ORIGINAL RESEARCH

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

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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
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, SM (32:1) to be associated with incident stroke, and urobilin and SM (30:1) to be linked to incident heart failure, 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.

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, a low left ventricular (LV) ejection fraction and thick LV walls, as well as an impaired transmitral early/late ratio, 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. 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. 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. 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 sub-cohort (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
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 R24 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/andgant/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...
LV mass was determined from the Penn conversion: LV mass was then indexed for height^{2/3} 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. SNPTTEST 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 multiajusted 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 ($P<0.00025$; 1-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/m² | 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/m²² | 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.
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 Phenoscaner (http://www.phenoscaner.medsc.hl.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 ($R^2$ 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 =0.0069, SE =0.0081, $P$ 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.

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...
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. 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. 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. 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. 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, and intentional weight loss is known to affect levels of ACs. Decenoylcarnitine and other ACs have also been linked to an impaired glucose tolerance in previous studies. Of particular interest is that 2 other medium-chained ACs (decanoylcarnitine and octanoylcarnitine) have been associated with cardioembolic stroke, a diagnosis mainly caused by AF.

In our secondary analysis, another medium-chained AC, L-octanoylcarnitine, 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, 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 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               | 0.000020 | 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.

### 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-  | 0.31             | 0.20      | 0.11    | 0.131            | 0.191     | 0.49    |
| diastolic diameter     | 0.008            | 0.003     | 0.019   | 0.006            | 0.003     | 0.079   |
| Relative wall thickness| 1.62             | 0.52      | 0.0019  | 0.79             | 0.42      | 0.064   |
| Left ventricular mass  | 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.
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 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.

ARTICLE INFORMATION
Received May 19, 2020; accepted November 16, 2020.

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Sources of Funding
The study was supported by the Swedish Heart and Lung Foundation and Uppsala University Hospital (ALF-meal).

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.

| Metabolite                                                                 | Description                                                                                   |
|---------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|
| 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  |                                               |
| Chemical Name                                |
|---------------------------------------------|
| 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                                    |
| Chemical Name                        |
|-------------------------------------|
| Creatine                            |
| Creatinine                          |
| D-Urobilinogen, l-Urobilin          |
| D-erythro-sphingosine                |
| DL-2-Aminoocctanoic acid            |
| Decanoyl-L-carnitine                |
| Deoxycylic acid                     |
| Deoxycylic acid glycine conjugate   |
| Deoxycylic 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 acid                  |
| Hippuric acid                       |
| Hyodeoxycholic acid                 |
| Compound                     |
|------------------------------|
| Indoleacetic acid            |
| Indolelactic acid            |
| L-Acetyl carnitine           |
| 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) |
|                  |                                              |
|------------------|----------------------------------------------|
| Lysophosphatidyl| Lysophosphatidylcholine(22:4)                |
| choline(22:4)    |                                              |
| Lysophosphatidyl| Lysophosphatidylcholine(22:5)a               |
| choline(22:5)a   |                                              |
| Lysophosphatidyl| Lysophosphatidylcholine(22:5)b               |
| choline(22:5)b   |                                              |
| Lysophosphatidylethanolamine(16:0) | Lysophosphatidylethanolamine(16:0) |
| Lysophosphatidylethanolamine(18:0) | Lysophosphatidylethanolamine(18:0) |
| Lysophosphatidylethanolamine(18:1) | Lysophosphatidylethanolamine(18:1) |
| Lysophosphatidylethanolamine(18:2) | Lysophosphatidylethanolamine(18:2) |
| Lysophosphatidylethanolamine(20:4) | Lysophosphatidylethanolamine(20:4) |
| N-(15Z-tetracosenoyl)-sphinganine-1-phosphocholine | N-(15Z-tetracosenoyl)-sphinganine-1-phosphocholine |
| N-(9Z-octadecenoyl)-sphing-4-enine-1-phosphocholine | N-(9Z-octadecenoyl)-sphing-4-enine-1-phosphocholine |
| N-(octadecanoyl)-sphing-4-enine-1-phosphocholine | N-(octadecanoyl)-sphing-4-enine-1-phosphocholine |
| N-palmitoyl-D-erythro-sphingosylphosphorylcholine | N-palmitoyl-D-erythro-sphingosylphosphorylcholine |
| Oleamide         |                                              |
| Oleoyl-L-carnitine hydrochloride | Oleoyl-L-carnitine hydrochloride |
| Ornithine        |                                              |
| Palmitic acid    |                                              |
| Palmitoleic acid |                                              |
| Pantothenic acid |                                              |
| Paraxanthine, Theophylline | Paraxanthine, Theophylline |
| Pentadecanoic acid |                                              |
| Phenylalanlyphenylalanine | Phenylalanlyphenylalanine |
| Phosphatidylcholine(28:1) | Phosphatidylcholine(28:1) |
| Phosphatidylcholine(28:2) | Phosphatidylcholine(28:2) |
| Phosphatidylcholine(29:1) | Phosphatidylcholine(29:1) |
| Phosphatidylcholine(30:1) | Phosphatidylcholine(30:1) |
| Phosphatidylcholine(30:2) | Phosphatidylcholine(30:2) |
| Phosphatidylcholine(32:1) | Phosphatidylcholine(32:1) |
| Phosphatidylcholine(33:1), Phosphoethanolamine(36:1) | Phosphatidylcholine(33:1), Phosphoethanolamine(36:1) |
| Phosphatidylcholine(34:0) | Phosphatidylcholine(34:0) |
| Phosphatidylcholine(34:0), Phosphoethanolamine(37:3) | Phosphatidylcholine(34:0), Phosphoethanolamine(37:3) |
| Phosphatidylcholine(34:2), Phosphoethanolamine(37:2) | Phosphatidylcholine(34:2), Phosphoethanolamine(37:2) |
| Compound                          |
|----------------------------------|
| 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                      |
| Compound                                      |
|----------------------------------------------|
| 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.

| Metabolite Pathway                               | 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    | 1      |
| Phenylalanine metabolism                        | 10                        | 3              | 0.013076    | 1.8835    | 0.35714 |
| Phenylalanine, tyrosine and tryptophan biosynthesis | 4                        | 2              | 0.015477    | 1.8103    | 0.69231 |
| 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 authentic 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 authentic 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 authentic 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.