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ORIGINAL RESEARCH

Multicohort Metabolomics Analysis Discloses 9-Decenoylcarnitine to Be Associated With Incident Atrial Fibrillation

Lars Lind, MD, PhD; Samira Salihovic, PhD; Johan Sundström, MD, PhD; Corey D. Broeckling, PhD; Patrik K. Magnusson, PhD; Jessica Prenni, PhD; Tove Fall, PhD; Johan Ärnlöv, MD, PhD

BACKGROUND: The molecular mechanisms involved in atrial fibrillation are not well known. We used plasma metabolomics to investigate if we could identify novel biomarkers and pathophysiological pathways of incident atrial fibrillation.

METHODS AND RESULTS: We identified 200 endogenous metabolites in plasma/serum by nontargeted ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry in 3 independent population-based samples (TwinGene, n=1935, mean age 68, 43% females; PIVUS [Prospective Investigation of the Vasculature in Uppsala Seniors], n=897, mean age 70, 51% females; and ULSAM [Uppsala Longitudinal Study of Adult Men], n=1118, mean age 71, all males), with available data on incident atrial fibrillation during 10 to 12 years of follow-up. A meta-analysis of ULSAM and PIVUS was used as a discovery sample and TwinGene was used for validation. In PIVUS, we also investigated associations between metabolites of interest and echocardiographic indices of myocardial geometry and function. Genome-wide association studies were performed in all 3 cohorts for metabolites of interest. In the meta-analysis of PIVUS and ULSAM with 430 incident cases, 4 metabolites were associated with incident atrial fibrillation at a false discovery rate <5%. Of those, only 9-decenoylcarnitine was associated with incident atrial fibrillation and replicated in the TwinGene sample (288 cases) following adjustment for traditional risk factors (hazard ratio, 1.24 per unit; 95% CI, 1.06–1.45, *P*=0.0061). A meta-analysis of all 3 cohorts disclosed another 4 significant metabolites. In PIVUS, 9-decenoylcarnitine was related to left atrium size and left ventricular mass. A Mendelian randomization analysis did not suggest a causal role of 9-decenoylcarnitine in atrial fibrillation.

CONCLUSIONS: A nontargeted metabolomics analysis disclosed 1 novel replicated biomarker for atrial fibrillation, 9-Decenoylcarnitine, but this acetylcarnitine is likely not causally related to atrial fibrillation.

Key Words: atrial fibrillation ■ carnitine ■ epidemiology ■ gene ■ metabolomics

Atrial fibrillation (AF) is a very common cardiovascular disorder. AF at extreme pulse rates is a potentially life-threatening condition, but in the majority of subjects a stable heart rhythm can be managed by appropriate medication. However, AF is still associated with a substantial increased risk of cardioembolic stroke, as well as of heart failure.[1,2]

Traditional risk factors for atherosclerotic cardiac disease, such as hypertension, diabetes mellitus, obesity, and smoking, are risk factors also for AF. In addition, apart from these established clinical risk factors, a number of biomarkers have also been identified as potential AF risk factors. NT-proBNP/BNP (N-terminal pro–brain natriuretic peptide),[3] cardiac troponins,[4] white blood cell count,[5] low testosterone levels in men,[6] advanced glycation end-products and their receptor,[7] FGF23 (fibroblast growth factor 23)[8] and C-reactive protein,[9] FABP4 (fatty acid-binding protein 4), GDF15 (growth differentiation factor 15), and interleukin-6[10] have all been identified as biochemical AF risk factors, but none of those are used in clinical practice.

Correspondence to: Lars Lind, MD, PhD, Department of Medical Sciences, Dag Hammarskjöldsv. 10 B, Uppsala Science Park, 75237 Uppsala, Sweden. E-mail: lars.lind@medsci.uu.se

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CLINICAL PERSPECTIVE

What Is New?

- In a multicohort analysis of metabolomics, we showed that 1 acetylcarnitine, 9-decenoylcarnintine, was related to incident atrial fibrillation using a discovery/validation approach in independent samples.

What Are the Clinical Implications?

- Although a Mendelian randomization analysis did not show a clear causal role of this metabolite in atrial fibrillation, it is worthwhile to further investigate the role of acetylcarnitines in the pathogenesis of atrial fibrillation.

Nonstandard Abbreviations and Acronyms

AC	acetylcarnitines
ACADM	acyl-CoA dehydrogenase medium chain gene
FDR	false discovery rate
PIVUS	Prospective Investigation of the Vasculature in Uppsala Seniors
SM	sphingomyelin
ULSAM	Uppsala Longitudinal Study of Adult Men

Recent technological advances have made measurements of small molecules in biological samples, so called metabolomics, available for use in epidemiological studies. Using this approach, we and others have found lysophosphatidylcholine 18∶1 and 18∶2, monoglyceride 18∶2, and sphingomyelin (SM) 28∶1 to be related to incident coronary heart disease,[11] SM (32:1) to be associated with incident stroke,[12] and urobilin and SM (30:1) to be linked to incident heart failure,[13] showing metabolomics to be a way forward to discover novel biomarkers for cardiovascular disease. Previous studies investigating the association of plasma metabolomics and incident AF in the community are scarce.[14–16]

The aim of the present study was to identify novel biomarkers for incident AF using large-scale metabolomics data in 3 independent Swedish cohorts, in which we have recorded incident AF for 10 to 12 years. For this primary aim, we used a discovery/validation approach in independent cohorts. Because AF has been linked to specific myocardial pathology such as enlargement of the left atrium,[17] a low left ventricular (LV) ejection fraction and thick LV walls,[18] as well as an impaired transmitral early/late ratio,[19] we also investigated how metabolites of interest were associated with myocardial geometry and function at echocardiography as a mechanistic explorative aim. Furthermore, we used Mendelian randomization analysis to evaluate causality.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Samples

In the PIVUS (Prospective Investigation of the Vasculature in Uppsala Seniors), all 70-year-old residents of Uppsala County, Sweden, were invited to participate in a health survey and detailed clinical assessment between 2001 and 2004, as described in detail previously.[20] Of 2025 invited, 1016 (50.2%) participated in the baseline assessment within 1 month of their 70th birthday. Following exclusion of 38 subjects with known AF at baseline and subjects without metabolomics measurements, 897 subjects with metabolomic data were used in the analyses.

In the ULSAM (Uppsala Longitudinal Study of Adult Men), all men born in Sweden between 1920 and 1924 and living in Uppsala were invited to participate in a health assessment between 1970 and 1973, as described in detail previously.[21,22] Of 1681 invited, 1221 (72.6%) participated in the follow-up assessment at age 70 between 1991 and 1995 that serves as the baseline examination for the present study. Following exclusion of 20 subjects with known AF at baseline and subjects without metabolomics measurements, 1118 subjects with metabolomic data were used in the analyses.

TwinGene is a longitudinal study of 12 591 men and women nested within the Swedish Twin Registry.[23] Metabolomics was performed in a subset of TwinGene using a case-cohort design, where all incident cases of type 2 diabetes mellitus (n=218), coronary artery disease (n=282), ischemic stroke (n=186), and dementia (n=114) before December 31, 2010 were included, and a subcohort (controls) of 1643 individuals (43% women) stratified on age and sex was included. In the analyses, we used data on incident AF in both cardiovascular disease cases and controls. A total of 1935 individuals without prevalent AF at baseline were used in the analyses.

A flow chart of the 3 samples is given in Figure 1.

Approval from the Ethics Committee at Uppsala University was obtained for the PIVUS and ULSAM studies and from Karolinska Institutet for the TwinGene cohort. All participants gave their informed consent to the study.

Traditional Cardiovascular Risk Factors

The investigations in PIVUS and ULSAM were performed using standardized methods including

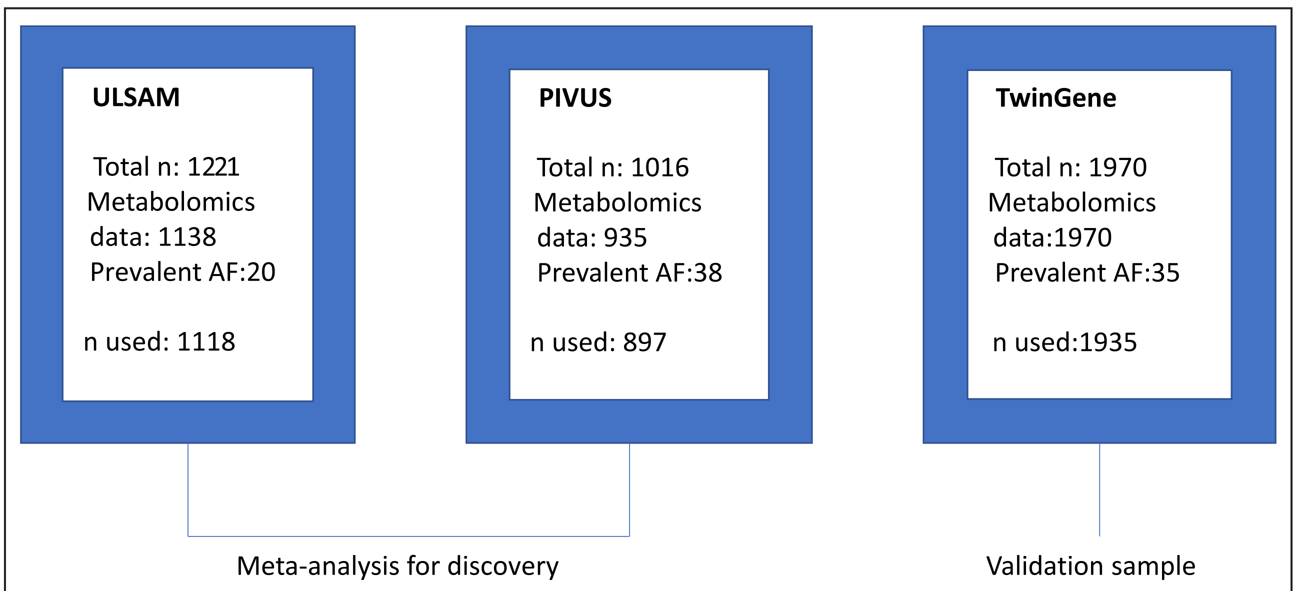

Figure 1. Flow chart on the use of the 3 samples.
A meta-analysis of all 3 cohorts was also performed as a secondary analysis. AF indicates atrial fibrillation; PIVUS, Prospective Investigation of the Vasculature in Uppsala Seniors; and ULSAM, Uppsala Longitudinal Study of Adult Men.

measurements of blood pressure (mean of 2 measurements in the supine position), biochemistry (lipids and glucose), and anthropometry. Participants in TwinGene went to their local healthcare center for a health checkup. In all cohorts, information on lifestyle and medication at baseline was collected through questionnaires. Diabetes mellitus was defined as having a fasting plasma glucose >7.0 mmol/L or taking antidiabetic medication.

Blood Sampling

In ULSAM and PIVUS, participants were investigated in the morning after an overnight fast. Venous blood samples were frozen immediately after separation of plasma (ULSAM) or serum (PIVUS) and stored at −80°C until analysis. Participants in TwinGene were sent blood sampling kits and then they went to their local healthcare center for blood sampling. Participants were instructed to perform the sample collection in the morning after an overnight fast, and serum samples were sent by overnight mail to the Karolinska Biobank where they were frozen at −80°C until analysis. However, some nonfasting individuals contributed with samples, and these were excluded in the present study.

Metabolomics

Metabolomics profiling in ULSAM, PIVUS, and TwinGene was performed using a Waters Acquity ultra-performance liquid chromatography system coupled to a Waters Xevo G2 Quadrupole Time-Of-Flight-Mass Spectrometry platform at Colorado State University (Fort Collins, CO, USA). Data acquisition using positive electrospray ion mode with a mass-to-charge ratio range of 50–1200 at 5 scans per second was alternately performed at collision energies of 6 V and 15–30 V. Details on sample handling and data processing by XCMS in R[24] have been published previously.[11,25] In total, 10 162 (ULSAM), 9755 (TwinGene), and 7522 (PIVUS) features were detected and adjusted for factors of external variability (plate effect, analysis date, retention, time drift, and sample collection) by analysis of variance-type standardization and/or log-transformation; by removal of spectra with abnormal intensities and/or low interduplicate correlations and/or retention times. This procedure has been described in detail (https://github.com/andgan/metabolomics_pipeline). For each feature, retention time, m/z, and fragmentation pattern were compared with in-house and public database reference libraries and matched according to Metabolomics Standard Initiative guidelines. Metabolites in common among ULSAM, PIVUS, and TwinGene were identified by matching m/z and retention time, followed by manual inspection of spectra. The metabolomic data are expressed in arbitrary units. Only the 200 endogenous metabolites being detected and annotated in all 3 samples are considered in this study. These metabolites are given together with a pathway analysis in Tables S1 through S2 and Figure S1.

Further confirmation of the metabolites of interest was performed to ensure data quality. The experimental confirmations were performed using the same method on the same instrument (ultra-performance

liquid chromatography-Quadrupole Time-Of-Flight Mass Spectrometry) as the samples from PIVUS, TwinGene and ULSAM. When synthetic standards were available, metabolites were annotated by matching of mass spectra and retention time to the mass spectra and retention time obtained from experiments of external standards (Metabolomics Standard Initiative level 1). When synthetic standards were not available, experimental confirmations were performed by parallel reaction monitoring of the different metabolites in question using random serum samples from the 3 cohorts or by experimentally obtained mass spectra obtained from public database reference libraries such as, for example, METLIN (Metabolomics Standard Initiative level 2). The confirmatory annotation information is provided in Figures S2 through S6.

Follow-Up

During the follow-up periods, incident cases of AF (*International Classification of Diseases, Eighth Revision* [*ICD-8*] code 427.9, *International Classification of Diseases, Ninth Revision* [*ICD-9*] 427D, and *International Classification of Diseases, Tenth Revision* [*ICD-10*] I48) were obtained by record linkage with the Swedish Patient register (inpatient) and the Swedish Cause of Death registry. The participants were followed to the first AF event, death, or censor date for the registers.

Echocardiography

A 2-dimensional echocardiography was performed with an Acuson XP124 cardiac ultrasound unit (Acuson, Mountain View, CA, USA) in the PIVUS study only. A 2.5 MHz transducer was used.

LV dimensions were measured with M-mode online from the parasternal projection, using a leading edge to leading edge convention. Measurements included left atrial diameter, interventricular septal thickness, posterior wall thickness, and LV diameter in end diastole and end systole. LV relative wall thickness (RWT) was calculated as (interventricular septal thickness + posterior wall thickness)/LV diameter in end diastole.

LV mass was determined from the Penn conversion. LV mass was then indexed for height$^{2.7}$ to obtain LV mass index.

LV volumes were calculated according to the Teichholz formula ($7*D^3/(2.4 + D)$) and from those values LV ejection fraction was calculated.

Statistical Analysis

Prevalent cases of AF known at baseline were deleted from the analyses. In the discovery step, we analyzed the relationships between 200 metabolites and incident AF one by one in the PIVUS and ULSAM separately using Cox proportional hazard regression analysis. Adjustments were performed for age and sex. Using the betas, SEs, and *P* values from these analyses, we performed an inverse-variance weighted, fixed-effects, meta-analysis of the PIVUS and ULSAM results. Metabolites passing the *P* value threshold estimated to yield a false discovery rate (FDR, Benjamini and Hochberg) of 5% in this meta-analysis was taken further to the replication step.

TwinGene was used for validation. Cox proportional hazard regression analysis weighted for the inverse of the sampling probability was used to relate the 200 metabolites to incident AF one by one adjusting for age and sex and in the second step also adjusted for the traditional risk factors systolic blood pressure, diabetes mellitus, smoking, low-density lipoprotein- and high-density lipoprotein-cholesterol, body mass index, as well as myocardial infarction and heart failure occurring before the AF event. Metabolites showing an FDR of 5% were considered significant in this validation analyses.

As a secondary analysis to maximize statistical power, we also performed an inverse weighted meta-analysis of all 3 cohorts using the same procedure as described previously. In this case, we adjusted for age and sex, as well as for the traditional cardiovascular risk factors, and used a strict Bonferroni adjustment for 200 tests (P=0.00025) for the age- and sex-adjusted *P* value in order to take into account the multiple testing, because no replication was performed in this secondary analysis.

In a mechanistic, exploratory analysis, we related replicated metabolites to left atrial diameter and different indices of LV geometry and function in the PIVUS cohort. These analyses were performed by linear regression with 2 levels of adjustment, first with age and sex only and then using also traditional risk factors, as previously. In this exploratory analysis, we reported associations at nominal significance (P<0.05).

In a publicly available database of genome-wide association studies (GWAS) for metabolites (http://mips.helmholtz-muenchen.de/proj/GWAS/gwas/index.php?task=advanced_search), we did not find any significant associations for 9-Decenoylcarnitine, our validated top finding. We therefore meta-analyzed GWAS results from ULSAM, PIVUS, and TwinGene for this metabolite. TwinGene and PIVUS participants were genotyped with Illumina Human OmniExpress (≈700 000 single-nucleotide polymorphisms [SNPs]), and ULSAM participants were genotyped with Illumina Human Omni2.5M (≈2 500 000 SNPs). Samples were excluded based on call rate <95%, extreme heterozygosity (>3 SD from the mean), sex discordance, duplicated samples, close relatives, or ethnic outliers. Variants with a call rate <0.99 and SNPs with effective allele frequency <1% were excluded from the scaffold before imputation. All the samples underwent the same quality control and imputation of polymorphic 1000 genome CEU SNPs (Phase

I, version 3) performed using IMPUTE2. SNPTEST 2.5 was used for the GWAS analyses. Only SNPs with effective allele frequency >0.05 and IMPUTE2 info >0.4 were included in the analyses. A cutoff of $P<5\times10^{-8}$ was used to denote genome-wide significance.

The *P* values reported in the tables and text are the original *P* values, not being adjusted for FDR. Analyses were performed using Stata 14.1 if not otherwise stated (Stata Corp., College Station, TX, USA).

Results

Basic characteristics of the 3 samples are given in Table 1.

Incident Cases of Atrial Fibrillation

During a follow-up period of a median 10.0 years in PIVUS (range 0.3–10.9 years), 148 incident cases of AF occurred during 8726 person-years at risk. In ULSAM 282 incident cases were recorded during median follow-up of 12.9 years (range 0.1–17.3 years, 13 373 person-years at risk), and in TwinGene 288 incident cases occurred during median follow-up of 10.1 years (range 0.1–12.6 years, 18 386 person-years at risk).

Primary Analysis

In a meta-analysis of the PIVUS and ULSAM samples (discovery samples), 3 metabolites were associated with incident AF with FDR <5%. Of those, only 9-decenoylcarnitine (hazard ratio, 1.24 per unit; 95% CI, 1.06–1.45, *P*=0.0061) was associated with incident AF in the TwinGene sample (replication sample) also following adjustment for traditional risk factors (for details see Table 2 and Figure 2). The mean values (and SD) for 9-decenoylcarnitine in the 3 cohorts were 13.4 (0.83) in TwinGene, 10.8 (1.11) in ULSAM, and 12.8 (0.85) in PIVUS, all expressed in arbitrary units.

Because antihypertensive treatment per se, and in particular beta-blocking agents, as well as heart rate could theoretically influence both metabolites and risk of incident AF, we performed a sensitivity analysis in the PIVUS sample, the only sample with data on these potential confounders. However, neither the addition of heart rate, nor antihypertensive treatment or beta-blocking agents to the multiadjusted models changed the relationships between the metabolites and incident AF to any substantial degree.

Secondary Analysis

In the exploratory meta-analysis of the 3 samples, 5 metabolites, including 9-Decenoylcarnitine, were related to incident AF when adjusted for age and sex and when the *P* value was adjusted for 200 tests according to Bonferroni (*P*<0.00025; l-octanoylcarnitine, bilirubin, urobilin, and SM (28:1)(inverse)). All of those showed FDR <5% when further adjusted for traditional risk factors (see Table 3 for details).

Table 1. Means (SD) or Proportions of Cardiovascular Risk Factors in the 3 Cohort Studies

	PIVUS (n=897)	ULSAM (n=1118)	TwinGene (n=1935)
Age, y	70.1 (0.1)	71.2 (0.6)	68.3 (8.2)
Sex (% female)	51	All males	43
Smokers, %	11	21	14
High-density lipoprotein-cholesterol, mmol/L	1.52 (0.42)	1.28 (0.35)	1.35 (0.40)
Low-density lipoprotein-cholesterol, mmol/L	3.40 (0.88)	3.89 (0.89)	3.75 (1.02)
Body mass index, kg/m^2	26.9 (4.3)	26.3 (3.4)	26.3 (4.0)
Diabetes mellitus, %	11	15	12
Systolic blood pressure, mm Hg	149 (22)	146 (18)	142 (20)
Antihypertensive medication, %	31	42	25
Heart rate, beats/min	62 (9)		
Prevalent myocardial infarction, %	7.2	9.0	8.3
Prevalent stroke, %	3.8	3.5	5.6
Prevalent heart failure, %	5.8	1.7	4.5
Left atrial diameter, mm	39 (7)		
Left ventricular end-diastolic diameter, mm	47 (6)		
Relative wall thickness	0.44 (0.08)		
Left ventricular mass index, g/m$^{2.7}$	43 (13)		
N-terminal pro–brain natriuretic peptide, pg/mL	Median: 106 Interquartile range: 62–174		

PIVUS indicates Prospective Investigation of the Vasculature in Uppsala Seniors; and ULSAM, Uppsala Longitudinal Study of Adult Men.

Table 2. Associations Between Metabolites and Incident Atrial Fibrillation in the TwinGene Sample for the 3 Metabolites That Showed a False Discovery Rate <0.05 in the Meta-Analysis of the PIVUS and ULSAM Results

Metabolite	Age and Sex Adjusted				Multiple Adjusted			
	HR	95% CI Lower Limit	95% CI Higher Limit	*P* Value	HR	95% CI Lower Limit	95% CI Higher Limit	*P* Value
9-decenoylcarnitine	1.20	1.03	1.41	0.015	1.24	1.06	1.45	0.0061
Bilirubin	1.05	.91	1.20	0.51	1.01	.87	1.14	0.95
L-octanoylcarnitine	1.11	.97	1.28	0.12	1.15	.98	1.34	0.074

Associations are given at 2 levels of adjustment. Multiple adjustment includes age, sex, systolic blood pressure, diabetes mellitus, smoking, low-density lipoprotein- and high-density lipoprotein-cholesterol, and body mass index. The HRs are given for a unit change in metabolite levels. HR indicates hazard ratio; PIVUS, Prospective Investigation of the Vasculature in Uppsala Seniors; and ULSAM, Uppsala Longitudinal Study of Adult Men.

Mechanistic Analyses

9-decenoylcarnitine was related to left atrium diameter, relative wall thickness, and LV mass index in the age- and sex-adjusted analyses, but following adjustment also for the traditional risk factors, these relationships were of borderline significance (*P*=0.06–0.08, see Table 4 for details).

Mendelian Randomization Analysis

In our own GWAS analyses, we found several genetic SNPs in the region of the acyl-CoA dehydrogenase medium chain gene *(ACADM,* chromosomal position chr1*:* 76190036–76253260) associated to 9-decenoylcarnitine at a genome-wide level ($P<5\times10^{-8}$). The strongest association was seen for rs74339586 (also denoted rs121226481 in some databases), position Chr1:76222640 (using HRCh37, hg19), being located in an intron of *ACADM* (see Table 5 for details). According to Phenoscanner (http://www.phenoscanner.medschl.cam.ac.uk) and the GWAS catalogue (https://www.ebi.ac.uk/gwas/), no phenotype has previously been associated with that SNP.

Other SNPs in the ACADM locus have been linked to other acetylcarnitines (AC), such as rs1146588 (chr1:76229787), being related to hexanoylcarnitine, octanoylcarnitine, cis-4-decenoyl carnitine, decanoylcarnitine, as well as isobutyrylcarnitine. Our top hit for 9-decenoylcarnitine was in close linkage disequilibrium (R2 0.92) with that SNP (https://snipa.helmholtz-muenchen.de/snipa3/?task=variant_browser).

In a Mendelian randomization analysis, our top hit for 9-Decenoylcarnitine, rs74339586, was not associated with AF (beta .0069, SE .0081, *P* value=0.39) using publicly available results of the largest published GWAS for AF by Roselli et al.[26]

For the SNPs within the region of *ACADM* (±50k bp) only 1 SNP showed *P*<0.01 (rs76671446, effect allele A, position chr1: 76251884, beta −0.062, SE 0.023, *P* value=0.0089) in the GWAS for AF. Our top hit was not in close linkage disequilibrium with that locus.

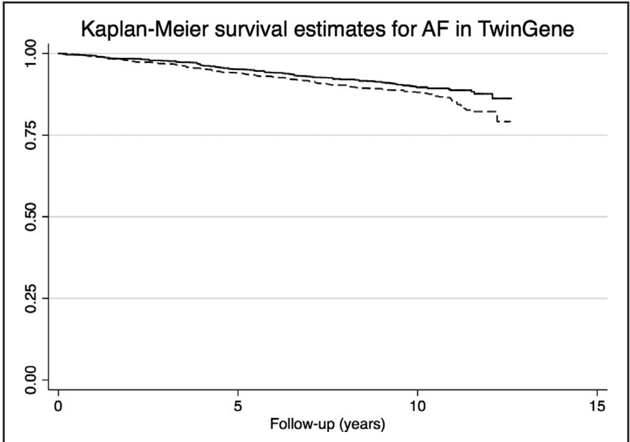

Figure 2. Kaplan-Meier survival curve for atrial fibrillation (AF) in the TwinGene sample regarding 9-decenoylcarnitine levels.

The dashed line indicates subjects with levels of 9-decenoylcarnitine above the median and the solid line indicates subjects below the median (*P*<0.015 for difference between the 2 groups).

DISCUSSION

The primary analysis of the present study showed 1 replicated metabolite, 9-Decenoylcarnitine, to be related to incident atrial fibrillation. A meta-analysis of all 3 cohorts disclosed additional 4 metabolites that were associated with AF. 9-decenoylcarnitine was furthermore related to left atrial size at echocardiography, but our Mendelian randomization analysis did not support a causal role for circulating 9-decenoylcarnitine in the development of incident AF.

Comparison With the Literature

There have been a few small-scale studies comparing the metabolomics profile of patients with prevalent AF with that of controls without AF.[27,28] However, because patients with AF most often receive medications with possible effects on the metabolome, such studies are hard to interpret.

We have identified 3 studies that have reported associations between untargeted circulating metabolomics

Table 3. Associations Between Metabolites and Incident Atrial Fibrillation in a Meta-Analysis of the TwinGene, ULSAM, and PIVUS Samples

Metabolite	Age and Sex Adjusted				Multiple Adjusted			
	HR	95% CI Lower Limit	95% CI Higher Limit	*P* Value	HR	95% CI Lower Limit	95% CI Higher Limit	*P* Value
9-decenoylcarnitine	1.18	1.09	1.27	0.000012	1.17	1.08	1.26	0.000042
Bilirubin	1.14	1.07	1.22	0.000013	1.11	1.04	1.20	0.0025
Sphingomyelin (28:1)	0.77	0.69	0.87	0.000029	0.80	0.70	0.91	0.0014
L-octanoylcarnitine	1.15	1.07	1.24	0.000045	1.16	1.08	1.25	0.000053
Urobilin	1.11	1.05	1.17	0.00020	1.09	1.02	1.16	0.0047

Associations are given at 2 levels of adjustment. Multiple adjustment includes age, sex, systolic blood pressure, diabetes mellitus, smoking, low-density lipoprotein- and high-density lipoprotein-cholesterol, and body mass index. Only associations with an age- and sex-adjusted *P* value<0.00025 (Bonferroni adjustment) are shown. The HRs are given for a unit change in metabolite levels. HR indicates hazard ratio; PIVUS, Prospective Investigation of the Vasculature in Uppsala Seniors; and ULSAM, Uppsala Longitudinal Study of Adult Men.

and incident AF. The first, performed in 1919 Blacks in the ARIC (Atherosclerosis Risk in Communities) study, identified associations between 2 conjugated bile acids (glycolithocholate sulfate and glycocholenate sulfate) and incident AF.[16] However, in a subsequent analysis in a larger portion of the ARIC study (n=3922) only glycocholenate sulfate was confirmed as an AF predictor. Also, that study identified 2 metabolites related to pyrimidine metabolism (pseudouridine and uridine) and 1 metabolite related to polyamine metabolism, acisoga, as AF predictors.[14] In contrast, none of 217 metabolites were associated with incident AF at the prespecified Bonferroni corrected level of significance in 2458 participants in the Framingham heart study.[15] Thus, none of the 6 metabolites disclosed in the present study were identified in previous studies relating untargeted metabolomics to incident AF.

9-decenoylcarnitine belongs to the family of ACs being oxidative metabolites with a fatty acid esterified to a carnitine molecule.[29] These compounds are generated by the enzymes CPT1 and CPT2 (carnitine palmitoyltransferase 1 and 2), in order to facilitate the transport of fatty acids across the mitochondrial membranes to the matrix space. There, the AC carnitines are split into acyl-CoA and carnitine. The carnitine molecule is shuttled back across the membrane while acyl-CoA is used for ATP production by the beta-oxidation pathway.

The heart tissue could use different sources for energy production. However, after a normal mixed meal, 60% to 70% of the ATP production in myocardial tissue is due to beta-oxidation of fatty acids. Thus, ACs have a major role in the daily energy production is the heart.

Medium- and long-chain ACs have been found to be linked to visceral obesity,[30] and intentional weight loss is known to affect levels of ACs.[31] Decenoylcarnitine and other ACs have also been linked to an impaired glucose tolerance in previous studies.[32] Of particular interest is that 2 other medium-chained ACs (decanoylcarnitine and octanoylcarnitine) have been associated with cardioembolic stroke,[33] a diagnosis mainly caused by AF.

In our secondary analysis, another medium-chained AC, L-octanoyncarnitine, was related to incident AF, suggesting that it may be ACs as a group rather than specific ACs that could be of interest for AF.

As reviewed by Huang et al,[34] it is commonly believed that for the maintenance of AF both a trigger and a substrate is needed. The trigger could be an increased tone in the autonomic nerve system or

Table 4. Relationships Between 9-Decenoylcarnitine Levels and Indices of Myocardial Function and Geometry in the PIVUS Study

	Age- and Sex-Adjusted Analysis			Multiple-Adjusted Analysis		
	Beta	SE	*P* Value	Beta	SE	*P* Value
Left atrial diameter	0.67	0.24	0.0064	0.39	0.21	0.063
Left ventricular end-diastolic diameter	0.31	0.20	0.11	0.131	0.191	0.49
Relative wall thickness	0.008	0.003	0.019	0.006	0.003	0.079
Left ventricular mass index	1.62	0.52	0.0019	0.79	0.42	0.064
Left ventricular ejection fraction	0.001	0.003	0.86	0.001	0.003	0.81

The betas are given for a unit change in 9-decenoylcarnitine levels. PIVUS indicates Prospective Investigation of the Vasculature in Uppsala Seniors.

Table 5. Genome-Wide Association Study (GWAS) for 9-Decenoylcarnitine as Being Related to Incident Atrial Fibrillation

rs-Number	rs74339586 (Also Denoted rs121226481)
Position (hg19)	1:76222640
Effect/other allele (EAF)	A/G (0.28)
Beta, SE, and *P* value	−0.280, 0.025, $P<1.0 \times 10^{-21}$
Nearest gene	*ACADM*

The most significant locus is given.

changed dynamics of Ca^{2+}. The substrate is typically an enlarged left atrium with fibrosis but also includes changed properties of ion channels. GWAS studies on AF have disclosed a number of genes being related to the disease.[25] A pathway analysis on those genes showed striated muscle development and cardiac muscle contraction to be the major pathways. Because 9-Decenoylcarnitine is involved in the generation of ATP from fatty acids, it is likely that this AC is involved in this cardiac muscle contraction pathway.

SM is the second most abundant phospholipid in human plasma and is present in all cellular membranes, in the myelin sheath, and in plasma lipoproteins. SM metabolites that are present or derived from lipoproteins have been implicated in various vascular cell changes that contribute to atherogenesis.[35]

Bilirubin and urobilin were also identified in the secondary analysis. Both are breakdown products of hemoglobin and have recently been linked to incident heart failure.[13] However, the inclusion of heart failure before AF in the models had only a marginal effect on the effect estimate, suggesting that the link between SM and incident AF is not mediated by heart failure to a major degree.

An important risk factor for AF is heart failure,[36] as well as myocardial infarction. Both of these disorders lead to cardiac remodeling with fibrosis development, and heart failure is a common complication of a myocardial infarction.

In the present study, we adjusted the analyses for both heart failure and myocardial infarction that presented before the AF. However, these analyses was based on hospitalized cases for both diseases, and silent myocardial infarction and less severe heart failure, not demanding in-hospital care, will not be covered by our approach. It could therefore not be ruled out completely that our top finding, 9-Decenoylcarnitine, is linked to AF through an association with mild heart failure or silent myocardial infarction.

Because of a lack of publicly available genetic instruments for 9-Decenoylcarnitine, we performed a meta-analysis of GWAS results from the 3 samples (around 4000 individuals) to search for a powerful genetic instrument to be used in Mendelian randomization analysis. We identified an SNP in the *ACADM* gene being associated with 9-decenoylcarnitine with a rather low *P* value (10^{-20}). In the Mendelian randomization analysis, we used a large GWAS study on AF with >65 000 cases as outcome data. It should be acknowledged that our own GWAS could have been underpowered in order to be able to show causality for 9-Decenoylcarnitine, and we have to await future larger GWASs for metabolomics to finally solve the causality issue. Because of the lack of causality for 9-decenoylcarnitine so far, this metabolite is not likely to be an important biomarker for AF to be used in the clinic in the future but might nevertheless shed a light on the role of carnitines in AF.

The strengths of the present study are that metabolomics was measured with the same instruments in 3 independent cohorts in combination with genetic analyses, which enabled us to use a strict discovery/validation approach in combination with a Mendelian randomization analysis.

A limitation is the amount of only 200 annotated metabolites, so it is not a true untargeted approach even though the selection of metabolites were solely based on the availability of reliable annotations. Another limitation is that we almost exclusively have individuals with European descent in our Swedish samples, so the results have to be confirmed in other geographical and ethnic groups. Because the present study consists of mainly elderly subjects, further studies on this topic are needed in younger samples. In epidemiological studies, the incidence rate of AF is almost always underestimated, because many cases with mild paroxysmal of AF will not demand hospital care.

Two cohorts used plasma, and 1 study used serum for the metabolomics analyses. This might have created slightly different values for the metabolites, although the values between the 3 cohorts were harmonized before statistical analyses were performed (11,25). If anything, using a mix of plasma and serum in the cohorts could only drive the associations toward the null hypothesis and underestimate the true association between the metabolites and AF. In this case, no false positive associations would be induced by the mix of plasma/serum.

All population-based epidemiological studies are suffering from a selection bias toward healthy subjects. However, as long as there is a wide range in the exposure variable, a selection bias is usually not a major problem when estimating the degree of association between a certain exposure and an outcome. In the case of metabolites being the exposures, as in this case, it is unclear if all metabolites have an appropriate wide range. It was therefore appropriate that we used 3 different study samples and a discovery/validation approach to ensure that a possible selection bias in 1 of the samples did not produce false positive results.

In conclusion, a metabolomics analysis disclosed 1 novel replicated biomarker for AF, 9-Decenoylcarnitine. The genetic studies did, however, not suggest a causal role in AF for circulating 9-Decenoylcarnitine.

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Affiliations

From the Department of Medical Sciences (L.L., J.S.)and Department of Medical Sciences, Molecular Epidemiology and Science for Life Laboratory (S.S., T.F.), Uppsala University, Uppsala, Sweden; School of Medical Sciences, Örebro University, Örebro, Sweden (S.S.); Uppsala Clinical Research Center, Uppsala University, Uppsala, Sweden (J.S.); Proteomics and Metabolomics Facility, Colorado State University, Fort Collins, CO (C.D.B.); Department of Medical Epidemiology and Biostatistics (MEB), Karolinska Institutet, Stockholm, Sweden (P.K.M.); Department of Horticulture and Landscape Architecture, Colorado State University, Fort Collins, CO (J.P.); Division of Family Medicine and Primary Care, Department of Neurobiology, Care Sciences and Society, Karolinska Institutet, Huddinge, Sweden (J.Ä.); and School of Health and Social Sciences, Dalarna University, Falun, Sweden (J.Ä.).

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Disclosures

None.

Supplementary Material

Tables S1–S2
Figures S1–S6

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SUPPLEMENTAL MATERIAL

Table S1. List of the 200 metabolites used in the present study.

1,2-dilinolenoyl-sn-glycero-3-phosphocholine
1,2-dilinoleoyl-sn-glycero-3-phosphocholine
1,2-dioleoyl-sn-glycero-3-phosphocholine, 1,2-dipetroselenoyl-sn-glycero-3-phosphocholine
1,2-dipalmitoleoyl-sn-glycero-3-phosphocholine
1,2-dipalmitoyl-sn-glycero-3-phosphocholine
1,3,7-Trimethyluric acid
1-O-1'-(Z)-octadecenyl-2-hydroxy-sn-glycero-3-phosphoethanolamine
1-Stearoyl-2-Hydroxy-sn-Glycero-3-Phosphoethanolamine
1-arachidoyl-2-hydroxy-sn-glycero-3-phosphocholine
1-linoleoyl-2-stearoyl-sn-glycerol
1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine
1-oleoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine
1-oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine
1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine
1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine
1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine
1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine, C16-20:4 Phosphatidylcholine
1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine
1-vaccenoyl-2-palmitoyl-sn-glycerol
10-nitro-9E-octadecenoic acid, 9-nitro-9E-octadecenoic acid
17-phenyl trinor Prostaglandin E2, 17-phenyl trinor Prostaglandin D2
2,6 dimethylheptanoyl carnitine
2-Ketohexanoic acid
3-Indolepropionic acid
3-Pyridylacetic acid, trigonelline
3a,6b,7b-Trihydroxy-5b-cholanoic acid
4-Androsten-11Beta-ol-3, 17-dione, 11_-Hydroxy-4-androstene-3,17-dione

6-hydroxy-5-cholestanol, cholesterol
7-Ketocholesterol
9-Decenoylcarnitine
Acetaminophen
Alpha-Linolenic acid
Alpha-Tocopherol
Arachidonic acid
Arachidonic acid ethyl ester
Arachidonic acid methyl ester
Barogenin
Betaine
Biliribin I
Biliribin II
Biliverdin hydrochloride a
Biliverdin hydrochloride b
C12 Carnitine
C16 Carnitine
C16-20:5 Phosphatidylcholine
Caffeine
Ceramide phosphoethanolamine(33:1), Sphingomyelin(30:1)
Ceramide phosphoethanolamine(34:1)
Ceramide phosphoethanolamine(35:1), Sphingomyelin(32:1)
Ceramide phosphoethanolamine(35:2), Sphingomyelin(32:2)
Ceramide phosphoethanolamine(36:1), Sphingomyelin(33:1)
Ceramide phosphoethanolamine(37:2), Sphingomyelin(34:2)
Ceramide phosphoethanolamine(38:2)
Chenodeoxycholic acid
Choline
Cinnamic acid and derivatives
Corticosterone
Cortisol

Creatine
Creatinine
D-Urobilinogen, I-Urobilin
D-erythro-sphingosine
DL-2-Aminooctanoic acid
Decanoyl-L-carnitine
Deoxycholic acid
Deoxycholic acid glycine conjugate
Deoxycholic acid related metabolite
Dodecanedioic acid
Dodecanoic acid
Fatty acid C16:0
Fatty acid 22:5
Fatty acid C18:4
Fatty acid C19:2
Fatty acid C20:2
Fatty acid C20:3
Fatty acid C20:4
Fatty acid C20:5 methyl ester
Fatty acid C22:4
Fatty acid C22:6
Fatty acid C22:6 methyl ester
Flavone
Gamma-Caprolactone
Gamma-Tocopherol
Gamma-glutamyl-Leucine
Geranyl acetoacetate
Glycocholic acid
Heptadecanoic aicd
Hippuric acid
Hyodeoxycholic acid

Indoleacetic acid
Indolelactic acid
L-Acetylcarnitine
L-Aspartyl-L-phenylalanine
L-Carnitine
L-Leucine, L-Norleucine
L-Octanoylcarnitine
L-Phenylalanine
L-Proline
L-Tryptophan
L-Tyrosine, o-Tyrosine
Linoleic acid
Linoleyl carnitine
Lyso-PAF C-18
Lysophosphatidylcholine(0:0/16:0)
Lysophosphatidylcholine(0:0/16:1)
Lysophosphatidylcholine(0:0/18:0)
Lysophosphatidylcholine(0:0/18:2)
Lysophosphatidylcholine(0:0/20:4)
Lysophosphatidylcholine(0:0/20:5)
Lysophosphatidylcholine(16:1/0:0)
Lysophosphatidylcholine(18:1)a
Lysophosphatidylcholine(18:2/0:0)
Lysophosphatidylcholine(18:3)
Lysophosphatidylcholine(18e:0/0:0)
Lysophosphatidylcholine(20:1)
Lysophosphatidylcholine(20:2)
Lysophosphatidylcholine(20:3)a
Lysophosphatidylcholine(20:3)b
Lysophosphatidylcholine(20:4/0:0)
Lysophosphatidylcholine(20:5/0:0)

Lysophosphatidylcholine(22:4)
Lysophosphatidylcholine(22:5)a
Lysophosphatidylcholine(22:5)b
Lysophosphatidylethanolamine(16:0)
Lysophosphatidylethanolamine(18:0)
Lysophosphatidylethanolamine(18:1)
Lysophosphatidylethanolamine(18:2)
Lysophosphatidylethanolamine(20:4)
N-(15Z-tetracosenoyl)-sphinganine-1-phosphocholine
N-(9Z-octadecenoyl)-sphing-4-enine-1-phosphocholine
N-(octadecanoyl)-sphing-4-enine-1-phosphocholine
N-palmitoyl-D-erythro-sphingosylphosphorylcholine
Oleamide
Oleoyl-L-carnitine hydrochloride
Ornithine
Palmitic acid
Palmitoleic acid
Pantothenic acid
Paraxanthine, Theophylline
Pentadecanoic acid
Phenylalanylphenylalanine
Phosphatidylcholine(28:1)
Phosphatidylcholine(28:2)
Phosphatidylcholine(29:1)
Phosphatidylcholine(30:1)
Phosphatidylcholine(30:2)
Phosphatidylcholine(32:1)
Phosphatidylcholine(33:1), Phosphoethanolamine(36:1)
Phosphatidylcholine(34:0)
Phosphatidylcholine(34:0), Phosphoethanolamine(37:3)
Phosphatidylcholine(34:2), Phosphoethanolamine(37:2)

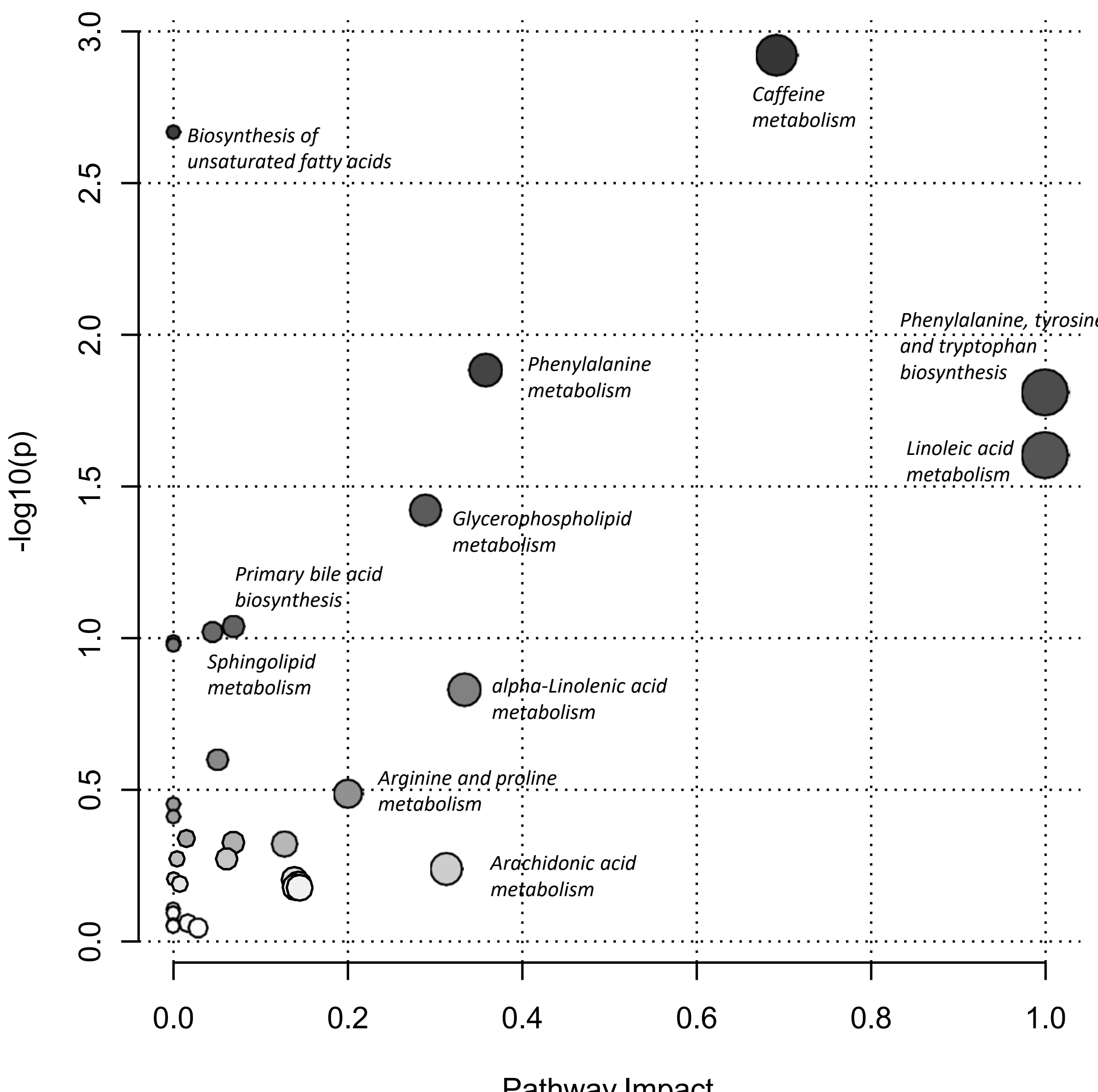
Phosphatidylcholine(34:3)
Phosphatidylcholine(34:4)
Phosphatidylcholine(34:5), Phosphoethanolamine(37:5)
Phosphatidylcholine(35:2), Phosphoethanolamine(38:2)
Phosphatidylcholine(35:4), Phosphoethanolamine(38:4)
Phosphatidylcholine(36:1)
Phosphatidylcholine(36:3), Phosphoethanolamine(39:3)
Phosphatidylcholine(36:5), Phosphoethanolamine(39:5)
Phosphatidylcholine(36:6)
Phosphatidylcholine(37:5), Phosphoethanolamine(40:0)
Phosphatidylcholine(38:2)
Phosphatidylcholine(38:3)
Phosphatidylcholine(38:4)
Phosphatidylcholine(38:5), Phosphoethanolamine(41:6)
Phosphatidylcholine(38:7)
Phosphatidylcholine(40:5)
Phosphatidylcholine(40:6)
Phosphatidylcholine(42:7)
Phosphatidylcholine(O-18:1/0:0), Phosphatidylcholine(P-18:1/0:0)
Phosphatidylethanolamine(19:1), Phosphatidylcholine(16:2)
Phosphatidylserine(18:0)
Piperine
Propranolol
Prostaglandin J2
Salicylic acid, Aspirin
Sphingomyelin(40:2)
Sphingomyelin(41:2)
Sphingomyelin(42:3)
Sphingomyelin(d18:2/18:1)
Stachydrine
Stearic acid

Theobromine
Treprostinil
Uric acid
Vitamin D3 and derivatives
Caprolactam
cis-5-Tetradecenoylcarnitine
cis/trans-Oleic acid
dehydroepiandrosterone sulfate (sodium salt)
monoacylglycerol(14:0)
monoacylglycerol(16:0)
monoacylglycerol(16:1)
monoacylglycerol(18:0)
monoacylglycerol(18:1)
monoacylglycerol(18:2)
monoacylglycerol(20:5)
myristic acid
sodium glycochenodeoxycholate
sum of Hexoses

Table S2. Metabolome pathway analysis was performed for 200 metabolites used in the present study using MetaboAnalyst 4.0. The analysis combines pathway enrichment analysis with pathway topology in order to simplify biological interpretation and pathway visualization. Metabolites from curated human metabolic pathways (Kyoto Encyclopedia of Genes and Genomes) were included in an over representation analysis which uses a hypergeometric test to evaluate whether the metabolite set of interest is represented more than expected by chance within that specific list.

	Total pathway metabolites	**Metabolite hits**	**Raw *P*-value**	**–log10(*P*)**	**Impact**
Caffeine metabolism	10	4	0.001198	2.9214	0.69231
Biosynthesis of unsaturated fatty acids	36	7	0.002147	2.6683	0
Phenylalanine metabolism	10	3	0.013076	1.8835	0.35714
Phenylalanine, tyrosine and tryptophan biosynthesis	4	2	0.015477	1.8103	1
Linoleic acid metabolism	5	2	0.024913	1.6036	1
Glycerophospholipid metabolism	36	5	0.03791	1.4212	0.28914
Primary bile acid biosynthesis	46	5	0.09152	1.0385	0.06847
Sphingolipid metabolism	21	3	0.095475	1.0201	0.04462
Neomycin, kanamycin and gentamicin biosynthesis	2	1	0.10304	0.98699	0
Aminoacyl-tRNA biosynthesis	48	5	0.1054	0.97715	0
alpha-Linolenic acid metabolism	13	2	0.14792	0.82999	0.33333
Glycine, serine and threonine metabolism	33	3	0.25206	0.59849	0.05034
Arginine and proline metabolism	38	3	0.32597	0.48683	0.20055
Valine, leucine and isoleucine biosynthesis	8	1	0.35328	0.45188	0
Ubiquinone and other terpenoid-quinone biosynthesis	9	1	0.38767	0.41153	0
Fatty acid biosynthesis	47	3	0.45786	0.33927	0.01473
Steroid hormone biosynthesis	85	5	0.47227	0.32581	0.06857
Porphyrin and chlorophyll metabolism	30	2	0.47781	0.32074	0.12753
Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	14	1	0.53432	0.2722	0.00399
Arginine biosynthesis	14	1	0.53432	0.2722	0.06091
Arachidonic acid metabolism	36	2	0.57743	0.2385	0.3135
Fatty acid degradation	39	2	0.62187	0.2063	0
Starch and sucrose metabolism	18	1	0.62616	0.20331	0.13851
Pantothenate and CoA biosynthesis	19	1	0.64617	0.18965	0.00714
Tryptophan metabolism	41	2	0.64948	0.18743	0.14305
Tyrosine metabolism	42	2	0.66269	0.17869	0.13972
Ether lipid metabolism	20	1	0.66512	0.1771	0.14458
Glutathione metabolism	28	1	0.7847	0.1053	0
Inositol phosphate metabolism	30	1	0.80728	0.092977	0
Purine metabolism	65	2	0.87027	0.060344	0.01651
Fatty acid elongation	39	1	0.88316	0.053959	0
Valine, leucine and isoleucine degradation	40	1	0.8895	0.050852	0
Steroid biosynthesis	42	1	0.90118	0.045187	0.0282

Figure S1. Metabolome pathway coverage for the 200 metabolites used in the present study.



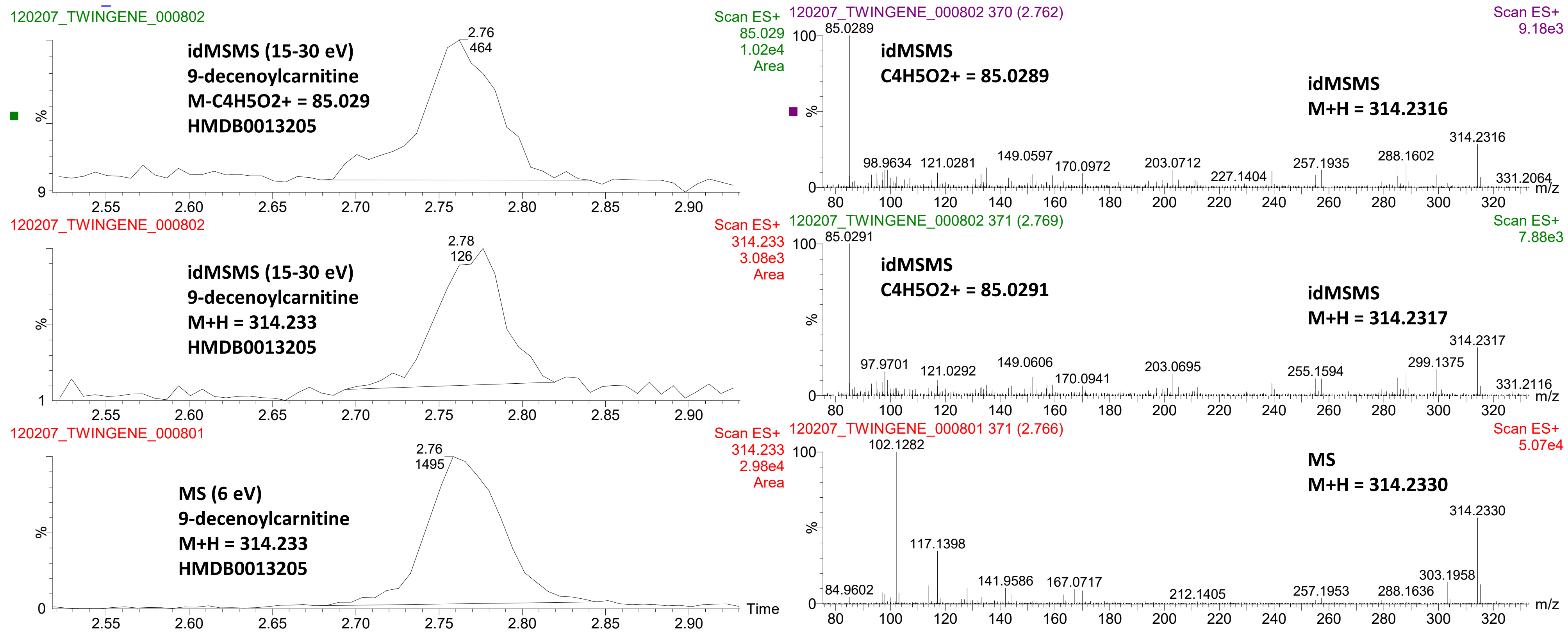


Figure S2. 9-decenoylcarnitine was annotated at metabolomics standards initiative (MSI) level 2 by database matching with accurate mass and fragmentation schemes reported for other acylcarnitines. These fragmentation schemes include the precursor of 85 fragment, or by detecting the neutral losses of either M-59 or M-161. We conclude that we selectively detect 9-decenoylcarnitine as shown in MS (collision induced dissociation at 6 eV) and idMSMS (collision induced dissociation ramp 15-30 eV) chromatograms obtained from an authenthic sample from TWINGENE. We consistently observe M+H = 314.233 in both MS and idMSMS mode as well as the major fragment C4H5O2+ = 85.0289 eluting at 2.76 minutes using UPLC-QTOFMS operated in positive ESI. The corresponding mass spectra are shown to the right.

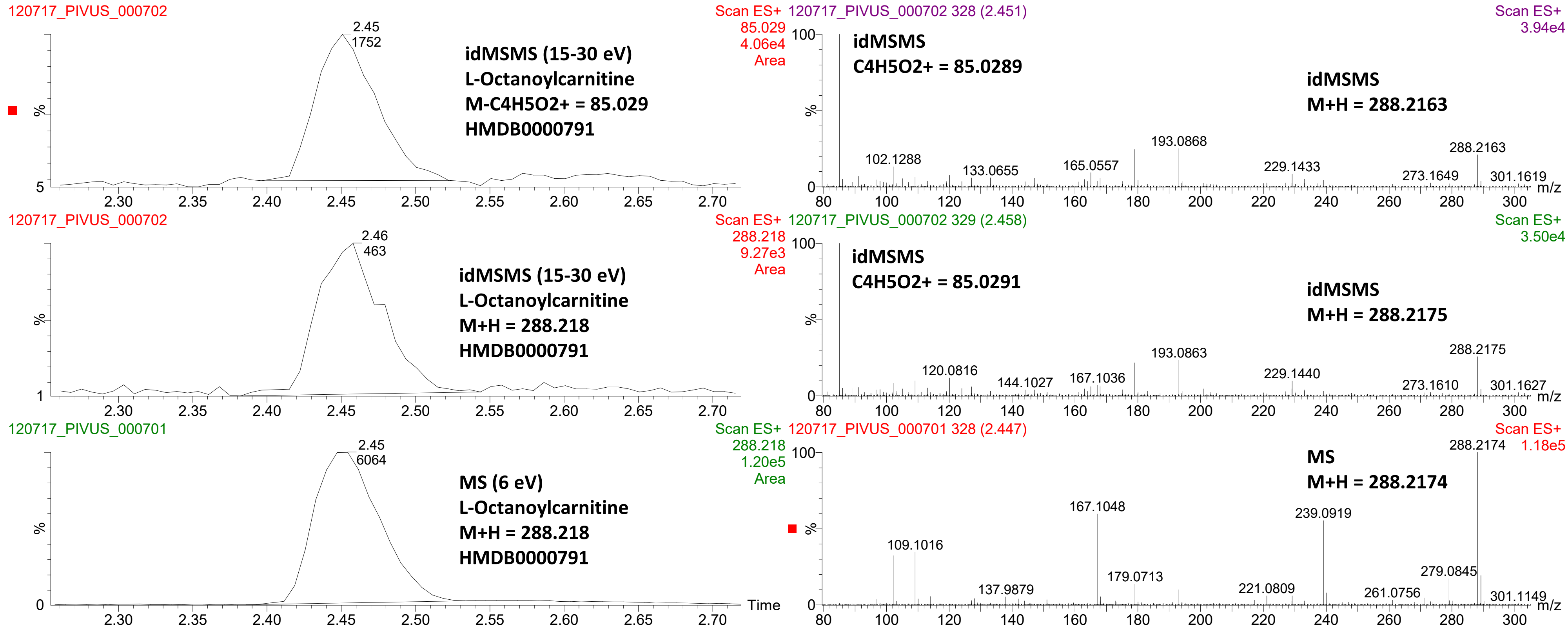


Figure S3. L-Octanoylcarnitine was annotated at MSI level 2 by database matching with accurate mass and fragmentation schemes reported for other acylcarnitines. These fragmentation schemes include the precursor of 85 fragment, or by detecting the neutral losses of either M-59 or M-161. We conclude that we selectively detect 9-decenoylcarnitine as shown in MS (collision induced dissociation at 6 eV) and idMSMS (collision induced dissociation ramp 15-30 eV) chromatograms obtained from an authenthic sample. We consistently observe M+H = 288.218 in both MS and idMSMS mode as well as the major fragment C4H5O2+ = 85.0289 eluting at 2.45 minutes using UPLC-QTOFMS operated in positive ESI. The corresponding mass spectra are shown to the right.

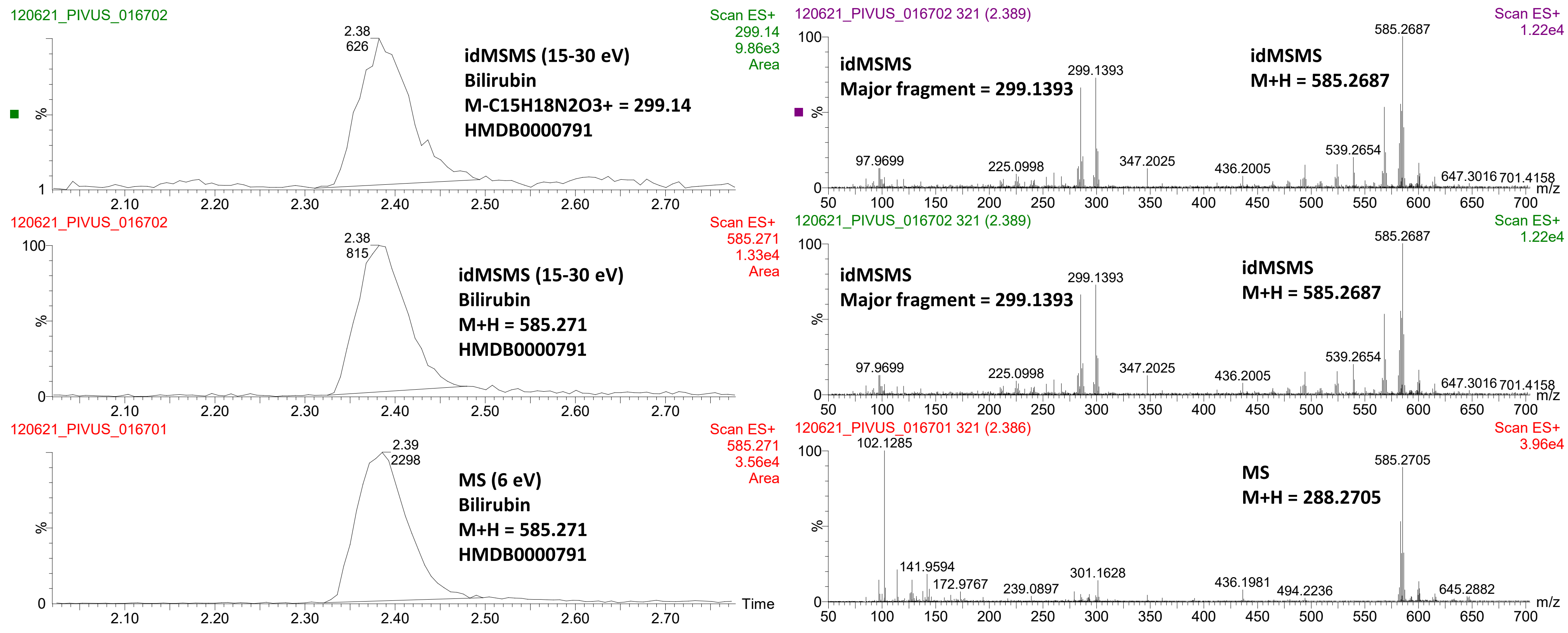


Figure S4. Bilirubin was annotated at MSI level 2 by database matching with accurate mass and major fragment observed experimentally. We consistently detect bilirubin as shown in MS (collision induced dissociation at 6 eV) and idMSMS (collision induced dissociation ramp 15-30 eV) chromatograms obtained from an authenthic sample. We consistently observe M+H = 585.271 in both MS and idMSMS mode as well as the major fragment M-C15H18N2O3+ = 299.14 eluting at 2.39 minutes using UPLC-QTOFMS operated in positive ESI. The corresponding mass spectra are shown to the right.

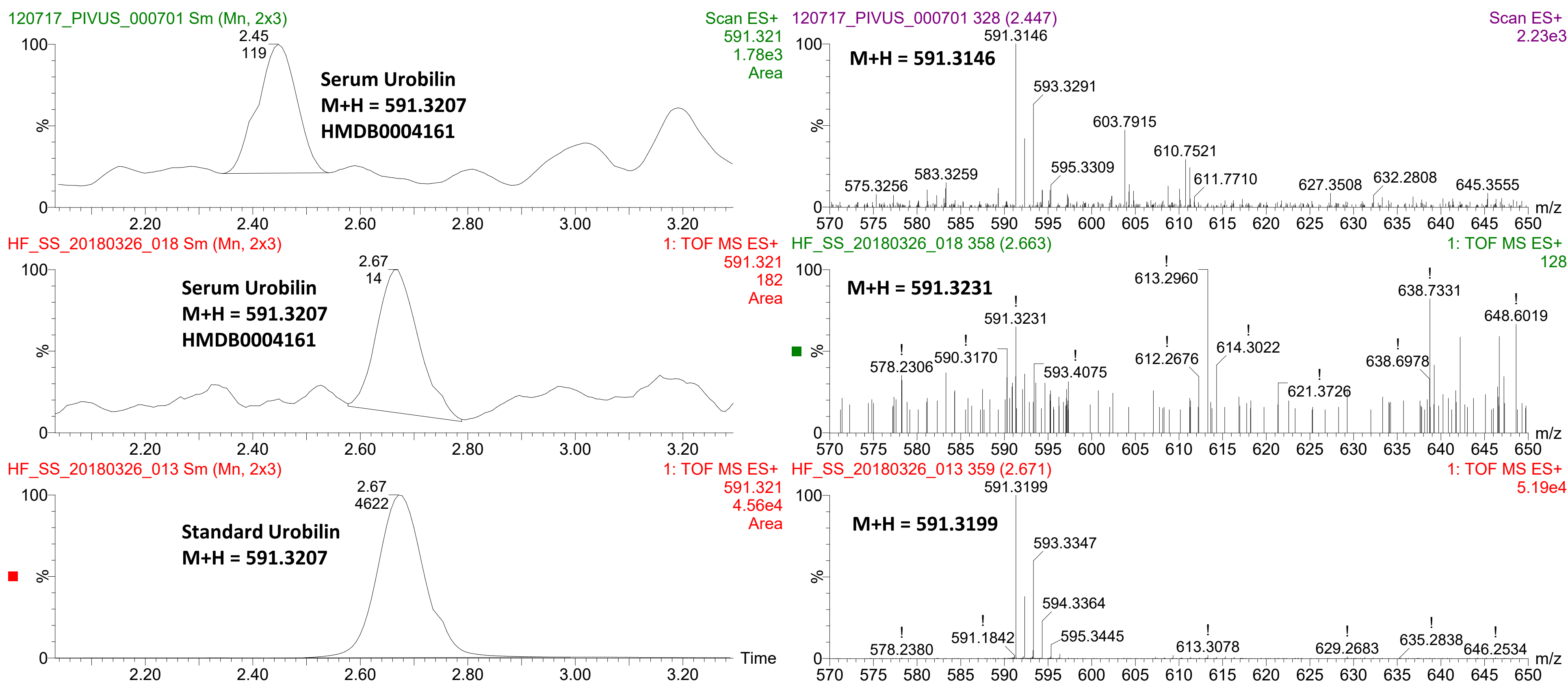


Figure S5. Urobilin was annotated at MSI level 1 by using an external standard. The data was acquired using UPLC-QTOFMS operated in positive electrospray ionization (ESI) mode. As shown in the bottom chromatogram, the urobilin standard eluted at 2.67 minutes using the method applied to all serum samples in 2012. The mid chromatogram shows M+H for urobilin in a random serum sample run alongside the standard. The top chromatogram shows a sample that was run in 2012, as can be seen there has been a shift in the rt time over this long time period. The selected M+H mass is shown to the right of each chromatogram. The mass spectra of M +H = 591.321 in both serum samples matches well with the spectra of the SM 30:1 standard. The human metabolome database (HMDB) accession number is also provided.

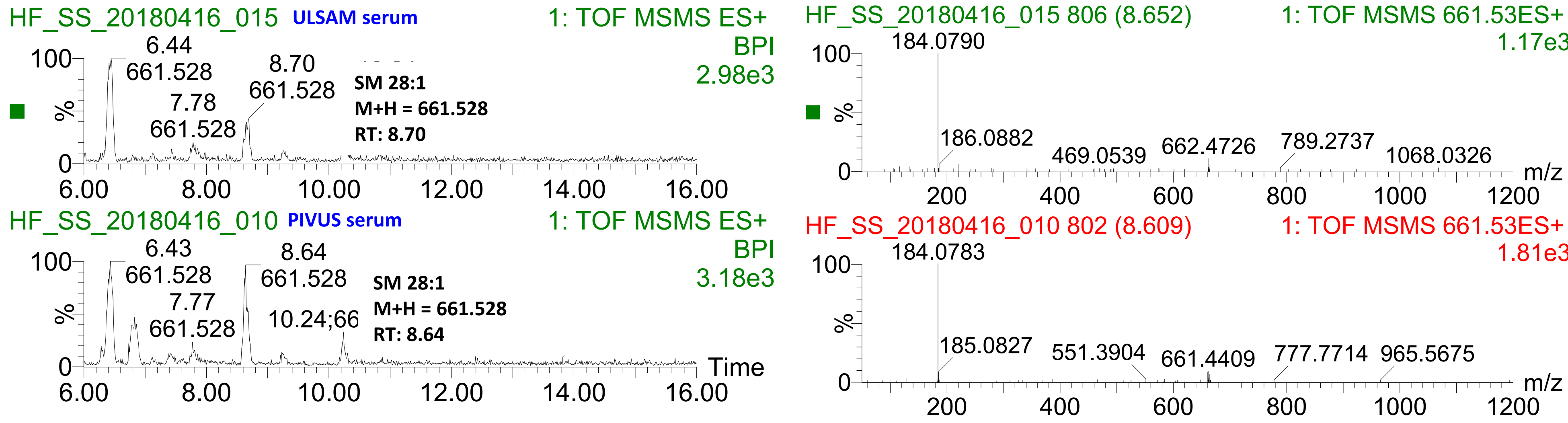


Figure S6. Parallel reaction monitoring (PRM) of sphingolipid SM 28:1 annotated at level MSI 2 in random samples from the ULSAM and PIVUS cohort. The data was acquired using UPLC-QTOFMS operated in positive electrospray ionization (ESI) mode. The selected precursor mass M+H = 661.528 is shown in the chromatogram. The corresponding mass spectra using collision induced dissociation (CID) at 30 eV are shown to the right.