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Original article

Determinants of blood acylcarnitine concentrations in healthy individuals of the European Prospective Investigation into Cancer and Nutrition

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SUMMARY

Background & aims: Circulating levels of acylcarnitines (ACs) have been associated with the risk of various diseases such as cancer and type 2 diabetes. Diet and lifestyle factors have been shown to influence AC concentrations but a better understanding of their biological, lifestyle and metabolic determinants is needed.

Abbreviations: AC, acylcarnitine; EPIC, European Prospective Investigation into Cancer and Nutrition; FFQ, food frequency questionnaire; LC-MS, liquid chromatography–mass spectrometry; BCAA, branched-chain amino acids.

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Methods: Circulating ACs were measured in blood by targeted (15 ACs) and untargeted metabolomics (50 ACs) in 7770 and 395 healthy participants of the European Prospective Investigation into Cancer and Nutrition (EPIC), respectively. Associations with biological and lifestyle characteristics, dietary patterns, self-reported intake of individual foods, estimated intake of carnitine and fatty acids, and fatty acids in plasma phospholipid fraction and amino acids in blood were assessed.
Results: Age, sex and fasting status were associated with the largest proportion of AC variability (partial-r up to 0.19, 0.18 and 0.16, respectively). Some AC species of medium or long-chain fatty acid moiety were associated with the corresponding fatty acids in plasma (partial-r = 0.24) or with intake of specific foods such as dairy foods containing the same fatty acid. ACs of short-chain fatty acid moiety (propionylcarnitine and valerylcarnitine) were moderately associated with concentrations of branched-chain amino acids (partial-r = 0.5). Intake of most other foods and of carnitine showed little association with AC levels.
Conclusions: Our results show that determinants of ACs in blood vary according to their fatty acid moiety, and that their concentrations are related to age, sex, diet, and fasting status. Knowledge on their potential determinants may help interpret associations of ACs with disease risk and inform on potential dietary and lifestyle factors that might be modified for disease prevention.

1. Introduction

Acylcarnitines (ACs) are esters of carnitine and fatty acids (FAs) enabling the transport of FAs across the membrane of the mitochondria [1] thereby playing an important role in energy metabolism in the cell. Based on the chain length of the fatty acid moiety, they can be divided in short-chain, medium-chain, and long-chain ACs. They can be found in blood and urine but the role of circulating ACs is not well understood. It has been speculated that circulating ACs are a consequence of a detoxification mechanism and facilitate the removal of excess FAs and the by-products of their metabolism [1]. Different ACs in plasma have been shown to be associated with risk of diabetes [2], cancer [3,4] and other diseases [5] and they have been proposed as markers of metabolic health [6]. More recently, a number of ACs have been shown to be associated with COVID-19 infection [7].

Biological factors such as sex, age and body mass index (BMI) have been associated with AC concentrations in blood [8,9]. Diet and intake of specific foods and nutrients such as meat [10], sunflower oil [11] or specific lipids [12] were also found to influence AC concentrations. Several metabolome wide association studies have reported AC concentrations to be associated with intake of foods; however, these studies lack adjustment for intake of other foods [13–15]. Carnitine, one of the two components of ACs, can be synthesized in human tissues but is also absorbed from the diet. Meat, the major dietary source of carnitine, has been linked to increased AC concentrations in blood in both intervention and observational studies [10,16,17], but it is not clear if this is due to increased carnitine intake or changes in FA metabolism. Intake of FAs, the other component of ACs, was also shown to impact the concentrations of ACs in blood in intervention studies, but data from observational studies, which are more representative of habitual diet than intervention studies, are limited [11,12,14]. Branched-chain amino acids (BCAA) which can be metabolized to short-chain FA have been reported to influence concentrations of some ACs [18]. Lastly, AC concentrations in blood have been shown to vary by the fasting status at blood collection, with several ACs, mainly with long-chain FA moiety, showing higher concentrations in fasting samples [13,17,19].

Most previous studies have not focused solely on ACs and only assessed a limited number of potential determinants such as diet or age and sex [9,17]. To better understand the role of ACs in the etiology of diseases, such as cancer and type 2 diabetes, and to interpret associations of circulating ACs with disease risk, an improved knowledge on the importance of different determinants is required. As ACs are linked with some modifiable risk factors such as BMI and diet, this knowledge could inform disease prevention.

The aim of this study was to identify and compare several different determinants of a wide range of circulating ACs in healthy adults from the European Prospective Investigation into Cancer and Nutrition (EPIC). In the first study (Study A), we applied a targeted metabolomics assay to measure a limited set of ACs in a large number of participants (n = 7770), allowing extensive subgroup analysis. In the second study (Study B), a large panel of ACs, including ACs of odd-chain and oxidized FA moieties, were measured by untargeted metabolomics (in 395 participants).

2. Methods

2.1. Study populations

The EPIC study is a prospective cohort conducted across 10 European countries that recruited over 520,000 healthy participants from 1992 to 2000 to identify risk factors for cancer and other chronic diseases [20]. Blood samples and detailed data on lifestyle and diet were collected at baseline. The study population, data and biospecimen collection have been described previously [20]. The study was approved by the IARC ethics committee and the institutional review boards of all participating institutions. All participants provided written informed consent prior to enrolment in the study. Two different subgroups of the cohort, for which blood samples and questionnaire data were collected as baseline, are included in this work. **Study A:** The study population consisted of 7770 healthy participants from the EPIC study that had previously been selected as matched controls in nested case–control studies on cancers of the prostate, endometrium, colorectum, breast, kidney, ovary, pancreas and liver [3,21–23]. Only controls for which endogenous metabolite levels using the Biocrates Absolute*IDQ* p150 or p180 kits (Biocrates Life Sciences AG, Innsbruck, Austria) were available were included in this study (Table 1) and different subsets of participants were chosen for different analyses depending on the availability of data on circulating amino acids and FAs (Supplemental Table 1, Supplemental Fig. 1). **Study B:** This study includes a subset of 395 participants from the cross-sectional EPIC calibration study [24] for which untargeted metabolomics data of serum samples was available (Table 1). Participants were from France, Germany and Italy. The participant selection is shown in Supplemental Fig. 2 and has been reported previously [25].

Table 1
Characteristics of the participants of the studies included in this work.

	Study A	Study B
n_{total}	7770	395
n_{female} (%)	3685 (47)	229 (56)
Age at blood collection (years)	56.1 ± 7.8[a]	53.6 ± 7.9
BMI (kg/m^2)	26.4 ± 3.9	25.5 ± 4.1
Fasting status, n (%)		
Non-fasting (>6 h)	3435 (44)	151 (38)
Partial (3–6 h)	1604 (21)	73 (18)
Fasting (<3 h)	2731 (35)	171 (43)
Sample type, n (%)		
Serum	1382 (18)	395 (100)
Plasma	6388 (82)	–
Country, n (%)		
France	249[b] (3.2)	66[b] (17)
Italy	1644 (21)	156 (39)
Spain	1407 (18)	–
UK	1367 (18)	–
Netherlands	688 (8.9)	–
Germany	1737 (22)	173 (44)
Sweden	50 (0.6)	–
Denmark	559 (7.2)	–
Norway	69 (0.9)	–

[a] Mean ± sd, all such values.
[b] This country included women only.

2.2. Anthropometry and dietary intakes

In EPIC, information on lifestyle, dietary intake and medical information as well as sociodemographic and anthropometric data were collected for all participants at inclusion in the study. Body weight and height were measured in all centers, except for Oxford, France and Norway where these were self-reported. Anthropometric characteristics were measured by trained observers using standardized methods. Diet was assessed at baseline using validated country/center-specific food questionnaires spanning the previous 12 months [26]. Relying on a common food classification system and standard handling of recipes, post-harmonization of all the questionnaire data was done to obtain a standardized food list for which the level of detail is more comparable between countries. Dietary FA intakes were calculated as previously described using the U.S. nutrient database food composition table [27]. Dietary intakes of FAs were log-transformed before statistical analysis. The dietary intake of total carnitine (both free and esterified in ACs) was calculated based on the intake of pork, veal, lamb, beef, poultry, other red meat, processed meat, offal, butter, milk, cheese, cream, yogurt and fish and the corresponding contents of carnitine in these foods as reported by Demarquoy et al. [28]. The median carnitine concentration was used if several values were available for a single food. Only foods with a carnitine content >1 mg/100 g were considered.

2.3. Metabolite measurements and data processing

2.3.1. Nomenclature of ACs

ACs vary depending on their FA moiety and are described as Cx:y, Cx:y-OH or Cx:y-DC, where x is the number of carbon atoms and y the number of double bonds in the FA moiety. The suffixes −DC and −OH indicate a dicarboxylic acid or a FA moiety with a hydroxyl group, respectively. Different isomers of ACs which are distinguished by the untargeted metabolomics method are indicated with an underscore and a number (e.g. _1). The exact structure of isomers could not be determined due to the lack of commercial standards. Semi-quantitative concentrations were derived from peak areas and were expressed in arbitrary units.

2.3.2. Targeted metabolomics

Serum or plasma samples in study A were analyzed for concentrations of 33 ACs (along with other metabolites, including amino acids) using the Biocrates Absolute*IDQ* p150 or p180 kits and tandem mass spectrometry following the procedure recommended by the vendor [3,21–23]. Participants with missing data on frequently detected ACs such as C3:0 and C18:0 were excluded (n = 28). Only ACs that were present in >80% of subjects were included in the statistical analysis. Details on the data-processing pipeline can be found elsewhere [29]. Acylcarnitines had a maximum of 35 missing values which were imputed with the median of the corresponding batch. As the metabolomics data originates from controls of several cancer-studies (some of which only included one sex), concentrations were transformed to the residuals of linear mixed-effect models of log-transformed concentrations on study and batch as random effects and sex as a fixed effect. The samples from each study were either all serum or all plasma so normalization by study was also performed to remove differences due to sample type.

2.3.3. Untargeted metabolomics

Serum samples in Study B were analyzed by liquid chromatography–mass spectrometry (LC-MS) at the International Agency for Research on Cancer, Lyon, using a method previously described that is developed to cover a broad range of metabolites and uses acetonitrile for protein precipitation and metabolite extraction [10]**.** Briefly, a 20 μL aliquot of serum was mixed with 200 μL of cold acetonitrile and the supernatant was then analyzed by LC-MS (1290 Binary LC system, 6550 quadrupole time-of-flight mass spectrometer equipped with a jet stream electrospray ionization source; Agilent Technologies). The analysis was conducted in positive ionization mode and ions across a mass range of 50–1000 Da were detected. For AC identification, data dependent and targeted MS/MS experiments were performed. ACs were identified by the Agilent Lipid Annotator 1.0 software [30], which searches MS/MS data for features matching those of a library by exact mass, isotope pattern and fragmentation. A few ACs that were identified in earlier work in our laboratory [10] but not identified by Lipid Annotator were annotated based on their exact mass, retention time compared to those in an in-house data base and characteristic fragments and neutral losses in MS/MS experiments. Signal intensity was extracted from the mass spectrometry raw data using the Profinder software (Agilent, version B08.00) as peak area (mass tolerance ± 8 ppm) using the find-by-formula method as described elsewhere [10]. Only ACs with missing values in less than 30% of samples measured were carried forward, missing values were replaced with 1 (which is less than 1% of the median peak height)and intensity data was log-transformed (ln) for statistical analysis.

2.3.4. Fatty acid analysis

Thirty-eight FAs were profiled in the phospholipid fraction of plasma samples in a subset of 854 participants in Study A, using an Agilent 7890 gas chromatograph instrument (Agilent, Santa Clara, CA, USA) as reported previously [31,32]. All FA analyses were performed at the International Agency for Research on Cancer. Concentrations were expressed as percentage of total FAs. Zero values were imputed with half of the lowest non-zero value and the data

was log-transformed before statistical analysis. Only FAs that had more than 70% non-zero values were retained for statistical analysis.

2.4. Statistical analysis

Baseline characteristics of participants were summarized by mean and standard deviation or frequency and proportion (Table 1).

2.4.1. PC-PR2 analysis and AC concentrations by sex and age

The association of different covariates with total variation of AC concentrations was estimated using the principal component partial R-squared (PC-PR2) method. PC-PR2 is a combination of principal component analysis and multivariable regression and estimates the proportion of variability in the matrix of metabolite measurements (expressed as $R_{partial}^2$) that is attributed to each of a selection of explanatory variables, participant characteristics and diet score in the present study [33]. This analysis was carried out in both studies separately. The explanatory variables included were BMI, sex, age, smoking status (current/former/never), fasting status at blood collection (yes/no/partial), physical activity (inactive/moderately inactive/moderately active/active) and the three first principal components of the food intake profiles. The principal component analysis (PCA) was performed on log-transformed and unit variance scaled intake of 40 food subgroups which were consumed by at least half of the volunteers (median intake > 1 g/day; Supplemental Table 2) The method was carried out as described by Fages et al. [33].

We calculated the mean and its 95% confidence interval for AC concentrations by age groups and sex. Participants with an age lower than 30 were excluded because they were all female (n = 5).

2.4.2. Associations of ACs with food intake

To assess the association between the intake of food groups and blood AC concentrations, least absolute shrinkage and selection operator (LASSO) analyses were conducted with AC levels as dependent variables, and participant characteristics and intake of all detailed food groups consumed by at least half of the participants as independent variables (Supplemental Table 2). The tuning parameter of the LASSO was selected by 10-fold cross-validation to optimize lambda and chose the largest value of lambda so that the error was within 1 standard error of the minimum of the cross-validated mean squared error. A 1000-fold bootstrap validation was used to identify covariates that were robustly associated with AC levels (>80% of the models). The participant characteristics were sex, age, BMI, smoking status (yes/no/former smoker/unknown), and fasting status at blood collection (yes/no/in between). Food intake data below 1 g/day was imputed with 1 and a Shapiro–Wilk test was performed beforehand for each food group to assess the normality of the non-transformed and log transformed data. If log-transformation of the data led to a higher p-value compared to the non-transformed data, then the intake data was log-transformed for statistical analysis. Non-binary categorical variables were coded as binary dummy variables with non-smoking, partial fasting and France being the reference groups for the variables cigarette smoking, fasting status at blood collection and country, respectively. The analysis was repeated separately for study A and study B. The analysis of study B was additionally carried out for German and Italian participants only. The analysis was not carried out for French participants due to small sample size.

2.4.3. Partial correlations of ACs and circulating amino acids, circulating fatty acids and dietary intake of fatty acids and carnitine

Partial correlations of circulating ACs in study A with FAs in the blood phospholipid fraction and amino acids in plasma, and dietary intake of FAs and carnitine were calculated after adjustment for the covariates sex, age, country, BMI and fasting status (for metabolites) using the residuals method. Partial correlations with associated FDR inferior to 0.05 were considered significant for each analysis. The subsets of study A used for these analyses are shown in Supplemental Fig. 1.

2.4.4. Determinants of selected ACs

We calculated r-squares of linear models including the variables that showed strong associations with AC levels for each group of determinants (i.e. amino acids, fatty acids, dietary intake, and participant characteristics) in the precedent analyses of this work to assess the influence on different ACs in study A. The variable BCAA is the sum of blood concentrations of leucine, isoleucine and valine.

All statistical analyses were carried out using the open source statistical software R, version 3.6.1 [34] and associations of ACs with circulating amino acids and dietary FAs were displayed using the complex heatmap package in R [35].

3. Results

3.1. Acylcarnitines measured in studies A and B

In Study A, we measured fifteen ACs with a targeted metabolomics assay in plasma and serum samples from 7770 healthy individuals from the EPIC cohort (Table 1). These fifteen ACs contain mainly even chain saturated or monounsaturated FA moieties (Supplemental Table 3). A correlation heatmap of their blood concentrations shows that they form 3 clusters of intercorrelated compounds, one containing carnitine and short-chain ACs except C2, one containing medium-chain ACs and one containing long-chain ACs (Supplemental Fig. 3).

In study B, serum samples from 395 participants of the EPIC cohort and originating from France, Germany and Italy (Table 1) were analysed by an untargeted metabolomics approach and 50 ACs were identified (Supplemental Table 4) which include the 15 AC measured with the targeted approach. These compounds represent a wide range of short-, medium- and long-chain ACs with different degrees of unsaturation and oxidation. A correlation heatmap shows that many ACs, especially even medium-chain AC, are highly correlated, whereas short-chain ACs (C3, C4 and C5) and some ACs with odd-chain FA moieties such as C9:0, C11:1 and C13:0 cluster apart (Supplemental Fig. 4).

3.2. Variability of acylcarnitine concentrations

We identified the factors that explain away most of the overall variability of AC profiles in both studies (Fig. 1). Fasting status at blood collection, sex, age and diet accounted for most of the variability. As the dietary patterns are highly associated with the country, the analysis was also performed including the variable country of the participants (Supplemental Fig. 5) which leads to a decrease in the variability associated with diet, especially in study B which included participants from three countries with distinct diets. In study A, variability of ACs was associated with country and in both studies inclusion of country led to a lower proportion of variance associated with the diet. Physical activity and cigarette smoking were associated with little of the AC variability.

We compared acylcarnitine levels in both sexes by age groups in study A. Generally, levels of acylcarnitines were higher in males than in females and increased with age (Supplemental Fig. 6). However, while 12 out of 15 AC species showed significantly higher levels in males compared to females in the age group of 40–50 years, differences of mean concentrations by sex decreased with

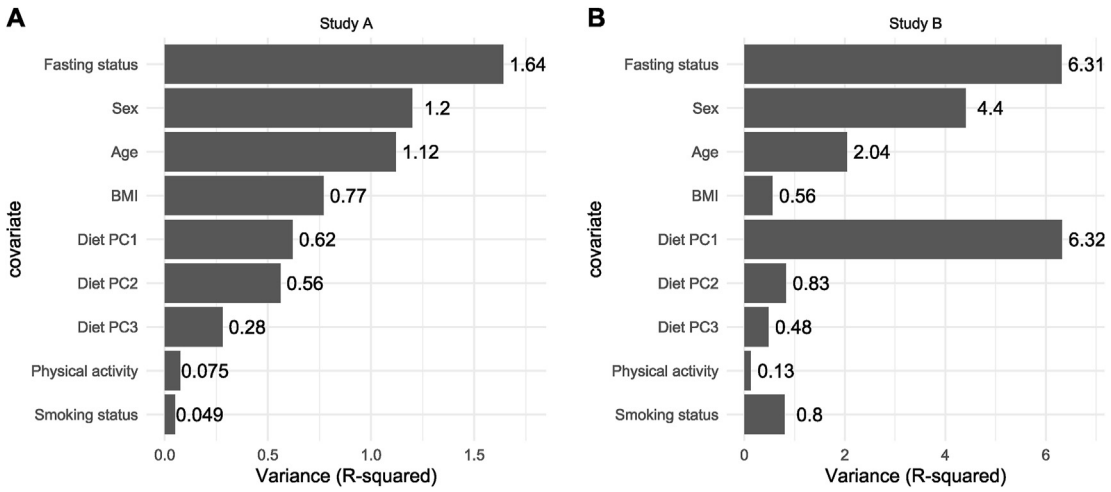

Fig. 1. Principal component partial R-squared (PC-PR2) analysis showing the variability due to different factors of blood levels of 15 and 50 ACs in study A (n = 7770, Fig. 1A) and in study B (n = 395, Fig. 1B), respectively. PCPR2 combines features of principal component analysis and multivariable linear regression to assess the amount of variability in omics data that is associated with different covariates.

age and only 6 AC species showed higher levels in males compared to females in the age group of 60–70 years.

3.3. Associations of acylcarnitines with dietary intake

3.3.1. Associations with intake of specific foods

In a bootstrapped Lasso applied in study B, butter intake was strongly associated with concentrations of C9:0 and C11:0, whereas vegetable oil intake was inversely associated with concentrations of C13:0 and C14:0 (Fig. 2). Concentrations of short-chain ACs were not associated with intakes of single foods. Many ACs showed higher concentrations in male participants and in fasting samples compared to intermediate fasting status at blood collection, whereas concentrations of many ACs were significantly lower in non-fasting samples compared to intermediate fasting status. When performing the same analysis within German or Italian participants only, butter was the only food that was associated with AC levels in both analyses (Supplemental Figs. 7 and 8). Fasting and sex were associated with AC levels in both analyses as well.

The same analysis with the targeted data from study A showed similar results (Supplemental Fig. 9). Covariates with the strongest associations were country of study, fasting state, age and BMI.

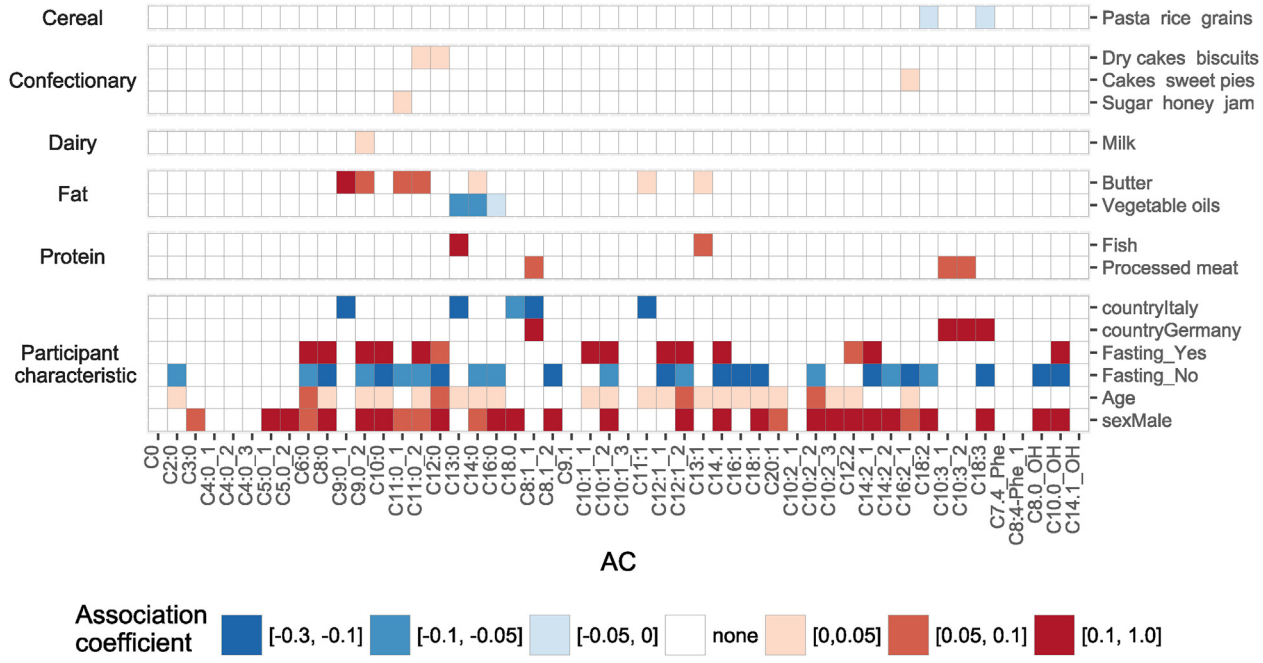

Fig. 2. Associations of participant characteristics and intakes of detailed food groups with blood levels of acylcarnitines in study B (n = 395). Least absolute shrinkage and selection operator (LASSO) analyses were used which contained AC levels as dependent and intake of all detailed food groups and participant characteristics as independent variables. Only associations are colored which had non-zero coefficients in more than 80% of a 1000-fold bootstrap iteration. No AC was associated with the covariates cigarette smoking, BMI and intake of vegetables, fruit, poultry, red meat, beverages, and other foods. For a full list of variables included in the model, see Supplemental Table 2.

While many ACs were elevated in fasting samples compared to non-fasting samples, C3 and C5 showed a decrease in fasting samples. Short chain ACs and C16 were associated with BMI. The strong associations with butter could not be reproduced because the corresponding odd-chain ACs were not included in the targeted assay used in study A.

3.4. Associations of acylcarnitines with acylcarnitine precursors

3.4.1. Fatty acids

The associations of ACs with 35 circulating FAs were assessed in 854 participants of study A (Supplemental Table 1). Out of the five ACs for which the FA with corresponding chain length and number of double bonds was measured, four showed a significant correlation with the corresponding FA (r = 0.14–0.26; Fig. 3). Additional associations were found between FAs and ACs that do not have the same FA chain length and number of double bonds, including associations of ACs C10:1 and C14:1 with FA 18:2 and FA 18:1, respectively (Fig. 3).

We also studied associations of ACs with estimated FA intake assessed by food frequency questionnaires in Study A (n = 7770, Supplemental Fig. 10). AC C18:0 was associated with the dietary intake of several saturated FAs (e.g. FA 18:0, FA 17:0, FA 10:0 and FA 14:0; R < 0.1) and the ACs C10:1, C14:2 and C18:2 were associated with the intake of linoleic acid (FA 18:2).

3.4.2. Amino acids

Moderate partial correlations (R = 0.30–0.47, p-values < 10^{-180}) between the circulating ACs C3 and C5 and the circulating BCAA valine, isoleucine and leucine, and the aromatic amino acids phenylalanine and tyrosine, lysine and methionine were observed in Study A (Fig. 4). C18:1 and C18:2 were positively associated with ornithine and glutamine (R = 0.4, *p*-values < 10^{-170}) and inversely with arginine (R = −0.42, *p*-values < 10^{-280}). These associations were almost identical after stratification by fasting status (data not shown).

3.4.3. Carnitine

Associations between estimated total dietary intake of carnitine (combined free carnitine and ACs) and blood AC concentrations in study A (Table 2) were in the same direction as those between the circulating BCAA and ACs (Fig. 4), but weaker (R < 0.06).

3.5. Main determinants of acylcarnitine concentrations by acylcarnitine species

We finally compared the influence of different variables on single ACs in study A by computing the partial R-squared of the different variables in linear models (Fig. 5). Concentrations of some ACs (e.g. C2 and C14:2) were highly associated with age or fasting status, whereas those of most short-chain ACs were associated with

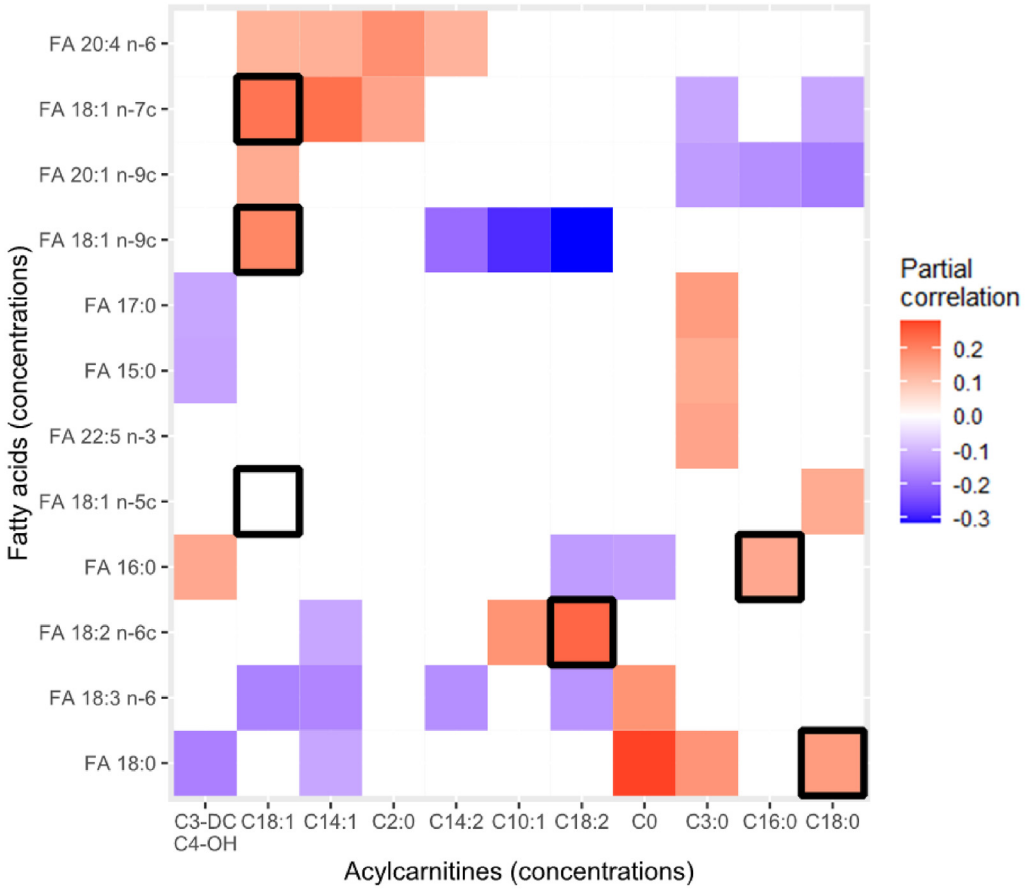

Fig. 3. Partial correlations of circulating phospholipid fatty acids (FA) with acylcarnitines (ACs) in healthy individuals of study A (n = 854). Pairs of ACs and FAs with corresponding chain length and number of double bonds are indicated with black boxes. Correlation of AC C16:1 with FA 16:1 (r = 0.08, nominal *p*-value = 0.02) did not remain significant after adjustment for multiple testing. Correlations are adjusted for study, sex, age, BMI, country and fasting status at blood collection. *p*-values are corrected for multiple testing using the FDR-method and only those correlations that have an adjusted *p*-value < 0.005 are colored. Displayed here are only those metabolites that show at least one significant positive association, and the metabolites on both axes are ordered by hierarchical clustering.

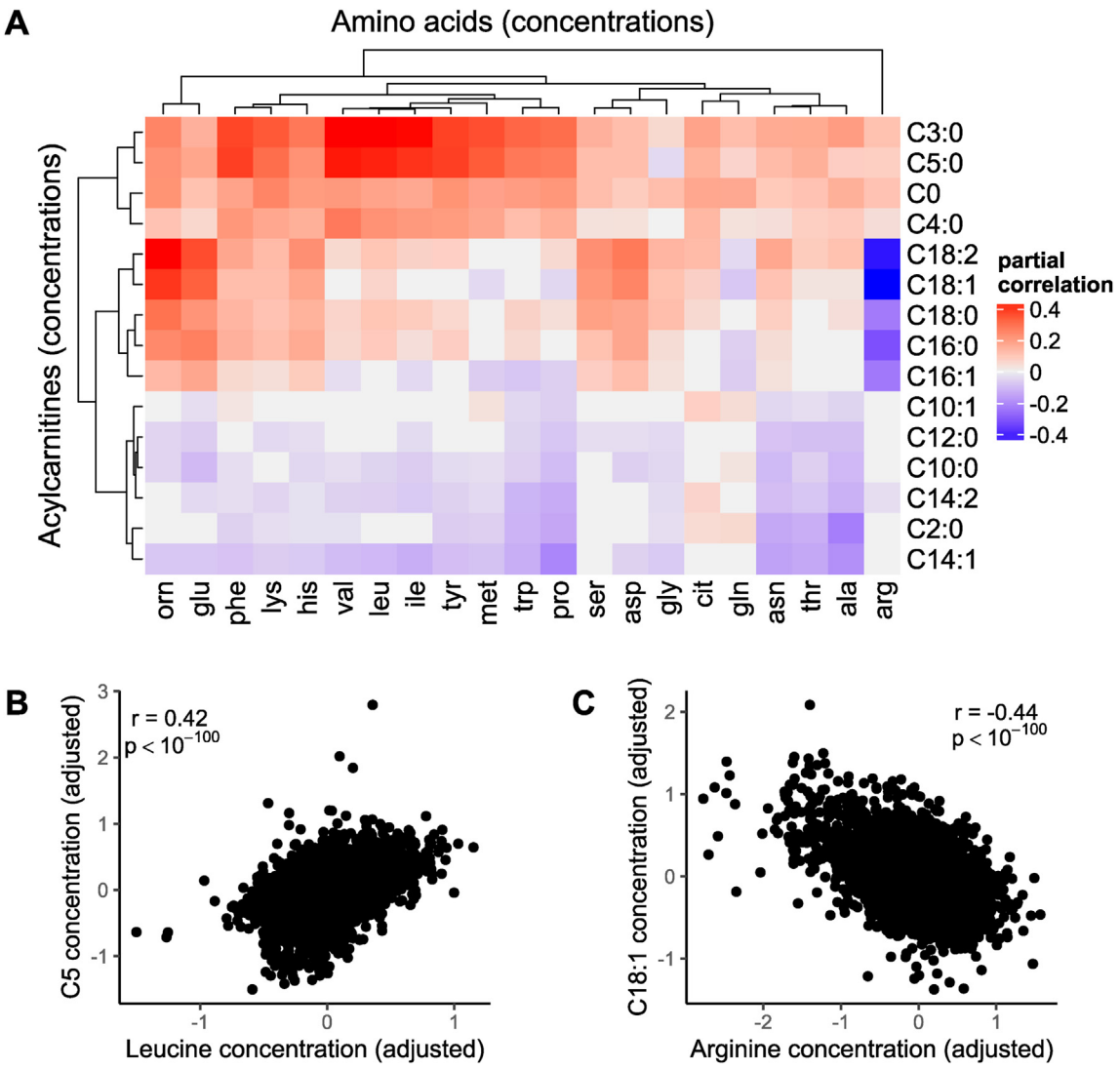

Fig. 4. Partial correlations of circulating ACs and amino acids in Study A (n = 6639). A: Heatmap showing partial correlations of all ACs and amino acids included in this analysis. Partial correlations were adjusted for the covariates country, sex, age, BMI, and fasting status. *p*-values were adjusted for multiple testing using the FDR-method and only correlations with an adjusted *p*-value < 0.05 are colored in the figure. B: Scatter plot of partial correlations of adjusted concentrations of C5:0 and leucine. B: Scatter plot of partial correlations of adjusted concentrations of C18:1 and arginine.

circulating BCAA. C18:0 and C18:2 were highly associated with their corresponding circulating FAs.

4. Discussion

4.1. Main findings

In this study we investigated the determinants of a wide range of ACs in blood. To this end we employed two complementary datasets acquired in the EPIC cohort: a targeted metabolomics dataset obtained from a large number of participants (n = 7770) for different subgroup analyses and a smaller untargeted metabolomics dataset obtained from 395 subjects, which included a wider range of ACs including oxidized and odd-chain ACs. Fasting status, age, sex and blood concentrations and intake of specific FAs and concentrations of BCAA were important determinants of AC concentrations, but the strength of associations varied greatly between AC species and some associations were specific for particular groups of ACs (scheme in Fig. 6). In addition, we show for the first time an association between blood ACs and circulating fatty acids in a large observational study.

Our findings are largely consistent with previous studies that found important determinants of circulating AC concentrations to be age, sex and fasting status [9,36–38]. In agreement with the literature, we found that AC concentrations increased with age. It has been suggested that AC concentrations increase with age due to decreased mitochondrial function which leads to impaired β-oxidation [9,37]. It has been shown that the sex-specific differences of circulating free [39] or total carnitine [36] are age dependent and differences between sexes decrease with higher age, which has been explained by changing levels of sex-hormones that influence transport and metabolism of ACs [36,39]. We show here for the first time that this pattern applies to several individual acylcarnitine species, although some differences between AC species can be observed. These findings highlight the complexity of different determinants of AC species and the importance of an adequate age and sex matched reference groups in studies on acylcarnitines and health outcomes. Except for a few specific foods, diet showed

Table 2
Partial correlations between estimated dietary intakes of carnitine and circulating levels of acylcarnitines (ACs) in study A, adjusted for potential confounders.

Acylcarnitine species	Partial correlation of blood AC concentration with carnitine intake[a]	
	R	*p*-value[b]
C18:2	−0.140	3.47×10^{-34}
C18:1	−0.082	4.19×10^{-12}
C14:2	−0.047	7.11×10^{-05}
C10:1	−0.039	0.001
C16:1	−0.016	0.203
C16:0	−0.005	0.760
C14:1	−0.004	0.760
C12:0	−0.004	0.760
C10:0	0.003	0.760
C18:0	0.026	0.033
C2:0	0.028	0.024
C0	0.052	1.10×10^{-05}
C3:0	0.053	1.04×10^{-05}
C4:0	0.056	2.58×10^{-06}
C5:0	0.057	2.23×10^{-06}

[a] Adjusted for country, study, sex, age, BMI, fasting status (blood AC levels only).
[b] Adjusted for multiple testing using the FDR method, FDR = 0.05.

weaker associations with ACs than sex, age, and fasting status. BMI has been reported as a determinant in smaller studies that included normal weight and obese individuals [8,18]. However, our findings suggest that the association between BMI and AC concentrations is specific for some AC species and less pronounced than the associations of other potential determinants such as sex and fasting status in our study population which is characterized by a more moderate BMI when compared with previous studies.

4.2. Findings by subgroups of acylcarnitines

We show that ACs form several groups of highly correlated species that are associated with specific anthropometric, dietary and metabolic variables as summarized in Fig. 6. In the following, the potential determinants of short, medium and long-chain ACs will be discussed.

Circulating FAs are major determinants of some blood long-chain ACs. The same long-chain ACs were also associated with intake of the corresponding FAs (Table 2) further supporting the link between these FAs and blood ACs.

Strong associations were found between the intake of butter or dairy foods and C9:0 and C11:0 (Fig. 2) and associations of butter intake and ACs were also seen within German or Italian participants only which increases confidence that these findings are not due to confounding. These ACs contain odd-chain FAs derived from fat in ruminant animal products [40] and C9:0 has been reported previously to be associated with butter intake in a metabolome-wide association study [15]. The findings combined suggest that the blood levels of long-chain ACs are strongly associated with the corresponding circulating FAs and some specific ACs containing diet-derived odd-chain FAs are associated with the corresponding dietary FA intake. Many diet-AC associations detected in study B were not found in analyses in the subsets of only the German or Italian participants. This suggests that some of the findings in the

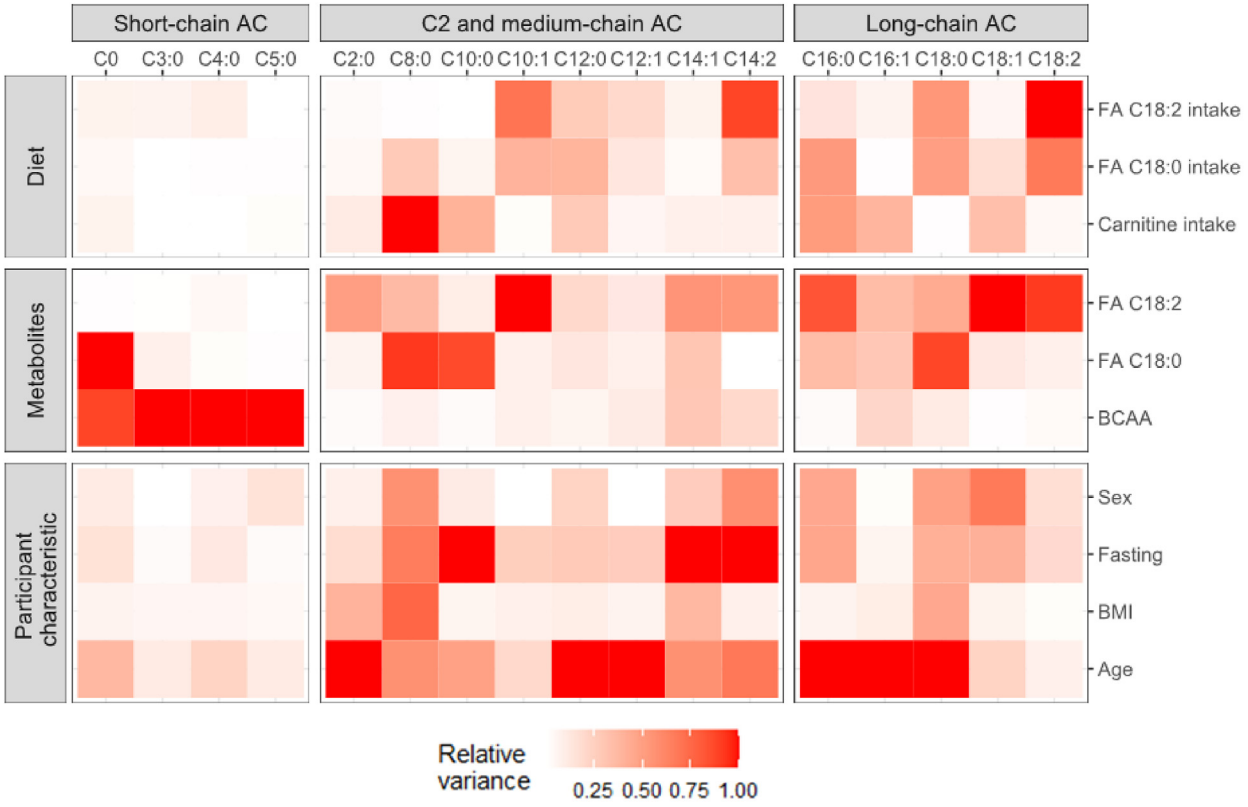

Fig. 5. Partial r-squares of regression models exploring the effect of the covariates that were found to be associated with AC concentrations on the variability of ACs in study A (n = 854). Each linear model included covariates on diet derived from the food frequency questionnaires (carnitine and C18:0 and C18:2 fatty acids), molecular data on circulating fatty acids and the sum of branched-chain amino acids (BCAA), and participant characteristics as independent variables and AC concentrations as dependent variable. The variability associated with the different covariates ($R_{partial}^2$) is expressed as a proportion of largest $R_{partial}^2$ for each AC.

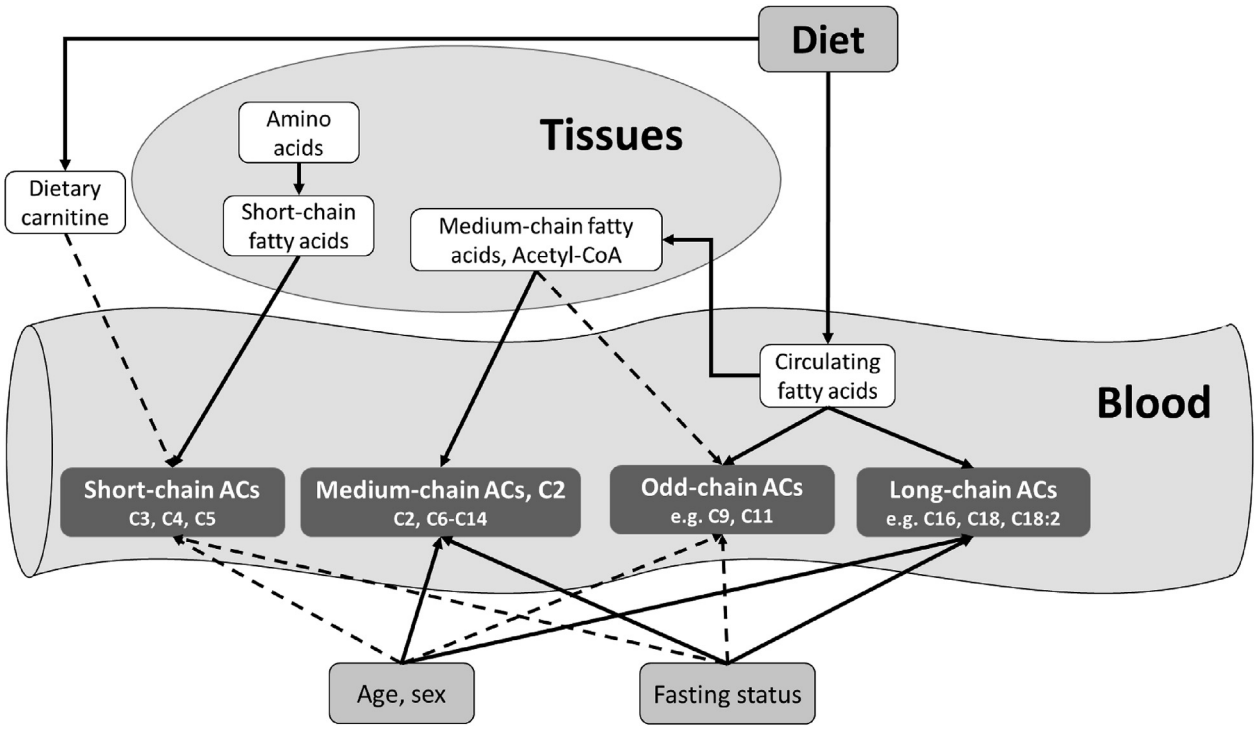

Fig. 6. Schematic representation of determinants of different groups of acylcarnitines (ACs) based on results derived from this study. Solid and dashed lines represent major and minor influences, respectively. Sex, age and fasting status at blood collection influence concentrations of most ACs. Dietary carnitine influences concentrations of short-chain ACs. Dietary fatty acids are absorbed through the gut barrier and are incorporated as such into ACs or enter fatty acid metabolism in the tissues. Accumulating fatty acid oxidation by-products such as medium-chain fatty acids can be exported from the tissues into blood as medium-chain ACs. Branched chain and aromatic amino acids are metabolized in tissues to form short-chain fatty acids, which can be incorporated into short-chain ACs.

whole population might be confounded by country. On the other hand, the sub populations might not have sufficient power to detect all associations and additional studies with larger populations from these countries would be needed to test this hypothesis.

Long-chain FAs were also associated with even-medium-chain ACs containing their downstream metabolites (e.g. the FA 18:2 and FA 18:1 with the ACs C10:1 and C14:1, respectively). This is in line with the finding that intake of linoleic acid (FA 18:2) was associated with blood concentrations of ACs C10:1 and C14:2. Medium-chain ACs are thus associated with the intake or circulating concentrations of their metabolic precursors and with fasting status and age.

Amino acids in blood were strongly associated with short-chain ACs except C2. C3 and C5 were highly associated with lysine, valine, leucine and isoleucine in blood ($R = 0.4$; p-value $< 10^{-100}$) and similar but weaker associations were found for C4:0. This is expected given that short-chain FAs are known amino acid metabolites [18,41]. The same associations between C3 and C5 and amino acids were reported in another study on 1765 individuals [42]. C18:1 and C18:2 were positively associated with levels of ornithine and inversely with arginine. We do not have a biological explanation for the latter associations and they might result from consumption of common food sources. We found short-chain ACs positively associated with dietary carnitine but these associations were much weaker than those between amino acids and ACs. The main food sources of carnitine are meat and other animal products. Intake of these foods have been shown to be associated with BCAA concentrations in blood [43] and it is possible that the association between intake of carnitine and circulating short-chain ACs is explained by high concentrations of BCAAs in meat [44]. Earlier work from our group showed that meat intake results in a major increase in urinary excretion of several short-chain and medium-chain ACs but little change in circulating ACs [10]. The difference between blood and urine was explained by homeostatic control of circulating AC concentrations. Concentrations of ACs associated with meat intake in blood appear to be influenced by FA and BCAA concentrations rather than by carnitine intake.

Most ACs were significantly increased in fasting samples, while the opposite was true for short-chain ACs (C3, C4, C5). Our data support earlier studies showing decreased levels of C3 as well as increased levels of long-chain ACs in fasting blood samples [13,45]. Increased concentrations of medium and long-chain ACs might be related to increased lipid metabolism during fasting. This data shows that the amino-acid derived ACs clearly form a group apart from other ACs with unique determinants.

Our findings have important implications for the interpretation of associations between ACs and disease risk. For example, the inverse associations observed between C18:1 and C18:2, and prostate cancer risk [4] could be partly explained by a high intake of the corresponding unsaturated FAs. Acetylcarnitine was associated with risk of breast cancer in a recent study [3] and findings of this present study suggest that increased acetylcarnitine levels might be an indicator of disrupted fatty acid metabolism rather than being linked to dietary factors. Similar to the two previous examples, findings of this present study may help to interpret other associations of AC concentrations with disease risk and understand the differences of particular AC species by providing knowledge about potential determinants. It is not clear whether ACs are a causal factor in the pathway linking the exposure (e.g. diet, BMI) and disease risk or whether ACs merely inform about exposures and the state of metabolism. In either case, knowledge of the determinants of AC concentrations is essential to interpret associations with disease risk and identify modifiable risk factors.

4.3. Strengths and limitations

This study has several strengths, firstly the large sample size and the wide variety of ACs measured. Associations between AC levels and potential determinants have previously been reported, but to the best of our knowledge, the present work is the largest and most complete study of various potential determinants of AC concentrations in healthy individuals so far. The EPIC study is well suited for this analysis as the dietary intake data has been thoroughly validated and harmonized between countries and the study setting provides a wide range of food intakes. The availability of data on both ACs and circulating FAs allowed the identification of correlations between these 2 classes of metabolites which to the best of our knowledge has not been reported in other observational studies yet. When permitted by samples size and availability of measurements, we were able to show similar results in both study A and B, e.g. the importance of age and sex and determinants of ACs or the direction of associations of different AC species with fasting status.

The study has also some limitations. Some ACs are highly associated with the country of study and it is not clear whether this is due to the differences in diet or to some other country specific factors that we were not able to adjust for. Another limitation is that we included data obtained from serum and plasma samples. Some differences in AC concentrations have been reported in the two specimen types [46], but we have adjusted the data to account differences between studies performed with serum or plasma. Not all AC species were covered by the targeted metabolomics method applied to study A which prevented the replication of some of the associations identified in study B. The untargeted method including sample extraction liquid chromatography was developed to cover a broad range of metabolites and is therefore not optimized for the measurement of single acylcarnitine species. The literature on carnitine content of foods is limited which might lead to imprecise estimates of carnitine intake in this study and thereby attenuating associations of ACs with dietary carnitine.

5. Conclusions

In conclusion, we have shown that the levels of most ACs are mainly associated with fasting status at blood collection, sex, age and diet. Specific determinants such as dietary factors or circulating FAs and amino acids for the different subgroups of ACs could be identified. Our findings on these determinants have important implications as they may inform on potential modifiable risk factors for diseases.

Disclaimer

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Availability of data and materials

For information on how to submit an application for gaining access to EPIC data and/or biospecimens, please follow the instructions at http://epic.iarc.fr/access/index.php.

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Authors' contributions

The authors' responsibilities were as follows - RW, IH, AS designed research; RW and NR conducted untargeted metabolomics analyses; RW analysed data and performed statistical analysis; Data interpretation: RW, JAR, PK-R, VV, JS, MBS, MJG, IH, AS; RW drafted the manuscript; AS had primary responsibility for final content; VC, VK, TJ, MSDM, VK, PA, CS, DR-S, JMH, AT, PP, PJ, RT, EA, TMS, AW, JH, MBS, EW, MJG: recruitment, dietary data collection, biological sample collection, and follow-up or management of the EPIC cohort; and all authors: critical revision and approval of the final version of the manuscript.

Conflict of interest

All authors have declared no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2022.05.020.

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