity C-Reactive Protein in milligrams per liter; hsTnI, High-sensitivity Troponin I in picograms per milliliter.

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Table S4. **Baseline characteristics of patients with NICM and ICM in the Validation cohort.**

	NICM (n=131)	ICM (n=85)	P-value
Demographics			
Age, years	54.53 ± 10.98	60.31 ± 11.64	<0.001
Male sex	76 (58.0)	69 (81.2)	<0.001
Black race	67 (51.1)	31 (36.5)	0.03
Clinical data			
Diabetes	36 (27.5)	34 (40.0)	0.06
Hypertension	81 (61.8)	63 (74.1)	0.06
Hyperlipidemia	52 (39.7)	58 (68.2)	<0.001
Smoking history	18 (13.8)	10 (11.8)	0.66
BMI, kg/m^2	31.35 ± 7.89	29.89 ± 6.77	0.16
Serum creatinine, mg/dL	1.32 ± 0.72	1.33 ± 0.44	0.89
Ejection fraction, %	23.99 + 11.73	22.69 ± 9.19	0.37
Medication history			
ACE/ARB	104 (79.4)	70 (82.4)	0.59
BB	120 (91.6)	82 (96.5)	0.16
Biomarkers			

BNP, pg/mL	195.00 [49.00, 617.00]	247.00 [112.25, 786.25]	0.10

Data are mean ± standard deviation, median (interquartile range), or N (%). ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; BB, Beta blocker; BMI, body mass index; BNP, B-type natriuretic peptide in picograms per milliliter; CAD, Coronary Artery Disease; EF, Ejection Fraction; HF, Heart Failure; ICM, Ischemic Cardiomyopathy; NICM, Non Ischemic Cardiomyopathy.

Table S5. **Metabolites that significantly correlated (FDR q<0.01) with N8AS by global correlation mWAS.**

m/z_RT(sec)	Spearman correlation coefficient	P-value	FDR Q-value	HMDB Confidence*	HMDB Name	HMDB ID	Adduct
mz241.1547_t42	0.37	1.09E-16	3.11E-13	2	Pirbuterol	HMDB15407	M+H
mz207.1105_t42	0.36	4.27E-16	8.14E-13	NA	NA	NA	NA
mz185.1285_t46	0.34	1.88E-14	2.69E-11	NA	NA	NA	NA
mz229.1547_t42	0.34	5.02E-14	5.74E-11	3	Several metabolite matches	Several matches to HMDB IDs	
mz189.1597_t36	0.29	6.19E-11	5.90E-08	2	N6N6N6-Trimethyl-L-lysine	HMDB01325	M+H

mz397.3101_t427	0.29	8.72E-11	6.46E-08	2	Several metabolite matches	Several matches to HMDB IDs	
mz136.0482_t39	0.29	9.05E-11	6.46E-08	NA	NA	NA	NA
mz384.2738_t285	0.29	1.02E-10	6.46E-08	NA	NA	NA	NA
mz223.0844_t39	0.29	1.79E-10	9.99E-08	NA	NA	NA	NA
mz369.2824_t327	0.29	1.92E-10	9.99E-08	3; 2	Several metabolite matches	Several matches to HMDB IDs	
mz230.158_t43	0.28	2.70E-10	1.29E-07	3	Several metabolite matches	Several matches to HMDB IDs	
mz129.0658_t40	0.28	4.65E-10	2.04E-07	2	Several metabolite matches	Several matches to HMDB IDs	

mz438.3207_t342	0.28	5.20E-10	2.13E-07	NA	NA	NA	NA
mz371.298_t347	0.27	1.21E-09	4.62E-07	NA	NA	NA	NA
mz386.2895_t308	0.27	1.98E-09	7.06E-07	NA	NA	NA	NA
mz389.3085_t325	0.27	2.75E-09	9.25E-07	3; 2	2-Hydroxymyristoylcarnitine; Methyl 2-(10-heptadecenyl)-6-hydroxybenzoate	HMDB13166; HMDB38523	M+H_[+1]; M+H
mz146.1175_t41	0.27	3.85E-09	1.22E-06	2	3-Dehydroxycarnitine	HMDB06831	M+H
mz343.2668_t308	0.26	1.20E-08	3.60E-06	3	trans-2-Dodecenoylcarnitine	HMDB13326	M+H_[+1]
mz358.2577_t202	0.26	1.34E-08	3.84E-06	NA	NA	NA	NA
mz186.0186_t36	0.26	1.41E-08	3.84E-06	NA	NA	NA	NA

mz372.3015_t347	0.26	1.74E-08	4.51E-06	NA	NA	NA	NA
mz344.2791_t329	0.25	2.25E-08	5.60E-06	3	Dodecanoylcarnitine	HMDB02250	M+H
mz230.1865_t50	0.25	2.45E-08	5.83E-06	NA	NA	NA	NA
mz368.2795_t327	0.25	3.36E-08	7.69E-06	NA	NA	NA	NA
mz340.2476_t291	0.25	4.04E-08	8.88E-06	NA	NA	NA	NA
mz186.1317_t47	0.25	5.80E-08	1.23E-05	2	Several metabolite matches	Several matches to HMDB IDs	
mz204.9579_t34	0.24	1.45E-07	2.96E-05	NA	NA	NA	NA
mz342.2635_t308	0.23	2.55E-07	5.04E-05	3	trans-2-Dodecenoylcarnitine	HMDB13326	M+H

mz142.9379_t87	0.23	2.68E-07	5.11E-05	NA	NA	NA	NA
mz395.2979_t346	0.23	3.61E-07	6.66E-05	2	78-Dehydro-beta-micropteroxanthin	HMDB38506	M+H
mz115.0695_t40	0.23	3.95E-07	7.06E-05	NA	NA	NA	NA
mz330.2274_t47	0.23	4.11E-07	7.12E-05	2	6-Keto-decanoylcarnitine	HMDB13202	M+H
mz399.3258_t293	0.23	4.45E-07	7.48E-05	NA	NA	NA	NA
mz396.3103_t359	0.23	4.77E-07	7.80E-05	NA	NA	NA	NA
mz302.1955_t48	0.23	4.95E-07	7.87E-05	NA	NA	NA	NA
mz185.1149_t75	0.23	5.69E-07	8.79E-05	NA	NA	NA	NA

mz253.1272_t38	0.23	6.28E-07	9.45E-05	2	Several metabolite matches	Several matches to HMDB IDs	
mz370.2946_t347	0.23	7.13E-07	1.03E-04	NA	NA	NA	NA
mz399.3292_t376	0.23	7.23E-07	1.03E-04	3	Trans-hexadec-2enoyl carnitine	HMDB06317	M+H
mz568.2331_t409	-0.22	8.49E-07	1.18E-04	NA	NA	NA	NA
mz261.6174_t410	-0.22	1.02E-06	1.38E-04	NA	NA	NA	NA
mz372.3102_t366	0.22	1.09E-06	1.45E-04	NA	Tetradecanoyl carnitine	HMDB05066	M+H
mz236.9982_t45	0.22	1.24E-06	1.61E-04	NA	NA	NA	NA
mz388.3051_t326	0.22	1.32E-06	1.67E-04	3	2-Hydroxymyristoylcarnitine	HMDB13166	M+H

mz453.3758_t410	0.22	1.44E-06	1.79E-04	2	23-Dihydro-phytomenadione; Phylloquinol	HMDB34839; HMDB60502	M+H
mz394.2946_t347	0.22	2.29E-06	2.75E-04	NA	Tetradecanoyl carnitine	HMDB05066	M + Na
mz270.9583_t42	0.22	2.31E-06	2.75E-04	NA	NA	NA	NA
mz315.2361_t159	0.21	2.40E-06	2.80E-04	NA	NA	NA	NA
mz172.0403_t39	0.21	2.47E-06	2.83E-04	NA	L-methionine	HMDB00696	M+Na
mz1091.7035_t445	-0.21	2.53E-06	2.84E-04	3	LysoPC(20:3(5Z8Z11Z)); LysoPC(20:3(8Z11Z14Z))	HMDB10393; HMDB10394	2M+H
mz381.3155_t293	0.21	2.78E-06	3.06E-04	NA	NA	NA	NA
mz304.2119_t46	0.21	3.08E-06	3.32E-04	NA	NA	NA	NA
mz159.1493_t36	0.21	3.27E-06	3.46E-04	NA	NA	NA	NA

mz266.1217_t55	0.21	3.51E-06	3.61E-04	3; 2	Several metabolite matches	Several matches to HMDB IDs	
mz420.8851_t38	0.21	3.53E-06	3.61E-04	NA	NA	NA	NA
mz153.066_t46	0.21	3.98E-06	4.00E-04	2	N1-Methyl-2-pyridone-5-carboxamide; N1-Methyl-4-pyridone-3-carboxamide	HMDB04193; HMDB04194	M+H
mz469.3111_t407	-0.21	4.34E-06	4.28E-04	NA	NA	NA	NA
mz491.2935_t408	-0.21	4.51E-06	4.36E-04	NA	NA	NA	NA
mz549.3641_t444	-0.21	4.58E-06	4.36E-04	3	LysoPC(20:3(5Z8Z11Z)); LysoPC(20:3(8Z11Z14Z))	HMDB10393; HMDB10394	M+H_[+3]
mz227.1251_t44	0.21	6.61E-06	6.20E-04	NA	NA	NA	NA
mz398.3258_t376	0.2	7.18E-06	6.62E-04	3	trans-Hexadec-2-enoyl carnitine	HMDB06317	M+H

mz260.9725_t417	-0.2	7.30E-06	6.63E-04	NA	NA	NA	NA
mz541.3096_t414	-0.2	7.41E-06	6.63E-04	3	Several metabolite matches	Several matches to HMDB IDs	
mz564.2914_t423	-0.2	7.99E-06	7.03E-04	NA	NA	NA	NA
mz424.3414_t384	0.2	8.17E-06	7.05E-04	NA	Linoelaidyl carnitine	HMDB06461	M+H
mz519.3272_t413	-0.2	8.26E-06	7.05E-04	3	Several metabolite matches	Several matches to HMDB IDs	
mz174.9533_t473	0.2	8.56E-06	7.20E-04	NA	NA	NA	NA
mz273.6277_t422	-0.2	8.84E-06	7.33E-04	NA	NA	NA	NA

mz258.1096_t428	-0.2	1.06E-05	8.64E-04	2	Several metabolite matches	Several matches to HMDB IDs	
mz367.2836_t403	0.2	1.11E-05	8.92E-04	NA	NA	NA	NA
mz596.3705_t453	-0.2	1.24E-05	9.85E-04	NA	NA	NA	NA
mz1042.6821_t433	-0.2	1.35E-05	1.05E-03	NA	NA	NA	NA
mz1026.66_t431	-0.2	1.41E-05	1.09E-03	NA	NA	NA	NA
mz482.6125_t42	-0.2	1.48E-05	1.13E-03	NA	NA	NA	NA
mz86.096_t43	-0.2	1.52E-05	1.14E-03	2	Piperidine	HMDB34301	M+H
mz129.0519_t37	0.2	1.53E-05	1.14E-03	NA	NA	NA	NA

mz565.309_t414	-0.2	1.62E-05	1.18E-03	NA	NA	NA	NA
mz144.0802_t64	0.2	1.63E-05	1.18E-03	2	Several metabolite matches	Several matches to HMDB IDs	
mz206.0477_t109	0.2	1.65E-05	1.18E-03	NA	NA	NA	NA
mz548.3616_t445	-0.19	2.07E-05	1.46E-03	3	LysoPC(20:3(5Z8Z11Z)); LysoPC(20:3(8Z11Z14Z))	HMDB10393; HMDB10394	M+H_[+2]
mz572.2435_t415	-0.19	2.09E-05	1.46E-03	NA	NA	NA	NA
mz384.3102_t362	0.19	2.22E-05	1.53E-03	NA	NA	NA	NA
mz286.6253_t416	-0.19	2.31E-05	1.56E-03	NA	NA	NA	NA
mz521.7852_t422	-0.19	2.31E-05	1.56E-03	NA	NA	NA	NA

mz564.305_t415	-0.19	2.40E-05	1.59E-03	NA	NA	NA	NA
mz212.9996_t41	0.19	2.43E-05	1.60E-03	3	Uric acid	HMDB00289	M+2Na-H
mz353.0548_t429	-0.19	2.63E-05	1.71E-03	NA	NA	NA	NA
mz207.1492_t43	0.19	2.76E-05	1.78E-03	2	Agrocybenine; Monoethylglycinexylidide	HMDB41445; HMDB60656	M+H
mz124.9997_t435	-0.19	2.84E-05	1.81E-03	NA	NA	NA	NA
mz246.7838_t41	-0.19	3.06E-05	1.92E-03	NA	NA	NA	NA
mz279.1427_t41	0.19	3.28E-05	2.04E-03	2	Pentoxifylline	HMDB14944	M+H
mz1041.685_t444	-0.19	3.72E-05	2.29E-03	NA	NA	NA	NA
mz216.9227_t39	0.19	3.77E-05	2.29E-03	NA	NA	NA	NA

mz454.441_t449	0.19	3.93E-05	2.35E-03	NA	NA	NA	NA
mz152.0217_t39	0.19	3.95E-05	2.35E-03	NA	NA	NA	NA
mz148.1102_t36	0.19	4.03E-05	2.37E-03	NA	NA	NA	NA
mz866.3046_t43	-0.19	4.07E-05	2.37E-03	NA	NA	NA	NA
mz1043.692_t447	-0.19	4.16E-05	2.38E-03	NA	NA	NA	NA
mz283.1634_t39	0.19	4.16E-05	2.38E-03	NA	NA	NA	NA
mz819.4669_t433	-0.19	4.24E-05	2.40E-03	NA	NA	NA	NA
mz413.3051_t440	0.19	4.43E-05	2.48E-03	3	Several metabolite matches	Several matches to HMDB IDs	

mz180.0656_t68	0.19	4.55E-05	2.52E-03	2	Hippurate	HMDB00714	M+H
mz170.0929_t38	0.19	4.78E-05	2.63E-03	2	1-Methylhistidine; 3-Methylhistidine	HMDB00001; HMDB00479	M+H
mz582.2524_t423	-0.19	4.99E-05	2.71E-03	NA	NA	NA	NA
mz219.1724_t47	0.19	5.05E-05	2.71E-03	2	Several metabolite matches	Several matches to HMDB IDs	
mz1089.6888_t438	-0.19	5.07E-05	2.71E-03	NA	NA	NA	NA
mz284.1853_t45	0.18	5.19E-05	2.73E-03	2	alpha-Hydroxymetoprolol	HMDB60994	M+H
mz1025.6565_t431	-0.18	5.20E-05	2.73E-03	NA	NA	NA	NA
mz1042.6924_t447	-0.18	5.29E-05	2.75E-03	NA	NA	NA	NA

mz547.3486_t432	-0.18	5.34E-05	2.75E-03	NA	NA	NA	NA
mz493.3111_t403	-0.18	5.59E-05	2.85E-03	NA	NA	NA	NA
mz412.3056_t365	0.18	5.85E-05	2.94E-03	2	3-Hydroxyhexadecadienoylcarnitine	HMDB13335	M+H
mz259.0051_t41	0.18	5.85E-05	2.94E-03	NA	NA	NA	NA
mz578.2549_t424	-0.18	6.34E-05	3.15E-03	NA	NA	NA	NA
mz584.2504_t420	-0.18	6.42E-05	3.17E-03	NA	NA	NA	NA
mz144.9917_t32	0.18	6.78E-05	3.31E-03	NA	NA	NA	NA
mz366.2633_t330	0.18	6.99E-05	3.38E-03	NA	NA	NA	NA

mz258.2176_t43	0.18	7.03E-05	3.38E-03	NA	NA	NA	NA
mz492.3082_t408	-0.18	7.22E-05	3.44E-03	NA	NA	NA	NA
mz1090.6927_t437	-0.18	7.48E-05	3.53E-03	NA	NA	NA	NA
mz205.1263_t42	0.18	7.76E-05	3.63E-03	NA	NA	NA	NA
mz204.123_t42	0.18	7.86E-05	3.63E-03	3	L-Acetylcarnitine	HMDB00201	M+H
mz518.3238_t413	-0.18	7.86E-05	3.63E-03	3	LysoPC(18:3(6Z9Z12Z)); LysoPC(18:3(9Z12Z15Z))	HMDB10387; HMDB10388	M+H
mz470.2876_t416	0.18	7.95E-05	3.64E-03	NA	NA	NA	NA
mz362.7011_t41	-0.18	8.33E-05	3.78E-03	NA	NA	NA	NA
mz286.2376_t468	0.18	8.40E-05	3.78E-03	2	Myristoylglycine	HMDB13250	M+H

mz538.5741_t41	-0.18	8.48E-05	3.79E-03	NA	NA	NA	NA
mz558.2924_t432	-0.18	8.67E-05	3.83E-03	NA	NA	NA	NA
mz506.323_t416	-0.18	8.80E-05	3.83E-03	2	LysoPE(0:020:2(11Z14Z)); LysoPE(20:2(11Z14Z)0:0)	HMDB11483; HMDB11513	M+H
mz1041.675_t431	-0.18	8.84E-05	3.83E-03	NA	NA	NA	NA
mz330.2633_t297	0.18	8.85E-05	3.83E-03	NA	NA	NA	NA
mz1044.6487_t453	-0.18	8.99E-05	3.86E-03	NA	NA	NA	NA
mz795.973_t446	-0.18	9.43E-05	4.03E-03	NA	NA	NA	NA
mz302.7455_t41	-0.18	9.96E-05	4.22E-03	NA	NA	NA	NA
mz294.939_t37	0.18	1.05E-04	4.38E-03	NA	NA	NA	NA

mz452.3727_t409	0.18	1.05E-04	4.38E-03	NA	NA	NA	NA
mz346.863_t38	0.18	1.11E-04	4.61E-03	NA	NA	NA	NA
mz286.9072_t38	0.18	1.14E-04	4.70E-03	NA	NA	NA	NA
mz306.7397_t41	-0.18	1.16E-04	4.72E-03	NA	NA	NA	NA
mz818.9659_t433	-0.18	1.23E-04	4.99E-03	NA	NA	NA	NA
mz475.2436_t312	0.17	1.37E-04	5.53E-03	2	Rubraflavone B	HMDB30629	M+H
mz161.129_t38	0.17	1.39E-04	5.55E-03	2	Isoputreanine	HMDB06009	M+H
mz796.9789_t455	-0.17	1.41E-04	5.59E-03	NA	NA	NA	NA
mz332.2418_t118	0.17	1.45E-04	5.67E-03	NA	NA	NA	NA

mz206.0482_t143	0.17	1.45E-04	5.67E-03	NA	NA	NA	NA
mz280.9876_t38	0.17	1.46E-04	5.69E-03	NA	NA	NA	NA
mz292.6546_t444	-0.17	1.49E-04	5.76E-03	NA	NA	NA	NA
mz272.9446_t36	0.17	1.51E-04	5.80E-03	NA	NA	NA	NA
mz422.3266_t368	0.17	1.53E-04	5.83E-03	NA	L-palmitoyl carnitine	HMDB00222	M+Na
mz280.1485_t432	-0.17	1.62E-04	6.09E-03	NA	NA	NA	NA
mz546.3553_t445	-0.17	1.63E-04	6.09E-03	3	LysoPC(20:3(5Z8Z11Z)); LysoPC(20:3(8Z11Z14Z))	HMDB10393; HMDB10394	M+H
mz556.8594_t38	0.17	1.63E-04	6.09E-03	NA	NA	NA	NA
mz362.9265_t37	0.17	1.67E-04	6.19E-03	NA	NA	NA	NA

mz357.2823_t327	0.17	1.70E-04	6.27E-03	NA	NA	NA	NA
mz468.3082_t411	-0.17	1.72E-04	6.29E-03	NA	NA	NA	NA
mz234.9815_t41	0.17	1.80E-04	6.55E-03	NA	NA	NA	NA
mz122.9245_t38	-0.17	1.81E-04	6.56E-03	NA	NA	NA	NA
mz1066.6812_t433	-0.17	1.96E-04	7.00E-03	NA	NA	NA	NA
mz133.1049_t42	-0.17	1.96E-04	7.00E-03	2	2-Heptanethiol; Heptane-1-thiol	HMDB32303; HMDB32304	M+H
mz190.9121_t38	-0.17	2.03E-04	7.21E-03	NA	NA	NA	NA
mz862.3109_t42	-0.17	2.05E-04	7.22E-03	NA	NA	NA	NA

mz571.3583_t440	-0.17	2.07E-04	7.22E-03	3	Several metabolite matches	Several matches to HMDB IDs	
mz311.2032_t326	0.17	2.07E-04	7.22E-03	3	Mestranol; Gestodene; Menaquinol	HMDB15446; HMDB15668; HMDB60487	M+H
mz282.1308_t411	-0.17	2.11E-04	7.29E-03	NA	NA	NA	NA
mz806.965_t433	-0.17	2.13E-04	7.29E-03	NA	NA	NA	NA
mz1037.6556_t431	-0.17	2.13E-04	7.29E-03	NA	NA	NA	NA
mz392.279_t329	0.17	2.19E-04	7.47E-03	NA	NA	NA	NA
mz523.802_t432	-0.17	2.23E-04	7.53E-03	NA	NA	NA	NA
mz594.249_t421	-0.17	2.26E-04	7.58E-03	2	4-Hydroxy-17beta-estradiol-2-S-glutathione	HMDB60139	M+H

mz188.0706_t42	-0.17	2.27E-04	7.58E-03	3	Indoleacrylic acid; 6-Chloro-N-(1-methylethyl)-135-triazine-24-diamine	HMDB00734; HMDB33249	M+H
mz302.2152_t80	0.17	2.33E-04	7.76E-03	NA	NA	NA	NA
mz360.275_t248	0.17	2.36E-04	7.79E-03	NA	NA	NA	NA
mz205.0972_t43	-0.17	2.37E-04	7.79E-03	NA	NA	NA	NA
mz987.6412_t421	-0.17	2.42E-04	7.90E-03	NA	NA	NA	NA
mz185.9601_t41	0.17	2.43E-04	7.91E-03	NA	NA	NA	NA
mz570.3536_t439	-0.17	2.46E-04	7.93E-03	NA	NA	NA	NA
mz172.8514_t42	-0.17	2.48E-04	7.98E-03	NA	NA	NA	NA

mz559.2985_t433	-0.17	2.57E-04	8.20E-03	NA	NA	NA	NA
mz552.2605_t410	-0.17	2.59E-04	8.20E-03	2	Endoxifen O-glucuronide	HMDB60622	M+H
mz521.3422_t432	-0.17	2.60E-04	8.20E-03	3	LysoPC(18:2(9Z12Z))	HMDB10386	M+H_[+1]
mz261.1885_t49	0.17	2.68E-04	8.43E-03	3	Hexanoylcarnitine; L-Hexanoylcarnitine	HMDB00705; HMDB00756	M+H_[+1]
mz91.0538_t458	0.17	2.71E-04	8.48E-03	NA	NA	NA	NA
mz532.2797_t420	-0.17	2.77E-04	8.57E-03	NA	NA	NA	NA
mz516.9107_t432	-0.17	2.77E-04	8.57E-03	NA	NA	NA	NA
mz329.2505_t279	0.17	2.81E-04	8.64E-03	2	Several metabolite matches	Several matches to HMDB IDs	

mz518.2327_t433	-0.17	2.85E-04	8.73E-03	2	Pyronaridine	HMDB42003	M+H
mz542.3235_t415	-0.17	2.91E-04	8.86E-03	NA	NA	NA	NA
mz438.6302_t42	-0.17	2.97E-04	8.99E-03	NA	NA	NA	NA
mz628.2589_t421	-0.17	3.06E-04	9.19E-03	2	Tri-N-acetylchitotriose	HMDB06698	M+H
mz132.9983_t56	-0.17	3.07E-04	9.19E-03	NA	NA	NA	NA
mz1065.678_t432	-0.16	3.16E-04	9.41E-03	NA	NA	NA	NA
mz274.6254_t420	-0.16	3.20E-04	9.44E-03	2	Darunavir	HMDB15393	M+2H
mz860.3134_t43	-0.16	3.20E-04	9.44E-03	NA	NA	NA	NA
mz186.1123_t43	0.16	3.24E-04	9.46E-03	2	Pseudoecgonine; Ecgonine	HMDB06348; HMDB06548	M+H

mz273.608_t428	-0.16	3.24E-04	9.46E-03	NA	NA	NA	NA
mz546.3455_t432	-0.16	3.31E-04	9.56E-03	NA	NA	NA	NA
mz153.127_t77	0.16	3.31E-04	9.56E-03	2	Several metabolite matches	Several matches to HMDB IDs	
mz208.0393_t33	0.16	3.34E-04	9.59E-03	NA	NA	NA	NA
mz192.0739_t36	0.16	3.36E-04	9.59E-03	NA	NA	NA	NA
mz352.8976_t38	0.16	3.37E-04	9.59E-03	NA	NA	NA	NA
mz226.9516_t37	0.16	3.42E-04	9.67E-03	2	25-Dichloro-4-oxohex-2-enedioate	HMDB60363	M+H
mz104.1068_t430	-0.16	3.47E-04	9.78E-03	2	Neurine	HMDB31259	M+H

mz400.3414_t398	0.16	3.54E-04	9.92E-03	3	L-Palmitoylcarnitine	HMDB00222	M+H

HMDB ID, Human Metabolome Database Identification; m/z, mass-to-charge ratio; M+H, Hydrogen adduct; M+Na, Sodium adduct; RT, Retention time in seconds; NA, Not Annotated in HMDB

*Note(s):

1) Metabolite identification levels are adapted from the criteria proposed by Schymanski et al.:
    a. Level 1 confirmed by MS/MS and co-elution with authentic standards;
    b. Level 2 confirmed by MS/MS and matches with online databases or in-silico predicted spectra
    c. Level 3 confirmed by MS/MS at the chemical class level, but no evidence for a specific metabolite
    d. Level 4 computationally assigned annotation using xMSannotator (medium or high confidence)
    e. Level 5 accurate mass match