A metabolomic study of red and processed meat intake and acylcarnitine levels in human urine and blood

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Data described in the manuscript, code book, and analytic code will be made available upon request pending.

Running head: Meat intake and acylcarnitines

Abbreviations: AC, Acylcarnitine; CoA, Co-enzyme A; EPIC, European Prospective Investigation into Cancer and Nutrition; FDR, false discovery rate; FFQ, Food frequency questionnaire; IARC, International Agency for Research on Cancer; LC-MS, liquid chromatography-mass spectrometry; RT, retention time

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Abstract:

Introduction: Acylcarnitines (ACs) play a major role in fatty acid metabolism and are potential markers of metabolic dysfunction with higher blood levels reported in obese and diabetic individuals. Diet, and in particular red and processed meat intake, has been shown to influence AC levels but data on the effect of meat consumption on AC levels is limited.

Objectives: To investigate the effect of red and processed meat intake on AC levels in plasma and urine using a randomized controlled trial with replication in an observational cohort.

Design: In the randomized cross-over trial, 12 volunteers consumed successively two different diets containing either pork or tofu for 3 days each. A panel of 44 ACs including several oxidized ACs was analyzed by liquid chromatography−mass spectrometry in plasma and urine samples collected after the 3-day period. ACs that were associated with pork intake were then measured in urine (n = 474) and serum samples (n = 451) from the European Prospective Investigation into Cancer and nutrition (EPIC) study and tested for associations with habitual red and processed meat intake derived from dietary questionnaires.

Results: In urine samples from the intervention study, pork intake was positively associated with levels of 18 short and medium-chain ACs. Eleven of these were also positively associated with habitual red and processed meat intake in the EPIC cross-sectional study. In blood, C18:0 was positively associated with red meat intake in both the intervention study (q = 0.004, Student's t-test) and the cross-sectional study (q = 0.033, linear regression).

Conclusions: AC levels in urine and blood were associated with red meat intake in both a highly controlled intervention study and in subjects of a cross-sectional study. Our data on the role of meat intake on this important pathway of fatty acid and energy metabolism may help understanding the role of red meat consumption in the aetiology of some chronic diseases.

Keywords: Meat intake, Red and processed meat, acylcarnitines, urine, blood, metabolomics

Introduction:

Acylcarnitines (ACs) are esters of carnitine and fatty acids that are essential for the transport of fatty acids into the mitochondria. Fatty acids that are bound to Co-enzyme A (CoA) in the cells are esterified with carnitine, which enables them to cross the membrane of the mitochondria where they are converted back to the CoA ester to be oxidized for energy metabolism. ACs are also found in plasma and urine and are thought to participate in detoxification of fatty acid metabolism by-products(1,2). Their levels in blood have been found to be elevated in obese or diabetic individuals(3,4), which may indicate incomplete fatty acid oxidation, and have been proposed as potential biomarkers of metabolic dysfunction(1,5).

Diet is known to influence AC levels in both urine and blood. Intervention studies have shown that AC levels in blood and urine are influenced by intake of specific fatty acids (6), sunflower oil (2) or meat (7). In addition, specific AC profiles were associated with Western dietary patterns (8,9) and intake of specific foods in several observational studies (10–12). Red meat which includes beef, pork, lamb and game is the main dietary source of carnitine in omnivores (13) and has received particular attention with regard to its associations with AC levels. Indeed some of the most prominent metabolic changes associated with meat intake are related to ACs. Acetylcarnitine (C2:0), propionylcarnitine (C3:0) and (iso)valerylcarnitine (C5:0) were positively associated with red meat intake in 50 European individuals (14) and 5 ACs were elevated in meat eaters compared to vegans in a British study (15). Similarly, associations of ACs with insulin resistance (16) (medium chain ACs) or type 2 diabetes (4) (C2:0, C3:0 and C8:0) have been shown to be specific for particular ACs or groups of ACs. Considering the large diversity of ACs described in human blood or urine (17) and their importance in energy metabolism, a more

thorough investigation of the effects of red and processed meat (RPM) intake on AC profiles is needed to help understanding the links between RPM intake and risk of several major chronic diseases such as type 2 diabetes (18) and cancer (19), and all-cause mortality (20).

The current study investigated the effect of RPM intake on AC levels using a two-tiered approach. First, AC levels in blood and urine were measured in a randomized cross-over dietary intervention study in which 12 volunteers successively consumed a pork-containing and a tofu-containing diet for 3 consecutive days each. ACs that showed differential levels between the two diets were then tested for association with habitual RPM intake in free-living subjects from the European Prospective Investigation into Cancer and nutrition (EPIC) study.

Methods:

Intervention study:

Twelve healthy volunteers (6 male, 6 female, BMI: 22.4 +/-2.6 kg/m^2 (mean +/- SD), age: 31 +/- 5.2 years (mean +/- SD)) were recruited for a randomized cross-over dietary intervention in which each volunteer consumed during five successive intervention periods different types of meats (fried fresh pork strips, salami, bacon, hot dog) or tofu for 3 consecutive days each (**Figure 1**). In a washout period between each of the intervention periods, participants consumed their habitual diet for at least 10 days. The study was designed to identify biomarkers of processed meat intake (21). In the current analysis, a subset of samples only was included from

the intervention periods where participants consumed pork or tofu. Fried fresh pork was chosen over the other meats because it is richer in muscle tissue which is the main source of carnitine (13). Tofu was chosen as a control non-meat food low in carnitine. The medium fatty pork was prepared without any added fat; tofu was marinated with a small amount of olive oil before being fried. In each intervention period, the volunteers consumed the same standardized breakfast and the same side dishes for 3 days together with pork (135 g, fried) or tofu (178 g) for lunch (day 2 and 3) and dinner (day 1, 2 and 3). The amount of pork and tofu were standardized to provide 250 kcal per meal. Spot urine samples were collected 2 and 12h after the first intervention meal of each intervention period (day 1). A cumulative 12h urine sample starting after the last meal (day 3) and a fasting plasma sample on the morning after the last intervention meal (day 4) were also collected. A wash out period of at least 10 days in which the volunteers resumed their habitual diet separated the two intervention periods. The participants gave their informed consent prior to their participation and procedures were carried out according to the principles expressed in the Declaration of Helsinki. The study was approved by the IARC Ethics Committee (IEC Project 17-12). The study was registered at clinicaltrails.gov as NCT03354130.

Cross Sectional study:

The European Prospective Investigation into Cancer and nutrition (EPIC) is a multicentric prospective cohort study that includes more than 520,000 men and women from 10 European countries (22) who provided blood samples and answered food frequency questionnaires (FFQ) at recruitment. The samples used in this work

are from a subset of the calibration study nested in EPIC (23) in which one 24-hr urine sample and a 24-hr dietary recall (24HDR) were collected per subject (n = 1,103) (24). In this analysis we included 474 volunteers from Germany, Italy, France and Greece who gave the 24h urine sample and 24-hr dietary information on the same day. Of these, serum samples with known fasting status at blood collection were also available for 451 participants (**Supplemental Figure 1**). Details on participant selection can be found elsewhere (25). Urine samples were collected between 1995 and 1999 and stored at -20°C until analysis. Serum samples were stored in liquid nitrogen and retrieved from the biobank in 2014 for analysis. Food intake data and participant characteristics such as smoking status, body mass index (BMI), etc. were provided by the national study centres. The proportion of pork-based processed meats was estimated using the food description of the questionnaire data. The ethical review boards from the International Agency for Research on Cancer (IARC) and from all local centres approved the study. All participants signed an informed consent prior to their participation in the study.

Sample analysis:

Urine and blood samples were analyzed by liquid chromatography−mass spectrometry (LC-MS) using an untargeted metabolomics method optimized to cover a broad range of metabolites (14,26). Urine samples from the intervention study and the cross-sectional study were processed separately. Urine samples were diluted with ultrapure water to the lowest specific gravity of any urine sample in the experiment to normalize their concentrations (27), centrifuged (2000 x g) and an aliquot of the supernatant diluted 2-fold (intervention study) and 1.25-fold (cross-sectional study) with acetonitrile and stored at -80 °C until analysis. Blood samples (intervention study: 50 µl plasma, cross-sectional study 20 µl serum) were mixed

with cold acetonitrile (intervention study: 300 µl, cross-sectional study 200 µl), shaken for 2 minutes, centrifuged (2000 x g) and the supernatant filtered with 0.2 µM polypropene filter plates (Captiva, Agilent) and stored at -80 °C. Samples were then analysed by LC-MS on an Agilent 1290 Binary LC system coupled to an Agilent 6550 quadrupole time-of-flight (QTOF) mass spectrometer with jet stream electrospray ionization source (Agilent Technologies), as previously described (26). Samples from the different studies (intervention study/cross-sectional study) and sample type (blood/urine) were analysed separately (4 batches). Samples were ordered randomly within each batch (up to 560 injections). A quality control (QC) sample consisting of a pool of all samples in one batch was analysed for every twelve (cross-sectional serum analysis) or eight (all other analysis) study samples injected. Two microliters of sample extracts were injected onto a reversed phase C18 column (ACQUITY UPLC HSS T3 2.1 × 100 mm, 1.8 µm, Waters) maintained at 45^{o}C. A linear gradient made of ultrapure water and LC-MS grade methanol, both containing 0.05 % (v/v) of formic acid, was used for elution. The mass spectrometer was operated in positive ionization mode, detecting ions across a mass range of 50-1,000 daltons.

Annotation of acylcarnitines

Intensity data of ACs was created by a targeted screening approach using positive ionization full scan LC-MS data. ACs were annotated based on their exact mass (8 ppm tolerance) and an in-house database containing retention times of ACs previously annotated in our laboratory. ACs were identified by their characteristic fragments (m/z = 60.0808 and 85.0284) and neutral losses (m/z = 59.0735) and their retention time in comparison to their homologs with different fatty acid chain lengths. An extensive approach for AC annotation using data-dependent MS/MS has been published recently (17). We use here the same nomenclature as used in this

previous work. AC general structures are described as Cx:y, Cx:y-OH and Cx:y-DC where x is the number of carbon atoms and y the number of double bonds in the fatty acid moiety, where the suffix –OH indicates ACs with a hydroxyl group on the fatty acid moiety and DC indicates dicarboxylic acids. Annotations were performed by matching retention time and MS/MS fragmentation when spectra were available. Identities of all ACs that are reported as statistically significant in this work were confirmed by targeted MS/MS fragmentation (see **Supplemental Figures 2-20)**. Due to the lack of commercial standards for most ACs, many AC isomers of identical molecular mass differing in their retention time could not be fully identified. Therefore, the position of double bonds and hydroxyl groups as well as the number of carbon atoms in sidechains of the fatty acids could not be determined. Different levels of confidence in the annotations were defined as proposed by Sumner et al. (28). For level 1, the highest level of confidence, full match of retention time and MS/MS spectrum with those of an authentic chemical standard was required. For level 2, no standard was available, and annotation was based on exact mass, retention time, isotope pattern, and MS/MS spectra.

Compound intensities were extracted from the raw data with the Profinder software as peak area (Agilent, version B.08.00), using a targeted feature extraction based on formula (mass tolerance +/- 8 ppm). Feature intensity data was log2 transformed for statistical analysis. Only compounds with a relative standard deviation of less than 25 % in the quality control samples were used for statistical analysis.

Statistical analysis

For the urine and plasma samples obtained from the intervention study, a paired Student’s t-test was conducted for each dataset separately to identify ACs whose

concentrations were significantly different between the pork and the tofu diet group. As a first discovery analysis, p-values were adjusted for multiple comparisons using the Benjamini-Hochberg method with a false discovery rate (FDR) of 0.1.

To validate the findings of the intervention study within the observational study, habitual dietary intake based on FFQs was used. Linear regression models with intake of major food groups and potential confounding variables (BMI, age, sex and cigarette smoking status) as predictors and the intensity of ACs in serum and urine as dependent variable were built with the data of the cross-sectional study (see **Supplemental Table 1** for the covariates included in each model). Food groups included as potential confounders were those that were consumed by at least half of the study population according to questionnaires. Coefficients and 95% confidence intervals (CI) were computed for “red and processed meat intake”, which includes all fresh red meat (pork, beef, horse, veal, game, mutton) and processed meat (meat processed by curing, smoking, fermentation, canning or other processes that enhance taste or shelf life). Since the goal of the regression analysis was to assess if associations in the population based study were significant and in the same direction as in the intervention study, one-sided p-values were computed for the covariate “red and processed meat intake”. Q-values were calculated using the Benjamini-Hochberg method and values below 0.05 were considered significant. For sensitivity analyses, the same analysis was carried out for total meat intake (red and processed meat, offal and poultry) as well as for poultry and red meat only. All statistical analyses and visualization were carried out using the open-source R software, version 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Effect of red and processed meat intake on acylcarnitine levels in urine

In a first study, two diets containing either pork as an example of red meat, or tofu taken as control, were successively consumed during three days by 12 subjects in a randomized cross-over trial. Cumulative twelve-hour urine samples were collected at the end of each intervention period and analyzed by mass spectrometry. Forty-four different ACs corresponding to a total of 63 isomers could be annotated in pooled 12h urine samples (**Supplemental Table 2**). Eighteen ACs significantly differed in their intensities between the two diet groups in the 12-hr urine samples ($q < 0.1$ (FDR); **Figure 2A**, **Supplemental Table 3**). Of these, 14 ACs showed increased intensity in the meat group and 4 decreased intensities compared to the tofu group. Intensities were also compared in spot urine samples collected 2h and 12h after the first of five meals of each intervention period. Results for spot samples collected at 2h and 12 h were not significant (**Supplemental Table 4**).

The 18 ACs that showed significant differences in 12-hr urinary levels after intake of pork compared to tofu in the intervention study were tested for their association with habitual RPM intake in 24-hr urine samples from the EPIC cross-sectional study. **Table 1** shows the characteristics and meat intake of the 474 free-living subjects with 24-hr urine samples. Pork accounted for 54 % of the RPM intake (red meat: 28% pork; processed meat: 87% pork) and beef represented 25% of RPM intake. Eleven of the 18 ACs tested were positively associated with habitual meat intake in a linear model which included BMI, sex, age, cigarette smoking status and intake of other foods as covariates to control for potential confounding ($q < 0.05$ (FDR); **Figure 2B;** Supplemental Table 3). The correlation of their relative intensities is

shown in **Supplemental Figure 21**. C0, C2:0, C3:0, C4:0-OH are highly correlated to each other and C4:0 is highly correlated to C5:0. The remaining ACs are only moderately associated to each other. Sensitivity analysis showed that associations between total meat intake and AC levels or red meat intake and AC levels were similar in direction and strength to associations between RPM intake and AC levels (**Supplemental table 5**). Poultry intake was not associated with any urinary AC.

Effect of red and processed meat intake on acylcarnitines in blood

Twenty-three different ACs corresponding to a total of 33 AC isomers were annotated in plasma samples from the dietary intervention study (**Supplemental table 6**). Their concentrations were first compared in fasting plasma samples collected in the morning following the three days of each dietary intervention period. Two of them were found to be significantly different after pork intake compared to tofu intake (**Figure 3A and Supplemental Table 7**).

The two ACs associated with pork intake in the intervention study were tested for their association with habitual RPM intake in free-living subjects of the EPIC cross-sectional study (Figure 3B, Supplemental table 7). Serum levels of C10:2 showed no association with RPM intake. Levels of C18:0 showed significant associations with habitual RPM intake when adjusted for fasting status, age, sex, BMI and intake of major animal derived foods and fats (FDR, q = 0.033). Sensitivity analysis for different types of meat intake (**Supplemental table 8**) showed the same direction and similar strength of association for total meat intake, but no association was observed between poultry intake and serum levels of C18:0 (q = 0.99). Associations of RPM intake in the cross-sectional study with all ACs including the ones that were not increased in the intervention study can be found in **Supplemental table 9.**

Discussion

We show in this work that intake of pork increases urinary levels of several ACs (dietary intervention study) and that the same ACs were also associated with habitual RPM intake (cross-sectional study). We could confirm associations of RPM intake with several of ACs (C0, C2:0, C3:0, C4:0-OH and C5:0) described in previous work (7,10,14,29) but also show for the first time positive associations with several other ACs (C4:0, C7:0, C8:0-OH, C10:0-OH and C11:1). The intensities of newly identified ACs were only moderately correlated with the intensities of the ones already known which suggests that they do not share the same pathways.

These changes in urinary AC levels were observed in 12-hr urine samples collected after 5 successive intervention meals, but not in spot urine samples collected 2 and 12 hours after the first intervention meal. This suggests that the changes detected are only expressed after a certain duration and amount of RPM intake, changes that are compatible with the associations of ACs with habitual RPM intake observed in the cross-sectional study. Poultry intake was not associated with levels of any AC identified in the cross-sectional study which is in line with prior studies (14).

In blood samples collected in the intervention study, C10:2 and C18:0 levels were elevated after pork intake compared to tofu intake. In the EPIC cross-sectional study, C18:0 levels were positively associated with RPM intake but not with poultry intake. These results can be compared to those of previous studies. We showed in a previous study associations of C2:0 and C3:0 with red meat intake 2h and 24h after its consumption (14). Their levels were consistently higher after intake of red meat compared to chicken. We could not detect the associations with these two ACs in the present work and this could be explained by the use of fasting samples in the

present intervention study. Schmidt et al. (15) observed higher levels of C0, C3:0, C4:0, C5:0 and C16:0 in meat-eaters when compared to vegans and to a lesser extent when compared to vegetarians in a cross-sectional study. The low number of vegetarians in our study population (less than 1%) and the adjustment for intake of all major food groups might be the reason that we do not find the same associations. We do, however, observe a trend for a positive association between habitual RPM intake and blood levels of C0:0, C4:0 and C5:0 (Supplemental table 9). Wittenbecher et al (30) found plasma levels of C18:0 to be associated with red meat intake in German men (n = 790) from the EPIC-Potsdam cohort, results consistent with our own findings.

Overall, we show that urinary excretion of several ACs are strongly associated with RPM intake whereas there are only limited variations in AC blood levels. This difference might be explained by the tight regulation of AC levels in blood through homeostatic control, with the excess of carnitine and ACs being cleared in urine or in bile (31,32). The increased excretion of ACs in urine after RPM intake indicates that carnitine ingested with meat is involved in fatty acid metabolism and detoxification (1).

Alterations in the AC pathway have been linked to dysregulation of energy metabolism, inflammation and higher risk of type II diabetes and other adverse health outcomes (1,4,5,33). It is not completely clear whether these increased levels of ACs are merely an indicator of impaired fatty acid metabolism or if the increased AC levels themselves play a causal role in the aetiology of metabolic diseases. It has been proposed that ACs can activate pro-inflammatory pathways (4,33). Alterations of the AC pathway and fatty acid metabolism might be one of the mechanisms through which RPM intake increases risk of several diseases. Our study shows that

in contrast to RPM intake, the intake of poultry has no effect on the carnitine pathway. This might help in understanding the specificity of the association of risk of certain chronic diseases with RPM intake, and the lack of association with white meat intake. Long-term longitudinal studies with repeated measurements of ACs are needed to disentangle the role of AC pathways and RPM in the aetiology of metabolic diseases.

This work has several limitations. A first limitation is related to the different nature of meat considered in the intervention study (fresh pork) and in the cross-sectional study (RPM). Beef was not considered on its own in the intervention study whereas it constituted a significant fraction of RPM consumed in the cross-sectional study which means that no conclusions can be drawn on beef intake alone. However, pork accounted for a large fraction (54%) of the RPM consumed in the cross-sectional study as either fresh pork or processed pork. Inclusion of beef with its higher content of carnitine compared to pork (13) in the intervention study might have led to the identification of more associations with ACs. Poultry was also not included in the intervention study and therefore the null association of poultry intake and AC levels is based only on the cross-sectional data. However, data from a prior intervention study showed a trend with higher levels of three ACs in RPM when compared with chicken (14) which might be due to higher carnitine content (13). A second limitation of this work is linked to the time frame of our experiments. Pork or tofu were consumed during 3 days in the intervention study whereas habitual RPM intake was measured with a questionnaire over a whole year. Due to the short duration of the intervention study, some effects on ACs that take more than 3 days to manifest might have been missed. However, RPM was very regularly consumed in our population and associations of ACs with RPM intake may also be the result of

repeated short term exposure as considered in the intervention study and this likely explains the good agreement between the intervention and cross-sectional studies. Other limitations are related to the nature of the blood samples collected. In the intervention study, we only collected fasted samples and some effects only observed in the fed state may have been missed. In addition, blood samples collected in the intervention study (plasma) were different from those collected in the cross-sectional study (serum). However this should have little impact on the results, considering the high correlations of ACs concentrations in the two matrices (34). A last limitation of this work is the incomplete identification of some AC isomers, due to the lack of commercially available chemical standards. However, the exact mass as well as the characteristic MS/MS fragmentation pattern of the ACs give us high confidence in the proposed annotations.

This study has also several strengths. First, we assessed a broad range of different ACs which gave us the opportunity to report novel associations. Secondly, we conducted our study with both blood and urine samples, providing a more holistic view on the impact of RPM intake on AC levels and metabolism than previous studies. Thirdly, we use a multi-tiered approach. Discovery in an intervention study gives confidence in the biological plausibility of the association and allows causal inference whereas the confirmation in an observational study shows that RPM intake has an effect on AC levels in subjects following their habitual diet. The extensive correction for potential confounders and the coherent results from different models (see supplemental table 8) increase confidence for the associations that we report in this work.

Conclusion

We were able to confirm several associations between urinary levels of ACs and RPM intake that were already known and also report new associations hitherto not described in the literature (C4:0, C7:0, C8:0-OH, C10:0-OH and C11:1). We also found an association of C18:0 levels in blood with RPM intake. These significant effects of RPM on AC levels and the lack of effects of poultry should be further explored. They may help in understanding the specific role of RPM intake in the aetiologies of type II diabetes, some cancers and cardiovascular diseases.

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Declaration of Interest

The authors have declared no conflicts of interest.

Authors' contributions

The authors' responsibilities were as follows - RW, IH, AS designed research; AK developed the in-house data base and extracted data; RW extracted and analysed data and performed statistical analysis; Data interpretation: RW, PK-R, VV, IH, AS; RW drafted the manuscript; AS had primary responsibility for final content; MBS,TK, TJ, AT, EP, CLV, GM, RT, CS, CW, MSM, GS, FRM, 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.

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Tables

Table 1: Characteristics of participants of the European Investigation into Cancer and nutrition (EPIC) cross-sectional study included in this analysis.

Characteristic	Participants with 24-hr urine samples	Participants with serum samples[1]
Subjects, n (% total)		
Total	474	451
Male	195 (41)	193 (43)
Female	279 (59)	258 (57)
Germany	178 (38)	173 (38)
Italy	174 (37)	156 (35)
France	66 (14)	66 (15)
Greece	56 (12)	56 (12)
Age, years*	53.9 +/- 8.5[2]	54.2 +/- 8.5
BMI, kg/m^2*	26.1 +/- 4.3	26.0 +/- 4.3
Fasting status at blood collection, n (% of total)		
Fasted		189 (42)
Not fasted		170 (38)
In between		92 (20)
Meat intake (g/day)[3]		
Total	105.7 +/- 54.8	106.1 +/- 55.8
Red meat		
Beef	20.2 +/- 20.8	19.7 +/- 20.9
Veal	8.4 +/- 14.5	8.5 +/- 14.6
Pork	12.3 +/- 12.0	12.3 +/- 12.2
Lamb/mutton/horse	3.7 +/- 8.0	3.7 +/- 8.2
White meat		
Poultry	18.0 +/- 15.4	18.0 +/- 15.6
Offal	3.2 +/- 5.5	3.1 +/- 5.5
Processed meat[4]	36.6 +/- 33.4	37.5 +/- 33.9
Red and processed meat[5]	**81.1 +/- 46.5**	**81.7 +/- 47.2**

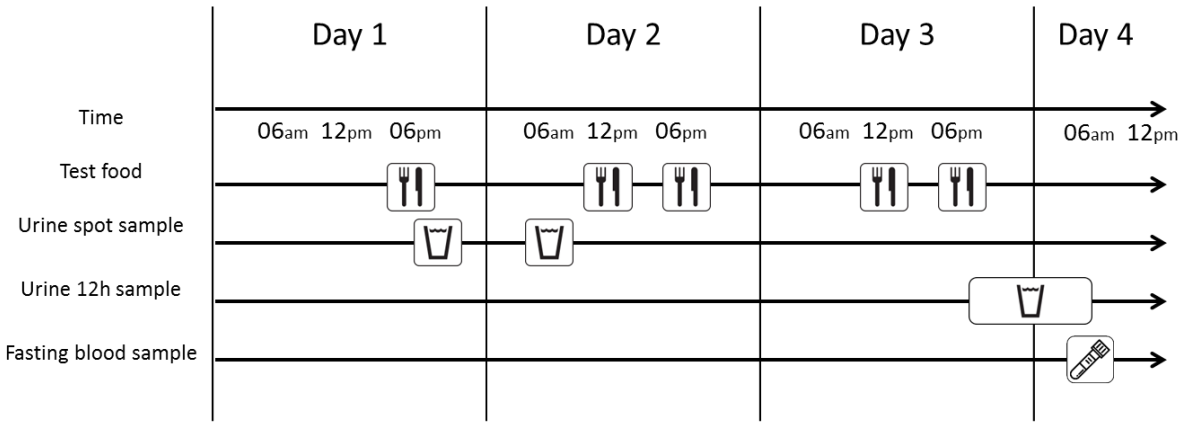
[1]For 451 out of the 474 subjects included in this study, serum samples and data on fasting status at blood collection were available.

[2]Mean +/- standard deviation, all such values

[3]Habitual intake as reported in food frequency questionnaire

[4]Processed meat was estimated to be made of 87% pork based on the food frequency questionnaires.

[5]Red and processed meat = Beef, veal, pork, lamb/mutton/horse, and processed meat.



- **Twelve healthy adults**
- **Two spot urine samples ( ), one 12h urine sample ( ) and one fasting blood sample ( ) were collected in each intervention period**
- **One period for each of the 5 test foods:** Tofu (178 g)
  Fried pork (135 g)
  Bacon (104 g)
  Salami (67 g)
  Hot dogs (107 g)

- **A standardized (vegetarian) breakfast was provided on day 2 and day 3**
- **Ten days minimum washout between intervention periods**
- **Food diary to assess compliance**

Figure 1: Design of the randomized cross-over dietary intervention study. Only one intervention period is shown but each participant completed 5 intervention periods that were identical except for the intervention food consumed (Tofu, fried pork, bacon, salami and hot dogs). This present study includes only samples from the tofu diet and the pork diet.

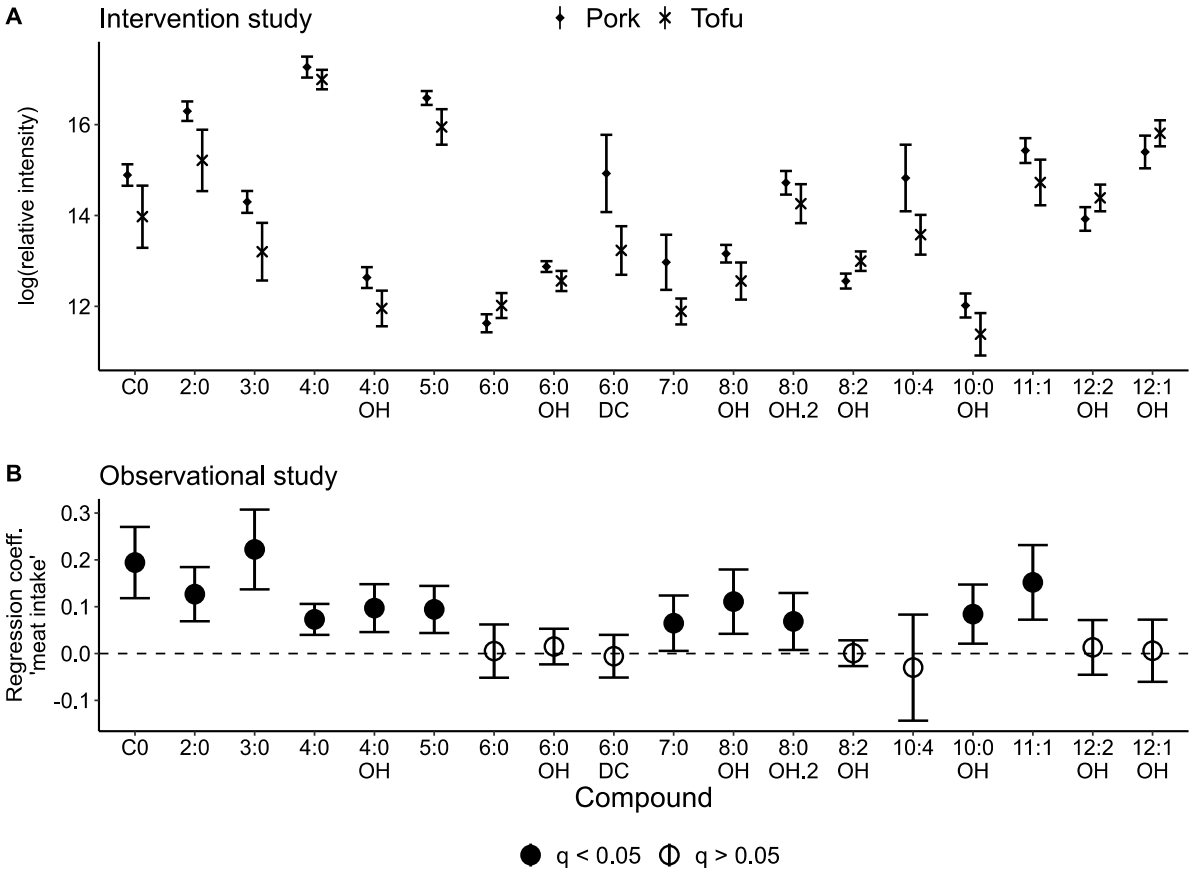


Figure 2: Urinary acylcarnitines (ACs) associated with red and processed meat intake **(A)** Intervention study: mean relative intensity of ACs with 95%-confidence interval in 12-hr urine samples after 3 days of intake of pork (circle, n = 12) or tofu (cross, n = 12). Shown are the eighteen ACs out of 63 tested that were significantly different between the two diets (FDR-adjusted q-values < 0.1). **(B)** Observational study: association of AC levels in 24-hr urine samples with habitual red and processed meat intake in the European Prospective Investigation into Cancer and nutrition (EPIC) cross sectional study (n = 474). Coefficients of the predictor “red and processed meat intake” (with 95%-confidence interval) in a linear regression model with urinary AC intensities as dependent variable are shown for each AC. The coefficient shows the change in acylcarnitine levels for an increase of one standard deviation of red and processed meat intake (46.5 g/day). Intake of major food groups

as well as subject characteristics (sex, age, BMI, smoking status, study center) are included as covariates in the linear models. Full circles indicate ACs for which habitual red and processed meat intake is a significant covariate in the model after adjustment for multiple testing (FDR-adjusted q-values < 0.05).

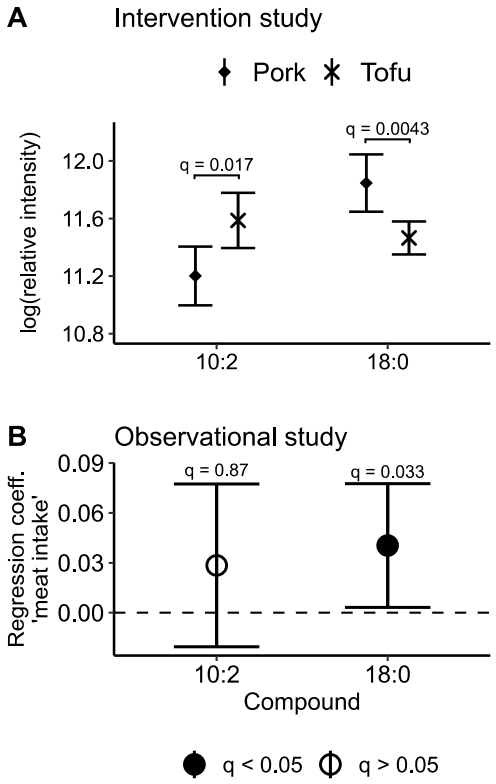


Figure 3: Blood acylcarnitines (ACs) associated with red and processed meat intake **(A)** Intervention study: mean relative intensity of ACs with 95%-confidence interval in fasting plasma samples after 3 days of intake of pork (circle, n = 12) or tofu (cross, n = 12). Shown are the 2 ACs out of 33 tested which were significantly different

between the two diets (q-value < 0.1) in a paired Student's t-test. **(B)** Observational study: association of AC levels in serum samples with habitual red and processed meat intake in the European Prospective Investigation into Cancer and nutrition (EPIC) cross sectional study (n = 451). Coefficients of the predictor "red and processed meat intake" (with 95%-confidence interval) in a linear regression model with serum AC intensities as dependent variable are shown for each AC. The coefficient shows the change in acylcarnitine levels for an increase of one standard deviation of red and processed meat intake (47.2 g/day). Intake of major food groups as well as subject characteristics (sex, age, BMI, smoking status, study center, fasting status at blood collection) are included as covariates in the linear models. Full circles indicate ACs for which habitual red and processed meat intake is a significant covariate in the model after adjustment for multiple testing (FDR-adjusted q-values < 0.05).