Amino acids, lipid metabolites, and ferritin as potential mediators linking red meat consumption to type 2 diabetes[1–4]

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ABSTRACT

Background: Habitual red meat consumption was consistently related to a higher risk of type 2 diabetes in observational studies. Potentially underlying mechanisms are unclear.

Objective: This study aimed to identify blood metabolites that possibly relate red meat consumption to the occurrence of type 2 diabetes.

Design: Analyses were conducted in the prospective European Prospective Investigation into Cancer and Nutrition–Potsdam cohort (n = 27,548), applying a nested case-cohort design (n = 2681, including 688 incident diabetes cases). Habitual diet was assessed with validated semiquantitative food-frequency questionnaires. Total red meat consumption was defined as energy-standardized summed intake of unprocessed and processed red meats. Concentrations of 14 amino acids, 17 acylcarnitines, 81 glycerophospholipids, 14 sphingomyelins, and ferritin were determined in serum samples from baseline. These biomarkers were considered potential mediators of the relation between total red meat consumption and diabetes risk in Cox models. The proportion of diabetes risk explainable by biomarker adjustment was estimated in a bootstrapping procedure with 1000 replicates.

Results: After adjustment for age, sex, lifestyle, diet, and body mass index, total red meat consumption was directly related to diabetes risk [HR for 2 SD (11 g/MJ): 1.26; 95% CI: 1.01, 1.57]. Six biomarkers (ferritin, glycine, diacyl phosphatidylcholines 36:4 and 38:4, lysophosphatidylcholine 17:0, and hydroxy-sphingomyelin 14:1) were associated with red meat consumption and diabetes risk. The red meat–associated diabetes risk was significantly ($P < 0.001$) attenuated after simultaneous adjustment for these biomarkers [biomarker-adjusted HR for 2 SD (11 g/MJ): 1.09; 95% CI: 0.86, 1.38]. The proportion of diabetes risk explainable by respective biomarkers was 69% (IQR: 49%, 106%).

Conclusion: In our study, high ferritin, low glycine, and altered hepatic-derived lipid concentrations in the circulation were associated with total red meat consumption and, independent of red meat, with diabetes risk. The red meat–associated diabetes risk was largely attenuated after adjustment for selected biomarkers, which is consistent with the presumed mediation hypothesis. *Am J Clin Nutr* 2015;101:1241–50.

Keywords: biomarkers, cohort study, type 2 diabetes mellitus, metabolomics, red meat

INTRODUCTION

Red meat consumption has consistently been associated with a higher risk of type 2 diabetes mellitus in several large cohort studies and summarized by several meta-analyses (1–3). Recently, change in red meat consumption was observed to be related to a change in diabetes risk among 3 large US cohorts (4), suggesting a dose-response relation. Furthermore, the red meat–associated diabetes risk was also observed in models in which red meat was substituted by foods with a similar macronutrient content (2), suggesting the relation to be independent of macronutrient composition of the diet. We have previously reported from the European Prospective Investigation into Cancer and Nutrition (EPIC)[5]–Potsdam study that with regard to type 2 diabetes risk assessment, a 150-g/d portion of red meat

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[3] Supplemental Tables 1–7 and Supplemental Figures 1–8 are available from the "Supplemental data" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

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[5] Abbreviations used: EPIC, European Prospective Investigation into Cancer and Nutrition; FA, fatty acid; FDR, false discovery rate; FFQ, food-frequency questionnaire.

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had a predictive value similar to smoking >20 cigarettes/d, having a 7.6-cm higher waist circumference, or having one parent with diabetes (5). Thus, available evidence from prospective cohorts indicates red meat consumption as a risk factor for the development of type 2 diabetes (6).

Yet, mechanisms underlying the relation of red meat consumption with diabetes risk remain largely unknown. Components of red meat that were proposed to affect diabetogenic pathways include heme iron, cholesterol, fatty acids (FAs), amino acids, and advanced glycation and lipoxidation end products (1), among others. Reported associations of red meat consumption with biomarkers of inflammation, glucose metabolism, and hepatocellular and oxidative stress were mostly explainable by differences in BMI (7, 8). Therefore, and because of potential residual confounding by meat-related lifestyle, skepticism prevails regarding red meat intake as a causal factor in diabetes development.

A growing body of observational studies and dietary interventions illustrates the impact of diet on systemic metabolic processes as reflected in metabolomic data (9). In addition, metabolomic approaches have provided deeper insight into early diabetogenic pathways (10). Integration of metabolomic data bears the potential here to focus on a systemic understanding of the observed diet-disease relations in nutritional epidemiology (11). In EPIC-Potsdam, metabolomic data were used to identify lipid metabolites and amino acids as biomarkers of diabetes risk (12). Furthermore, iron status, as reflected in circulating ferritin, was shown to be related to the risk of diabetes in EPIC-Potsdam (13). Changes in amino acid and lipid profiles and iron status might also help to identify mechanisms underlying the red meat–associated diabetes risk.

The present study aimed to identify metabolites that possibly relate habitual red meat consumption to the occurrence of type 2 diabetes and to estimate to what extent this association depends on such selected metabolites. To pursue that aim, we evaluated data from targeted metabolomics within a large prospective cohort study.

METHODS

Study population

The EPIC-Potsdam cohort is part of the multicenter EPIC study (14). In Potsdam and the surrounding area, in total, 27,548 persons were recruited from the general population (16,644 women and 10,904 men) between 1994 and 1998, with an age range of 35–64 y (15). The baseline examination included anthropometric measurements, a validated semiquantitative food-frequency questionnaire (FFQ) (16), a lifestyle questionnaire, and a personal interview. Written informed consent was obtained from all study participants a priori, and the study was approved by the ethics committee of the Medical Society of the State of Brandenburg (15). In course of active follow-up, participants were contacted every 2 y, with response rates ranging between 90% and 96% per follow-up round (17). Over a mean follow-up time of 7.0 y, 849 incident type 2 diabetes cases were identified until August 2005. For molecular phenotyping, a nested case cohort was constructed based on all participants who provided blood samples at baseline (n = 26,444) (18), including a random sample representative of the full cohort (2500 participants, from here on referred to as the subcohort) and all incident type 2 diabetes cases (n = 801), with an overlap of 74 cases (19). For the current analysis, participants with prevalent type 2 diabetes, cancer, myocardial infarction, or stroke at baseline (n = 359); implausible self-reported energy intake (<3.35 MJ/d or >25.12 MJ/d) and missing data for relevant variables (serum concentrations of metabolites and other biomarkers) (n = 104); incomplete follow-up information (n = 58); and outliers regarding the relation between total red meat consumption and metabolites (n = 25) (see "Statistical analysis" section) were excluded. Thus, the analytic sample comprised 2681 participants, including 2047 participants of the subcohort and 688 incident type 2 diabetes cases (with an overlap of 54 cases).

Dietary assessment

Mean daily intake of 158 food items in grams per day in the year before baseline examination was estimated for each participant from self-reports on habitual consumption in a semiquantitative FFQ. Total red meat intake was defined as the summed intake of unprocessed red meat (dishes prepared from unprocessed beef, veal, pork, and lamb) and processed meat (bacon, ham, sausages, and the like). A substudy assessment of unprocessed and processed meat intake showed high reproducibility with correlation coefficients of 0.77 and 0.73, respectively, between FFQs 6 months apart. Furthermore, a high relative validity of meat intake was shown if FFQ information was compared with repeated 24-h recalls (correlation coefficients: 0.65 unprocessed and 0.70 for processed red meat) (20). Total red meat consumption was computed as dietary density (g/MJ) (21). To define a realistic portion size as an exposure unit, we divided total red meat consumption by 2 SD (~11 g/MJ). This corresponds to an absolute amount of 93 g/d of total red meat consumption for a person with an energy intake similar to the populations mean.

Quantification of biomarkers

Blood samples were collected, fractioned, and aliquoted by qualified medical personnel and stored in liquid nitrogen (~−196°C) (14). A targeted metabolomic approach was conducted based on a commercial kit (AbsoluteIDQTM p 150 Kit; Biocrates Life Sciences AG). Serum samples were analyzed on high-throughput flow injection analysis–tandem mass spectrometry devices at the Helmholtz Center Munich. The measurement of the samples of this study as well as the assay procedure was described elsewhere in detail (22, 23). On the basis of a previous evaluation of the reliability of measurements (23), we excluded metabolites below the limit of detection (n = 30) and those with very high analytic variance (n = 6). We also did not consider hexoses, because hexose concentrations in the blood are mainly attributable to glucose. Elevated blood glucose, however, is hardly attributable directly to red meat intake but rather closely linked to the endpoint, type 2 diabetes. Thus, 126 metabolites were left for analyses (14 amino acids, 17 acylcarnitines, 34 diacyl phosphatidylcholines, 37 acyl-alkyl phosphatidylcholines, 10 lysophosphatidylcholines, and 14 sphingomyelins). For lipid metabolites, FA residues were abbreviated x:y, where x represented the cumulative number of carbon atoms and y the cumulative number of double bonds. Serum ferritin concentrations were determined by using an

ADVIA Centaur XP (Siemens Health Care) (13). Plasma concentrations of alanine aminotransferase, γ-glutamyltransferase, and high-sensitivity C-reactive protein were determined by using the automatic ADVIA 1650 analyzer (Siemens Medical Solutions). All assay procedures were performed as described by the manufacturer (24).

Identification of incident type 2 diabetes cases

Systematic information sources for the identification of incident cases of type 2 diabetes mellitus were self-reports of a diabetes diagnosis, diabetes-relevant medication, and dietary treatment due to diabetes during follow-up. Furthermore, hints from other information sources such as diagnoses from the tumor center or doctor or clinic or from the death certificate were used to identify participants who had likely developed type 2 diabetes. Potentially incident cases were validated by the treating primary care physician. Verification process included correct diagnosis (International Classification of Diseases, 10th revision, E11) and exact date of diagnosis. In the Methods and Results sections of this article, the terms *diabetes* and *type 2 diabetes*, respectively, refer to the verified diagnosis of type 2 diabetes mellitus as defined here.

Statistical analysis

We selected mediators of the red meat–diabetes association according to 4 predefined mediation criteria (25) (see **Supplemental Figure 1**A). These criteria were tested one after the other in distinct regression models for each potential mediator individually. Lipid metabolites, amino acids, and ferritin were included as potential mediators of the red meat–associated diabetes risk because these biomarkers were assumed to be likely influenced by total red meat consumption in a relatively direct manner based on its nutrient composition (Supplemental Figure 1B). To reduce the probability of spurious associations, we selected biomarkers only if they were associated with total red meat consumption in both sexes in stratified analyses. The associations with diabetes risk were in general similar for men and women, for total red meat consumption as well as for the selected biomarkers. Thus, for respective analyses, men and women were pooled.

Assessment of the red meat association with type 2 diabetes

Mediation criterion 1 (significant association between total red meat consumption and diabetes risk) was evaluated in Cox proportional hazards regression models with age as underlying time scale. Study exit was determined by diagnosis of diabetes, dropout, or censoring time, whichever came first. The case-cohort design was accounted for by Prentice weighting (26). Potential confounders to be included as covariates were a priori selected based on the literature on the association between red meat intake and diabetes risk (2, 3) and prior investigations in EPIC-Potsdam regarding risk factors of type 2 diabetes (5), dietary patterns (27), and determinants of metabolic profiles (28, 29). Model 1 was adjusted for total energy intake (MJ/d) and sex and stratified according to age at recruitment (rounded to the next full year). Model 2 also included risk factors for type 2 diabetes (5): sportive activity [sports (h/wk), biking (h/wk)]; smoking (4 stages: never smoker, former smoker, current smoker $<$20 units/d, or current heavy smoker $\geq$20 units/d); education (4 stages: no vocational training or in training, vocational training, technical school, or technical college or university); daily intake of alcohol, coffee, and sugar-sweetened beverages (g/d) and consumption of whole-grain bread (g/MJ); consumption of foods related to red meat intake (27) [refined-grain bread, cabbage, cooked vegetables, mushrooms, potatoes, sauce, and poultry (g/MJ)]; and factors related to serum metabolite concentrations (28, 29) [intake of margarine and butter (g/MJ), antihypertensive medication (yes/no), and antidyslipidemic medication (yes/no)]. To separately evaluate a possible dependency of selected mediators on body fatness, we also adjusted the latter model for BMI (in kg/m^2) (model 3).

Biomarker selection

Mediation criterion 2 (significant association of total red meat consumption with biomarker concentrations in the blood) was evaluated in the subcohort in multivariate linear regression models stratified by sex. Biomarkers were Box-Cox transformed and Z-standardized (mean = 0, SD = 1). Outliers regarding the relation of total red meat consumption with serum metabolites were detected based on multivariate regression models. Outliers were defined as observations with studentized residuals larger than 3.5 for $>$5 metabolites or larger than 5 for any of the investigated metabolites and high influence on estimates for the association according to Cook's D statistics and were excluded (see Study population section). The P values were controlled for false discovery rate (FDR) within metabolite class by using the linear step-up method by Benjamini and Hochberg (30). Covariates were the same as for model 3 described above. Further analyses were restricted to metabolites that were significantly associated with total red meat consumption in either men or women and showed at least a trend ($P < 0.1$) in the same direction in the other.

Mediation criterion 3 (significant associations of red meat–associated biomarkers with diabetes risk) was evaluated in Cox models for all biomarkers that were selected according to the second mediation criterion. Associations of serum metabolite concentrations with the risk of type 2 diabetes have been investigated in a previous study in EPIC-Potsdam (12). Cox models were adjusted as described for models 2 and 3 above, additionally including total red meat consumption as a covariate. Correction for multiple testing (control of FDR) was applied considering the number of biomarkers preselected according to mediation criterion 2. Biomarkers were further evaluated as mediators of the red meat–associated diabetes risk if the association with diabetes risk was significant and equally directed as the association with total red meat consumption.

Attenuation of the red meat–associated diabetes risk by selected biomarkers

Mediation criterion 4 (attenuation of the red meat–associated diabetes risk after adjustment for selected biomarkers) was evaluated by comparing Cox models without and with adjustment for biomarkers. The statistical significance of an attenuation was evaluated with a method introduced by Hoffmann et al. (31), applying a one-sided Wald test (H_0: β-coefficient for red meat from the corresponding reference model $\leq$ β-coefficient for red meat from the biomarker-adjusted model). In a sensitivity analysis, we

separately evaluated the attenuation after biomarker adjustment of the relation between subtypes of exposure (i.e., total red meat and processed meat) and the risk of diabetes.

The proportion of the excess diabetes risk associated with total red meat consumption that was explainable by selected biomarkers was quantitatively estimated based on the difference between the reference HR and the biomarker-adjusted HR relative to respective excess risk [$(HR_{Red\ meat} - HR_{Red\ meat\ adjusted\ for\ biomarkers})/(HR_{Red\ meat} - 1)$] (32). This estimate is a pseudo-proportion because it is not restricted to values between 0 and 1. Note that the term *explainable* refers to a statistical dependency here and does not necessarily implicate causal inference. Especially, complex confounding mechanisms such as not included metabolic variables that were influenced by red meat and associated with both the potential mediator and type 2 diabetes might have influenced the estimates. The explainable proportion and its stability were estimated as median and dispersion from a bootstrapping procedure (1000 bootstrap replicates).

Relation of biomarkers with other diabetes risk markers

We furthermore evaluated cross-sectional associations of the identified mediators with proteinaceous biomarkers of pathophysiologic processes (γ-glutamyltransferase, alanine aminotransferase, and high-sensitivity C-reactive protein) that have been shown to be related to red meat intake as well as diabetes risk in EPIC-Potsdam (7). Analyses were conducted in the

TABLE 1
Baseline characteristics according to tertiles of total red meat consumption stratified by sex, EPIC–Potsdam Study[1]

	Women (n = 1,257)			Men (n = 790)		
Characteristic	T1 (n = 419)	T2 (n = 419)	T3 (n = 419)	T1 (n = 263)	T2 (n = 264)	T3 (n = 263)
Meat intake, g/MJ						
Total meat	6.3 (0.6, 8.3)[2]	10.6 (8.7, 12.4)	16.1 (13.0, 23.9)	7.8 (3.1, 9.7)	12.2 (10.3, 13.9)	17.3 (14.6, 25.6)
Unprocessed meat	2.4 (0.2, 5.2)	4.3 (1.8, 7.6)	6.6 (2.9, 13.3)	3.3 (0.9, 5.5)	5.1 (2.1, 8.4)	6.8 (2.3, 13.2)
Processed meat	3.1 (0.2, 6.0)	6.1 (3.2, 9.0)	9.1 (4.7, 17.2)	4.1 (1.2, 6.8)	6.7 (3.7, 10.4)	10.8 (5.4, 19.6)
Age at baseline, y	47.7 ± 9.5[3]	49.2 ± 9.2	47.4 ± 8.9	52.2 ± 7.9	51.5 ± 8.4	49.9 ± 7.3
BMI, kg/m^2	25.1 ± 4.6	25.5 ± 4.2	25.9 ± 4.6	26.3 ± 3.2	26.4 ± 3.3	27.0 ± 3.4
Sports, h/wk	1.3 ± 1.9	0.9 ± 1.5	0.8 ± 1.4	1.1 ± 2.1	1.0 ± 1.7	1.0 ± 1.8
Biking, h/wk	2.2 ± 3.2	2.0 ± 2.9	1.5 ± 2.6	2.1 ± 2.9	1.6 ± 2.5	1.5 ± 2.4
Current smokers, %	15	14	22	22	25	29
Higher education,[4] %	65	57	58	68	73	64
HT-medic (yes), %	15	16	15	20	15	17
HL-medic (yes), %	3	2	4	3	5	6
Energy, MJ/d	7369 ± 1845	7428 ± 1664	7455 ± 1709	10,539 ± 2651	10,574 ± 2386	10,911 ± 2703
Beverages, g/d						
Alcohol	8.4 ± 10.9	8.1 ± 9.7	8.8 ± 9.6	22.2 ± 26.7	23.3 ± 20.5	25.5 ± 21.2
Coffee	360.5 ± 287.7	409.3 ± 268.2	442.1 ± 308.3	426.1 ± 354.0	459.2 ± 362.6	479.4 ± 358.8
Soft drinks	24.4 ± 98.7	27.5 ± 84.2	43.5 ± 167.2	80.1 ± 180.0	71.7 ± 175.9	64.9 ± 134.1
Food intake, g/MJ						
Whole-grain bread	8.8 ± 8.1	6.4 ± 6.7	5.1 ± 5.7	5.2 ± 6.7	4.0 ± 5.4	3.1 ± 4.5
Refined-grain bread	11.7 ± 8.2	14.4 ± 8.0	15.6 ± 8.1	15.6 ± 8.5	15.3 ± 7.6	15.8 ± 7.1
Butter	1.1 ± 1.5	1.1 ± 1.5	1.1 ± 1.4	1.0 ± 1.4	1.0 ± 1.3	1.0 ± 1.3
Margarine	1.7 ± 1.7	1.8 ± 1.7	2.0 ± 1.7	1.5 ± 1.4	1.6 ± 1.4	1.6 ± 1.4
Cabbage	1.9 ± 1.9	1.9 ± 1.7	2.3 ± 2.2	1.2 ± 1.2	1.4 ± 1.3	1.5 ± 1.5
Cooked vegetables	4.2 ± 3.1	3.8 ± 2.2	4.4 ± 2.6	2.4 ± 1.6	2.8 ± 1.7	2.9 ± 2.1
Mushrooms	0.3 ± 0.3	0.3 ± 0.3	0.3 ± 0.4	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2
Potatoes	8.9 ± 6.3	10.4 ± 6.2	11.7 ± 6.0	8.3 ± 4.9	9.5 ± 4.9	10.3 ± 5.9
Sauce	1.3 ± 1.5	1.4 ± 1.2	1.9 ± 1.5	1.0 ± 0.8	1.3 ± 1.1	1.6 ± 1.2
Poultry	1.2 ± 1.3	1.5 ± 1.3	1.9 ± 1.5	1.2 ± 1.1	1.5 ± 1.5	1.7 ± 1.5
Nutrient intake						
Carbohydrates, g/MJ	26.9 ± 3.7	26.0 ± 2.9	24.3 ± 3.0	24.3 ± 3.4	22.7 ± 3.1	20.7 ± 2.8
Monosaccharides, g/MJ	5.5 ± 2.0	5.4 ± 1.7	4.8 ± 1.9	4.2 ± 2.1	3.9 ± 2.0	3.2 ± 1.6
Disaccharides, g/MJ	9.3 ± 2.7	8.5 ± 2.1	7.5 ± 1.9	7.2 ± 2.6	6.4 ± 2.3	5.3 ± 2.2
Protein, g/MJ	7.8 ± 1.1	8.1 ± 1.0	8.7 ± 1.0	7.5 ± 1.1	8.1 ± 1.0	8.9 ± 1.1
Fat, g/MJ	9.6 ± 1.4	10.0 ± 1.2	10.6 ± 1.3	10.2 ± 1.9	10.8 ± 1.5	11.5 ± 1.5
Cholesterol, g/MJ	0.031 ± 0.01	0.035 ± 0.01	0.040 ± 0.01	0.032 ± 0.01	0.035 ± 0.01	0.038 ± 0.01
SFA, g/MJ	4.03 ± 0.87	4.20 ± 0.78	4.37 ± 0.76	4.29 ± 1.06	4.44 ± 0.94	4.62 ± 0.83
MUFA, g/MJ	3.17 ± 0.48	3.37 ± 0.41	3.64 ± 0.45	3.38 ± 0.62	3.66 ± 0.49	4.03 ± 0.54
PUFA, g/MJ	1.65 ± 0.61	1.74 ± 0.57	1.85 ± 0.56	1.85 ± 0.71	1.98 ± 0.62	2.11 ± 0.67
Iron, mg/MJ	1.55 ± 0.21	1.57 ± 0.18	1.62 ± 0.19	1.32 ± 0.23	1.35 ± 0.20	1.44 ± 0.25

[1]Baseline characteristics of the subcohort (n = 2047). EPIC, European Prospective Investigation into Cancer and Nutrition; HL-medic, medication due to dyslipidemia; HT-medic, medication due to hypertension; T, tertile.
[2]Median; 5th, 95th percentiles in parentheses (all such values).
[3]Mean ± SD (all such values).
[4]Professional school, a college of higher education, or a university.

subcohort by using multivariate linear regression models. All metabolites identified as potential mediators were mutually included in the same model as explanatory variables for the plasma concentration of each proteinaceous biomarker. The models were adjusted as described above for model 3, including total red meat consumption as an additional covariate.

All analyses were conducted with SAS version 9.4 (SAS Institute). In all statistical tests, an α level below 0.05 was considered statistically significant and an α level below 0.1 borderline significant. If multiple tests were conducted, FDR-corrected P values were used to judge significance.

RESULTS

Study population and baseline characteristics

Baseline characteristics of the EPIC-Potsdam subcohort according to categories (tertiles) of total red meat consumption and stratified by sex are summarized in **Table 1**. Briefly, in both sexes, persons with high total red meat consumption compared with those with low total red meat consumption tended to have a higher BMI, a lower level of sportive activity, and a lower level of formal education and were more likely to be smokers and to use lipid-lowering drugs. Participants with higher total red meat consumption tended to consume less whole-grain bread and more alcohol, coffee, margarine, refined-grain bread, cabbage, potatoes, sauce, and poultry compared with persons with lower total red meat consumption. Men with higher total red meat consumption were younger and less likely to use antihypertensive drugs. Consumption of soft drinks was higher in women while being lower in men with higher red meat consumption. Energy standardized total intake of protein and fat was higher, whereas intake of carbohydrates was lower among participants with higher total red meat consumption. Also, the dietary intake of specific sugars (mono- and disaccharides) was lower, but it was higher for different FA types (i.e., SFAs, MUFAs, and PUFAs) and cholesterol among participants with higher total red meat consumption. Furthermore, dietary iron intake was higher in participants with high total red meat consumption.

Biomarker selection

The cross-sectional associations between total red meat consumption and each of the 127 biomarkers were analyzed, stratified by sex. Among women, the number of P values found to be significant after correction for FDR within metabolite class was 32 (one of 14 amino acids, zero of 17 acylcarnitines, 10 of 34 diacyl phosphatidylcholines, 12 of 37 acyl-alkyl phosphatidylcholines, 4 of 10 lysophosphatidylcholines, and 5 of 14 sphingomyelins). Among men, the number of P values found to be significant after correction for FDR within metabolite class was 26 (zero of 14 amino acids, 2 of 17 acylcarnitines, 4 of 34 diacyl phosphatidylcholines, 11 of 37 acyl-alkyl phosphatidylcholines, 2 of 10 lysophosphatidylcholines, and 7 of 14 sphingomyelins). In addition, ferritin was significantly associated with total red meat consumption in both sexes (see **Supplemental Tables 1–6** for a detailed listing of β-coefficients and raw and FDR-corrected P values and **Supplemental Figures 2–7** for P value plots of all associations between total red meat consumption and single serum metabolite concentrations). For 21 of 127 biomarkers (glycine, 4 diacyl phosphatidylcholines, 11 acyl-alkyl phosphatidylcholines, 2 lysophosphatidylcholines, 2 sphingomyelins, and ferritin), the criterion of a significant association with total red meat consumption in either men or women and at least a trend ($P < 0.1$) in the same direction in the other after controlling for FDR was fulfilled (**Table 2**). Of these 21 biomarkers, 13 were significantly associated with diabetes risk after controlling for FDR (**Supplemental Table 7**). For 6 of these 13 biomarkers—namely, ferritin, glycine, diacyl phosphatidylcholines 36:4 and 38:4, lysophosphatidylcholine 17:0, and hydroxysphingomyelin 14:1—the associations with total red meat consumption and with diabetes risk were consistent with the mediation hypothesis (Table 2 and **Table 3**); that is, both associations were in the same direction. These 6 biomarkers were further investigated as potential mediators.

In a sensitivity analysis, we evaluated the impact of different selection strategies and P value adjustments on selecting the 6

TABLE 2
Association with total red meat intake for biomarkers that fulfilled mediation criterion 2[1]

	Women (n = 1257)			Men (n = 790)		
Biomarker	β	P_{raw}	P_{FDR}	β	P_{raw}	P_{FDR}
Glycine	−0.098	0.001	0.019	−0.099	0.009	0.067
Diacyl PC 36:0	0.124	<0.001	<0.001	0.140	<0.001	0.004
Diacyl PC 36:4	0.102	0.001	0.003	0.124	0.001	0.007
Diacyl PC 38:0	0.148	<0.001	<0.001	0.152	<0.001	0.002
Diacyl PC 38:4	0.108	<0.001	0.001	0.120	0.001	0.008
Acyl-alkyl PC 34:0	−0.097	0.001	0.002	−0.073	0.037	0.091
Acyl-alkyl PC 34:2	0.095	0.001	0.005	0.078	0.036	0.091
Acyl-alkyl PC 34:3	0.066	0.023	0.064	0.106	0.004	0.014
Acyl-alkyl PC 36:1	−0.099	<0.001	0.002	−0.079	0.026	0.080
Acyl-alkyl PC 36:3	0.127	<0.001	<0.001	0.137	<0.001	0.002
Acyl-alkyl PC 36:4	0.235	<0.001	<0.001	0.205	<0.001	<0.001
Acyl-alkyl PC 36:5	0.183	<0.001	<0.001	0.225	<0.001	<0.001
Acyl-alkyl PC 38:4	0.129	<0.001	<0.001	0.146	<0.001	<0.001
Acyl-alkyl PC 38:5	0.211	<0.001	<0.001	0.219	<0.001	<0.001
Acyl-alkyl PC 38:6	0.155	<0.001	<0.001	0.156	<0.001	<0.001
Acyl-alkyl PC 40:4	0.058	0.048	0.098	0.119	0.002	0.006
Lyso-PC 17:0	−0.057	0.043	0.087	−0.098	0.004	0.019
Lyso-PC 20:4	0.109	<0.001	0.002	0.115	0.002	0.017
Hydroxy-SM 14:1	−0.121	<0.001	<0.001	−0.069	0.053	0.092
SM 24:1	0.075	0.014	0.040	0.203	<0.001	<0.001
Ferritin	0.137	<0.001	<0.001	0.113	0.002	0.002

[1]Biomarkers were preselected based on the criterion of a significant ($P_{FDR} < 0.05$) association with total red meat consumption in either men or women and at least a similar trend ($P_{FDR} < 0.1$) in the other. Standardized β-coefficients (β) for a linear association of total red meat consumption with serum metabolite concentration in the subcohort (n = 2047). Regression models were adjusted for total energy intake (MJ/d), age (years), BMI (in kg/m^2), sports (h/wk), biking (h/wk), smoking (4 stages: never smoker, former smoker, current smoker <20 units/d, or current heavy smoker >20 units/d), education (4 stages: no vocational training or in training, vocational training, technical school, or technical college or university degree), antihypertensive medication (yes/no), antidyslipidemic medication (yes/no), intake of beverages (alcohol, coffee, sugar-sweetened beverages) (g/d), and intake of whole-grain bread, refined-grain bread, butter, margarine, cabbage, cooked vegetables, mushrooms, potatoes, sauce, and poultry (g/MJ). Raw P values and FDR-controlled P values (corrected within metabolite classes) from a 2-sided t test (H_0: $\beta = 0$). FDR, false discovery rate; PC, phosphatidylcholine; SM, sphingomyelin.

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TABLE 3
Association with type 2 diabetes risk for biomarkers that fulfilled mediation criteria 2 and 3[1]

Selected biomarker	HR (95% CI)[2]	p_{raw}	p_{FDR}
Glycine	0.66 (0.57, 0.77)	<0.001	<0.001
Diacyl PC 36:4	1.20 (1.07, 1.35)	0.002	0.003
Diacyl PC 38:4	1.24 (1.12, 1.38)	<0.001	<0.001
Lyso-PC 17:0	0.78 (0.68, 0.89)	<0.001	<0.001
Hydroxy-SM 14:1	0.83 (0.73, 0.94)	0.004	0.007
Ferritin	1.28 (1.15, 1.42)	<0.001	<0.001

[1]Biomarkers were selected based on the mediation criteria 2 and 3—that is, a significant ($P_{FDR} < 0.05$) association with total red meat consumption in either men or women and at least a similar trend ($P_{FDR} < 0.1$) in the other (criterion 2) and equally directed associations with type 2 diabetes risk (criterion 3). Raw *P* values and FDR-controlled *P* values (corrected for the 21 tests conducted among all metabolites that fulfilled mediation criterion 2) from a 2-sided Wald-test (H_0: $\beta = 0$). FDR, false discovery rate; PC, phosphatidylcholine; SM, sphingomyelin.

[2]Diabetes-HR per SD in serum concentration; the associations of 21 preselected metabolites with type 2 diabetes risk were evaluated in Cox models in the case cohort ($n = 2681$) adjusted for total red meat intake, total energy intake (MJ/d), age (years), sex, BMI (in kg/m^2), sports (h/wk), biking (h/wk), smoking (4 stages: never smoker, former smoker, current smoker <20 units/d, or current heavy smoker >20 units/d), education (4 stages: no vocational training or in training, vocational training, technical school, or technical college or university degree), antihypertensive medication (yes/no), antidyslipidemic medication (yes/no), intake of beverages (alcohol, coffee, sugar-sweetened beverages) (g/d), and intake of whole-grain bread, refined-grain bread, butter, margarine, cabbage, cooked vegetables, mushrooms, potatoes, sauce, and poultry (g/MJ).

above-defined potential mediators. Pooling men and women and selecting biomarkers based on a significant *P* value after controlling for FDR over all 127 biomarkers would have led to the selection of all 6 biomarkers; using Bonferroni correction over all biomarkers in the pooled analysis would have led to the selection of all metabolites but lysophosphatidylcholine 17:0 ($P = 0.198$); keeping the sex-stratified analysis and controlling FDR over all metabolites (instead of control within metabolite class) would have resulted in the selection of all metabolites but lysophosphatidylcholine 17:0 (men: $P = 0.138$; women: $P = 0.024$) and hydroxy-sphingomyelin 14:1 (men: $P < 0.001$; women: $P = 0.159$). Thus, results were robust to the choice of selection strategy and *P* value adjustment method for glycine, ferritin, and phosphatidylcholines 36:4 and 38:4 and were somewhat sensitive to that choice for lysophosphatidylcholine 17:0 and hydroxy-sphingomyelin 14:1.

Attenuation of the red meat–associated diabetes risk by selected biomarkers

A higher total red meat consumption was related to a higher risk of developing type 2 diabetes [HR for 2 SD (11 g/MJ): 1.47; 95% CI: 1.24, 1.75] in the minimally adjusted model 1 (**Table 4**). Additional adjustment for lifestyle, medication, and diet (model 2) attenuated the relation (HR: 1.33; 95% CI: 1.09, 1.62). This association was further attenuated after also adjusting for BMI and again remained significant (HR for 2 SD: 1.26; 95% CI: 1.01, 1.57). In Table 4, results are also presented from multiple Cox models constructed to assess to what extent the association between total red meat consumption and diabetes risk was attenuated after adjustment for selected biomarkers. Considering the selected biomarkers individually, the association of total red meat consumption with diabetes risk was significantly attenuated after adjustment for diacyl phosphatidylcholines 36:4 and 38:4, lysophosphatidylcholine 17:0, glycine, and ferritin, and it was borderline significantly attenuated after adjustment for hydroxy-sphingomyelin 14:1 ($P < 0.1$) when using model 2 as a reference model. When using the BMI-adjusted model 3 as reference, the attenuation was significant after adjustment for diacyl phosphatidylcholine 36:4, hydroxy-sphingomyelin 14:1, and glycine and borderline significant after adjustment for diacyl phosphatidylcholine 38:4 ($P = 0.05$) and ferritin ($P = 0.06$). Diacyl phosphatidylcholines 36:4 and 38:4 were highly correlated, and adjustment for diacyl phosphatidylcholine 36:4 did not further attenuate the association between total red meat consumption and type 2 diabetes risk if diacyl phosphatidylcholine 38:4 was also included in the same model (data not shown). Therefore, mutual biomarker-adjusted models did not include diacyl phosphatidylcholine 36:4. After mutually including the 5 other selected biomarkers as covariates, the HR (per 2 SD) of total red meat consumption was significantly attenuated from 1.33 (95% CI: 1.09, 1.62) to 1.04 (0.83, 1.30) for the non–BMI-adjusted model 2 and from 1.26 (95% CI: 1.01, 1.57) to 1.09 (0.86, 1.38) for the BMI-adjusted model 3.

The proportion of red meat–related diabetes risk explainable by selected biomarkers (median from 1000 bootstrap replicates) is shown in **Figure 1**. If the non–BMI-adjusted model 2 was defined as reference, the red meat–associated diabetes risk was attenuated by 89% (IQR: 69%, 121%) after simultaneous adjustment for the selected biomarkers (Figure 1A). If the BMI-adjusted model 3 was defined as reference, the red meat–associated diabetes risk was attenuated by 69% (IQR: 49%, 106%) after simultaneous adjustment for selected biomarkers (Figure 1B). Consistent with above-described models, diacyl phosphatidylcholine 36:4 was not contained in mutual biomarker-adjusted models. The explainable proportion ranged between 8% and 33% for single biomarker adjustments.

Our sensitivity analysis evaluating different subtypes of exposure revealed that the attenuation of the associated diabetes risk after mutual adjustment for all selected biomarkers was also significant if only unprocessed red meat ($P = 0.007$) or processed meat ($P = 0.002$) consumption was considered the exposure variable (reference: model 3). In the fully adjusted model 3, the proportion of associated diabetes risk explainable by simultaneous biomarker adjustment (median and IQR from 1000 bootstrap replicates) was 49% (IQR: 29%, 89%) for unprocessed red meat consumption, whereas it was 71% (IQR: 45%, 115%) for processed meat consumption (**Supplemental Figure 8**).

Relation of selected biomarkers with other diabetes risk markers

Cross-sectional associations between the selected biomarkers and plasma concentrations of C-reactive protein, γ-glutamyltransferase, and alanine aminotransferase are presented in **Table 5**. Directions of the associations were largely accordant with the direction of the relations of the selected mediators with the risk of type 2 diabetes.

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TABLE 4
Association of total red meat consumption with type 2 diabetes risk in mediator-adjusted vs. non–mediator-adjusted models[1]

	β-coefficient[2]	P value[3] for attenuation of β	HR (95% CI)[4]
Model 1	0.393		1.47 (1.24, 1.75)
Model 2	0.282	(referent)	1.33 (1.09, 1.62)
Model 2 + diacyl PC 36:4	0.229	0.002	1.26 (1.03, 1.54)
Model 2 + diacyl PC 38:4	0.210	0.002	1.23 (1.01, 1.52)
Model 2 + lyso-PC 17:0	0.229	0.015	1.26 (1.02, 1.55)
Model 2 + hydroxy-SM 14:1	0.265	0.099	1.30 (1.07, 1.59)
Model 2 + selected lipid-mediators[5]	0.142	<0.001	1.15 (0.93, 1.43)
Model 2 + ferritin	0.193	0.003	1.21 (0.98, 1.50)
Model 2 + glycine	0.197	0.002	1.22 (0.99, 1.50)
Model 2 + selected mediators[6]	0.038	<0.001	1.04 (0.83, 1.30)
Model 3 (model 2 + BMI)	0.228	(referent)	1.26 (1.01, 1.57)
Model 3 + diacyl PC 36:4	0.194	0.018	1.21 (0.97, 1.52)
Model 3 + diacyl PC 38:4	0.199	0.053	1.22 (0.97, 1.53)
Model 3 + lyso-PC 17:0	0.212	0.166	1.24 (0.99, 1.55)
Model 3 + hydroxy-SM 14:1	0.202	0.035	1.22 (0.98, 1.53)
Model 3 + selected lipid mediators[5]	0.163	0.011	1.18 (0.94, 1.48)
Model 3 + ferritin	0.189	0.057	1.21 (0.96, 1.52)
Model 3 + glycine	0.164	0.003	1.18 (0.94, 1.48)
Model 3 + selected mediators[6]	0.086	<0.001	1.09 (0.86, 1.38)

[1]An attenuation of the β-coefficient and the corresponding HR after adjustment for a biomarker indicates that this biomarker plays a role as mediator. Cox models were used to estimate the association between total red meat consumption and risk of type 2 diabetes in the case cohort (n = 2681). Model 1 was adjusted for age (years), sex, and total energy intake (MJ/d). Model 2 was also adjusted for sports (h/wk), biking (h/wk), smoking (4 stages: never smoker, former smoker, current smoker <20 units/d, or current heavy smoker >20 units/d), education (4 stages: no vocational training or in training, vocational training, technical school, or technical college or university degree), antihypertensive medication (yes/no), antidyslipidemic medication (yes/no), intake of beverages (alcohol, coffee, sugar-sweetened beverages) (g/d), and intake of whole-grain bread, refined-grain bread, butter, margarine, cabbage, cooked vegetables, mushrooms, potatoes, sauce, and poultry (g/MJ). Model 3 was also adjusted for BMI (in kg/m^2). PC, phosphatidylcholine; SM, sphingomyelin.

[2]β-Coefficient for 2 SD (11 g/MJ) of total red meat consumption.

[3]P value for the attenuation of β-coefficients from the biomarker-adjusted compared with the corresponding reference model [one-sided Wald-test (H_0: $\beta_{\text{mediator adjusted}} < \beta_{\text{non–mediator adjusted}}$)].

[4]HR for 2 SD (11 g/MJ) of total red meat consumption.

[5]Selected lipid mediators: diacyl phosphatidylcholine 38:4, lysophosphatidylcholine 17:0, and hydroxy-sphingomyelin C14:1.

[6]Selected mediators: lipid mediators, ferritin, and glycine.

DISCUSSION

In this prospective study of middle-aged men and women, total red meat consumption was related to an elevated risk of type 2 diabetes. Of 127 investigated biomarkers comprising lipids, amino acids, and ferritin, 33 among women and 27 among men were associated with total red meat consumption, with 21 showing similar associations in both sexes. Of these 21 biomarkers, 6 were equally associated with type 2 diabetes risk. Mutual adjustment for selected biomarkers revealed that the red meat–associated diabetes risk was largely dependent on the serum concentrations of ferritin, a small set of glycerophospholipids, and glycine.

Regarding lipid profiles, we found strong sex-consistent associations with total red meat consumption for 19 lipid metabolites from 4 classes. The detected enrichment of lipid metabolites that contained 16 or more carbon atoms in their FA residues in which one FA was contained (lysophosphatidylcholines and sphingomyelins) or 34 or more carbon atoms in which 2 FAs were contained (diacyl phosphatidylcholines and acyl-alkyl phosphatidylcholines) is plausibly related to the high proportion of even-numbered long- and very long-chained FA contained in red meats (33). Circulating lipid metabolites reflect synthesis activity of lipid classes as well as availability and metabolism of specific FAs in the liver. In the literature, several lines of experimental evidence relate lipid metabolism to the pathogenesis of type 2 diabetes, among them a challenged mitochondrial metabolism, altered cellular signaling processes at the membrane, and modulation of gene expression (34, 35). Consistently, endogenous FA composition in several lipid compartments has been related to the risk of type 2 diabetes in several prospective cohorts, including EPIC-Potsdam (36).

Among amino acids, glycine fulfilled predefined selection criteria. Several studies found an inverse relation between red meat intake and the glycine concentration in biofluids (37, 38). In a randomized controlled feeding trial, glycine concentrations were slightly lower in response to a meat protein–based diet compared with a plant protein–based diet, whereas the amount of glycine provided by the meat diet was about 50% higher (39). Hence, available evidence suggests elevated glycine utilization in response to red meat intake. Glycine is related to insulin resistance and oxidative stress by its essential role in gluconeogenesis and the formation of glutathione (40). An independent inverse association of glycine with diabetes risk was previously

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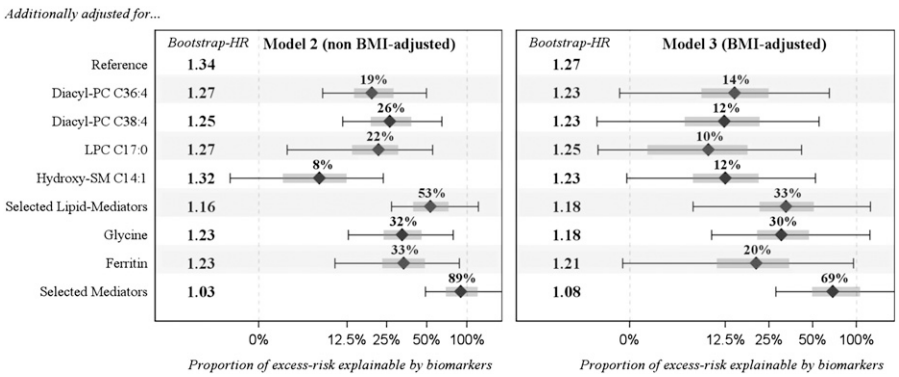

FIGURE 1 *Bootstrap-HR* is the median of diabetes-HR for 2 SD (11 g/MJ) of total red meat consumption from 1000 bootstrapping repetitions. Left panel: adjusted for age, sex, diet, and lifestyle. Right panel, additionally adjusted for BMI. *Proportion of excess risk explainable by biomarkers* was estimated as the difference of non–mediator-adjusted and mediator-adjusted HR divided by red meat–related excess diabetes risk; displayed are the median percentage, the IQR (gray box), and the 5th and 95th percentiles (edges of the line). Selected lipid mediators: diacyl phosphatidylcholine 38:4, lysophosphatidylcholine 17:0, and hydroxysphingomyelin 14:1. Selected mediators: lipid mediators, ferritin, and glycine. LPC, lysophosphatidylcholine; PC, phosphatidylcholine; SM, sphingomyelin.

described by us and others (12, 41). A Mendelian randomization study, however, did not provide evidence for a causal role of glycine in diabetes-related traits (42).

We observed a direct association of total red meat consumption with plasma ferritin levels. Heme iron intake from red meats was shown to induce higher ferritin levels in feeding trials (43). Interestingly, heme biosynthesis, which is triggered by high iron availability, is an important glycine-using pathway, and high expression of heme-synthesizing enzymes was associated with low circulating glycine concentrations in humans (41). Consistent with other studies (44), elevated iron status as assessed by ferritin was directly related to the risk of diabetes in EPIC-Potsdam (13). Underlying mechanisms seem to be manifold and likely include oxidative stress and modulation of intracellular signaling cascades (44).

The marked attenuation of red meat–associated diabetes risk after adjustment for selected biomarkers suggests that these biomarkers reflect metabolic processes that link habitual red meat consumption to diabetes risk. Given that an observational study is generally prone to confounding and other sources of bias, interpretation of our findings with regard to biological paths is rather hypothesis generating than able to proof their causal nature (45). Thus, our results generate and underline experimentally testable hypotheses that can inform future dietary interventions in terms of design and biomarker assessment (46).

Total red meat as defined in our study is a large and heterogeneous food group. Still, an elevated diabetes risk was consistently observed for both unprocessed and processed red meat (1–3). Thus, we focused on the investigation of metabolic traits that were related to red meat consumption in general. Our sensitivity analyses in subcategories of exposure (unprocessed and processed meat, respectively) suggest that the mutual set of selected biomarkers is relevant across subtypes of red meat. Still, identification of meat type–specific effects is of particular interest. For example, advanced glycation end products, *trans* FA, nitrites and nitrates, and methylamines are discussed as meat type–specific mediators (1). Here, metabolomic data might help to identify meat type–specific mechanisms, which could possibly account for differences in the associated diabetes risk between types of red meat.

Several valid alternatives to our regression-based approach to model metabolomic data exist (e.g., correlation networks and data reduction methods). By considering the potential biomarkers individually, we were able to correct the predefined significance

TABLE 5
Association of selected mediators with CRP, GGT, and ALT[1]

	CRP		GGT		ALT	
Selected mediator	Standardized β-coefficient (95% CI)	FDR-controlled P value	Standardized β-coefficient (95% CI)	FDR-controlled P value	Standardized β-coefficient (95% CI)	FDR-controlled P value
Ferritin	0.020 (−0.027, 0.066)	0.404	0.127 (0.084, 0.171)	<0.001	0.165 (0.121, 0.208)	<0.001
Diacyl PC 38:4	0.038 (−0.003, 0.08)	0.069	0.127 (0.088, 0.166)	<0.001	0.106 (0.067, 0.145)	<0.001
Glycine	−0.150 (−0.191, −0.110)	<0.001	−0.033 (−0.071, 0.005)	0.140	−0.012 (−0.051, 0.026)	0.529
Lyso-PC 17:0	−0.246 (−0.294, −0.199)	<0.001	−0.069 (−0.114, −0.023)	0.005	−0.063 (−0.108, −0.018)	0.007
Hydroxy-SM 14:1	0.125 (0.075, 0.174)	<0.001	0.009 (−0.038, 0.056)	0.697	0.044 (−0.003, 0.091)	0.098

[1]Associations of selected mediators (explanatory variable) with CRP, GGT, and ALT (outcome) were estimated based on the subcohort (n = 2047). Linear regression models were used, adjusted for other mediators, total red meat consumption, and total energy intake (MJ/d), age (y), sex, BMI (in kg/m^2), sports (h/wk), biking (h/wk), smoking (4 stages: never smoker, former smoker, current smoker <20 units/d, or current heavy smoker >20 units/d), education (4 stages: no vocational training or in training, vocational training, technical school, or technical college or university degree), antihypertensive medication (yes/no), antidyslipidemic medication (yes/no), intake of beverages (alcohol, coffee, and sugar-sweetened beverages) (g/d), and intake of whole-grain bread, refined-grain bread, butter, margarine, cabbage, cooked vegetables, mushrooms, potatoes, sauce, and poultry (g/MJ). FDR-controlled P values were from a 2-sided t test (H_0: $\beta = 0$). ALT, alanine transaminase; CRP, C-reactive protein; FDR, false discovery rate; GGT, γ-glutamyl transferase; PC, phosphatidylcholine; SM, sphingomyelin.

thresholds for multiplicity at each stage of the analysis and to compare significant findings across sexes. This should have reduced the likelihood of false discoveries. Furthermore, we evaluated the robustness of our results in a bootstrapping procedure. Still, the a priori design of our search strategy included arbitrary decisions, and *P* values lose their straightforward interpretability with regard to the probability of type I errors in a step-by-step selection procedure such as ours (47). Our sensitivity analysis applying different *P* value adjustments and differently defined selection criteria showed that selection was very robust for most potential mediators but was somewhat sensitive to these choices for lysophosphatidylcholine 17:0 and hydroxy-sphingomyelin 14:1. For these 2 metabolites, therefore, a nonneglectable probability of type I error might have remained even after *P* value adjustment. External validation of our results is warranted and should be facilitated by our decision to consider biomarkers individually.

To our knowledge, this is the first study evaluating a large set of metabolites as potential mediators of the association between red meat intake and diabetes risk, including several lipid metabolites, amino acids, and ferritin. The prospective design of our study should have minimized various sources of bias (selection bias, reverse causation), and we controlled for a large set of potentially confounding factors in our analyses, including lifestyle, medication, dietary factors, and BMI. Because of the observational nature of our study, however, the possibility of residual confounding cannot be ruled out. Further limitations of our study refer to the data reliability. Dietary information relied on estimates of habitual consumption over the past year by FFQs, and metabolites were measured at a single time point. However, FFQs showed good reliability and relative validity to assess meat intake, and investigated metabolites showed good reliability in validation studies (23). Still, random measurement error may have masked some associations. Albeit large, the set of targeted metabolites was not exhaustive with regard to potential mediating mechanisms. Furthermore, acylcarnitines have previously been linked to red meat intake (38). This effect, however, may reflect mitochondrial metabolism under acute dietary challenge (48) rather than effects of long-term habitual diet as assessed in our study. Furthermore, estrogen is a modulator of mitochondrial function, and associations of red meat consumption with acylcarnitines might be sex specific. In this study, however, we only selected biomarkers that were associated with red meat in both sexes to avoid spurious findings.

In addition, there is the possibility that cases remained undiagnosed during follow-up. However, this misclassification should not bias the associations between red meat consumption and diabetes given that false-positive case definitions should have been rare due to the strict verification procedures in EPIC-Potsdam (49).

In conclusion, high ferritin, low glycine, and altered hepatic-derived lipids in the circulation were associated with both total red meat consumption and diabetes risk. The large attenuation of the red meat–associated diabetes risk after adjustment for these potential mediators is consistent with the hypothesis that metabolic processes reflected in the circulating concentrations of these biomarkers take part in linking red meat consumption to type 2 diabetes incidence. Our results, however, cannot prove causality of the observed associations and, where possible, the suggested single relations should inform the design and the biomarker assessment of interventional studies.

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