



## Original Research Article

# Fasting plasma metabolites reflecting meat consumption and their associations with incident type 2 diabetes in two Swedish cohorts

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## ABSTRACT

**Background:** Consumption of processed red meat has been associated with increased risk of developing type 2 diabetes (T2D), but challenges in dietary assessment call for objective intake biomarkers.

**Objectives:** This study aimed to investigate metabolite biomarkers of meat intake and their associations with T2D risk.

**Methods:** Fasting plasma samples were collected from a case–control study nested within Västerbotten Intervention Program (VIP) (214 females and 189 males) who developed T2D after a median follow-up of 7 years. Panels of biomarker candidates reflecting the consumption of total, processed, and unprocessed red meat and poultry were selected from the untargeted metabolomics data collected on the controls. Observed associations were then replicated in Swedish Mammography clinical subcohort in Uppsala (SMCC) ( $n = 4457$  females). Replicated metabolites were assessed for potential association with T2D risk using multivariable conditional logistic regression in the discovery and Cox regression in the replication cohorts.

**Results:** In total, 15 metabolites were associated with  $\geq 1$  meat group in both cohorts. Acylcarnitines 8:1, 8:2, 10:3, reflecting higher processed meat intake [ $r > 0.22$ , false discovery rate (FDR)  $< 0.001$  for VIP and  $r > 0.05$ ; FDR  $< 0.001$  for SMCC) were consistently associated with higher T2D risk in both data sets. Conversely, lysophosphatidylcholine 17:1 and phosphatidylcholine (PC) 15:0/18:2 were associated with lower processed meat intake ( $r < -0.12$ ; FDR  $< 0.023$ , for VIP and  $r < -0.05$ ; FDR  $< 0.001$ , for SMCC) and with lower T2D risk in both data sets, except for PC 15:0/18:2, which was significant only in the VIP cohort. All associations were attenuated after adjustment for BMI ( $\text{kg}/\text{m}^2$ ).

**Conclusions:** Consistent associations of biomarker candidates involved in lipid metabolism between higher processed red meat intake with higher T2D risk and between those reflecting lower intake with the lower risk may suggest a relationship between processed meat intake and higher T2D risk. However, attenuated associations after adjusting for BMI indicates that such a relationship may at least partly be mediated or confounded by BMI.

**Keywords:** red meat, processed meat, diabetes mellitus, metabolomics, biomarkers

## Introduction

The consumption of processed red meat has previously been linked with a higher risk of cardiovascular disease [1] and type 2 diabetes (T2D) [2,3]. Habitual red meat intake has been linked with unfavorable cardiometabolic health risk markers, such as plasma C reactive protein, glucose, insulin, HbA1c, and blood lipids and cholesterol profiles [1,4,5], but with large differences in associations observed [2,3,6,7].

Several mechanisms for how red meat intake could be linked with T2D have been suggested. The heme iron content and advanced glycation end products induced by high-temperature cooking have been suggested to form reactive oxygen species, especially in individuals with disrupted regulation of iron absorption [8]. The reactive oxygen species have been suggested to disrupt metabolic balance and increase oxidative stress, which triggers proinflammatory responses and cell and organ damage [8]. Furthermore, nitrosamine from nitrite and nitrate used in cured meat may cause DNA damage and pancreatic  $\beta$  cells

**Abbreviations:** ESI, electrospray ionization; FDR, false discovery rate; FFQ, food frequency questionnaire; HILIC, hydrophilic interaction chromatography; LC-MS, liquid chromatography coupled with mass spectrometry; RP, reversed-phase; SMCC, Clinical subcohort of Swedish Mammography Cohort; TMAO, trimethylamine-N-oxide; T2D, type 2 diabetes; VIP, Västerbotten Intervention Program.

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toxicity, which subsequently lower insulin secretion [9]. Recent studies have also indicated a possible involvement of gut microbiota in the association between red meat and T2D risk. For example, *Alistipes shahii* has been shown to enhance the positive association between consumption of red meat and HbA1c [4]. The microbial metabolite trimethylamine N-oxide (TMAO) has also been associated with a higher risk of T2D [10]. Red meat consumption, in particular, has been associated with a higher concentration of TMAO in patients with T2D [11]. Noteworthy, fish intake could also increase blood TMAO concentrations [12,13], which makes the association between plasma TMAO and higher T2D risk complicated.

Besides interindividual variations in these molecular aspects, inconsistent findings across studies may also be attributable to reasons such as different study populations, locations [6], dietary habits, and T2D risk across sexes. Reported associations between diet and health are typically based on dietary assessments relying on participants' self-report [7,14–16] and are, therefore, prone to systematic and random measurement errors. Therefore, molecular studies oriented toward identification of objective, robust, and easily obtainable measurements that reflect real-life dietary intake with all its complexities [17] may help to complement current dietary measurements, improve accuracy in diet-health assessment [18,19], and identify metabolites on the causal path from exposure/intake toward health and disease.

Despite the rapid developments of food intake biomarkers fueled by advancements in metabolomics, studies on validated biomarkers of meat consumption related to T2D risk are still few [20,21]. Using untargeted plasma metabolomics from the Västerbotten Intervention Program (VIP), we have previously discovered metabolites [22] and several dietary exposures [23–25] related to T2D. In the same data set, in this study, we aimed to discover metabolite intake biomarkers of total, processed, and unprocessed red meat and poultry. We also investigated whether we could replicate the associations between biomarkers of meat consumption reported from the literature with participants' self-reported intake. We then replicated the biomarker candidates in another independent free-living cohort to obtain biomarker candidates with lower risk of false discovery. Finally, we aimed to investigate the associations between the biomarker candidates with risk of developing T2D in both cohorts.

## Methods

### Study populations and diagnoses

#### *Västerbotten Intervention Program*

VIP is a subcohort of a population-based prospective Northern Sweden Health and Disease Study Cohort that started in 1985 [26]. The study protocol for VIP was approved by the Regional Ethics Committee in Uppsala with registration number 2014/011. All participants provided written informed consent prior to participation. The details about the cohort have been reported previously [27,28]. The inhabitants of Västerbotten County were invited at 30 (recruited until 1995), 40, 50, and 60 years of age to a screening of cardiovascular disease risk factors and health counseling about increasing physical activity and diet modification [29]. Dietary recommendations included lowering the intake of total fat, replacing saturated fats with polyunsaturated fats, and encouraging the consumption of vegetables, legumes, fruit, fish, and wholegrains [29]. During the study visit, information about socioeconomic conditions, health-related family history, quality of life, physical activity, dietary habit in the past year

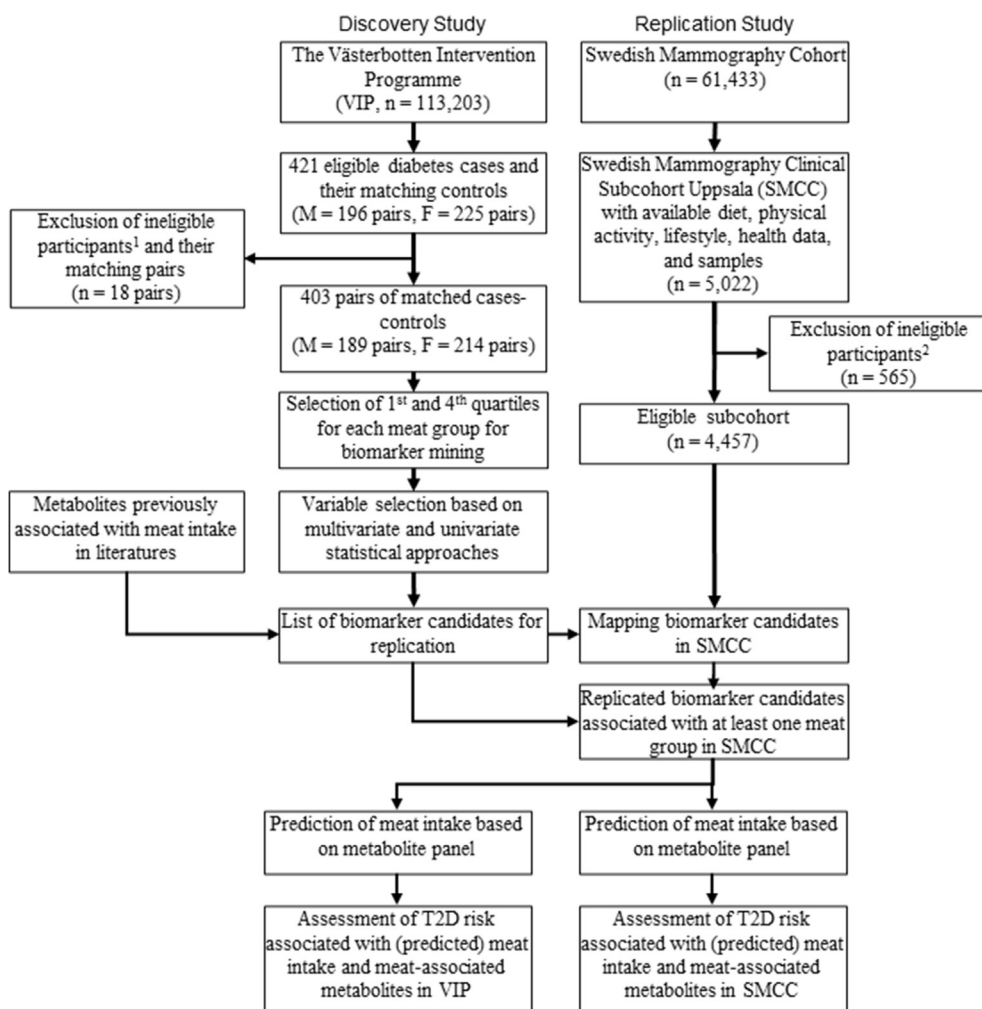
using food frequency questionnaire (FFQ), and other lifestyle habits related to health, in addition to anthropometric measures, were recorded [27–31]. Physical activity was calculated based on a modified [32] measure of Cambridge index of physical activity [33]. During the visit, venous blood samples were drawn after overnight fasting without stasis, followed by centrifugation at 1500g for 15 min [30]. The obtained plasma was aliquoted and stored at  $-80^{\circ}\text{C}$  at the Umeå University medical biobank [26,27].

Information on T2D diagnosis was taken from the Diabetes Register in Northern Sweden (DiabNorth) [34] on the VIP participants. Besides information on pre-existing T2D diagnosis based on record linkage with participants' pharmaceutical registry (usage of diabetic medications), participants were invited for the health examination, including a standardized 2-h oral glucose tolerance test after an overnight fasting. T2D diagnosis was established according to the guidelines from the World Health Organization (WHO) (plasma glucose concentrations  $\geq 7.0$  mmol/L at fasted condition and/or  $\geq 12.2$  mmol/L after oral glucose tolerance test [27]) and confirmed by a diabetes specialist.

Participants with any portion size question or  $>10\%$  of the FFQ questions left unanswered, energy intake estimated as lowest 5% or highest 2.5% in the Northern Sweden FFQ database, or missing height or weight data were excluded [29–31]. Among the remaining participants after such exclusion, 421 individuals (Figure 1) recruited between 1991 and 2005 with T2D diagnosis between the baseline examination and the follow-up (median time of 7 years) [24] and unfrozen fasting plasma samples were randomly selected as prospective T2D cases [22]. For selection of controls, 421 participants were matched individually for age ( $\pm 2$  y), sex, ethnic group, the season of blood collection (sampling date  $\pm 90$  d), version of questionnaire at baseline examination, and sample storage time. Within the matched case-control subset, participants with energy intake outside the 1st and 99th percentiles and their matching pairs were further excluded from this study, making up 403 pairs of matched cases-controls for the discovery study. Untargeted metabolomics analysis was conducted on fasting heparin plasma samples [35] taken during the baseline visit.

#### *Clinical Subcohort of Swedish Mammography Cohort*

Females born between 1914 and 1948 and living in Västmanland county between 1987 and 1989 or living in Uppsala County between 1988 and 1990 were invited to participate in a mammography screening program (Swedish Mammography Cohort [SMC],  $n = 90,303$ ), among whom 61,433 were included in the cohort [36]. Among them, 8311 cohort participants from SMC living in Uppsala aged 55–85 years were randomly selected and invited to take part in the clinical examination in 2003–2009; 5032 of those who participated in the clinical examination were then included in the clinical subcohort (SMCC), among whom samples were available for 5022 participants [37] (Figure 1). During the examination, anthropometric measurements, blood sample collection, and data recording of diet, lifestyle, and physical activity took place. Untargeted metabolomics analysis was conducted on lithium-heparin plasma samples. The cohort is managed by the Swedish Infrastructure for Medical Population-Based Life Course and Environmental Research (SIMPLER, [www.simpler4health.se](http://www.simpler4health.se)). The study protocol for SMCC was approved by the Regional Ethics Committee in Stockholm with registration number 2006/1490-31/1. The biobank and responsibility for SMCC were then moved to Uppsala under registration number 2017/232 and 2018/261. All participants provided written informed consent prior to participation.



**FIGURE 1.** The flowchart of the study design and data analysis for both discovery [Västerbotten Intervention Programme (VIP)] and replication [Swedish Mammography Cohort, Clinical subcohort Uppsala (SMCC)] studies. <sup>1</sup>Reasons for exclusion at VIP: 1st and 99th percentiles food intake level. <sup>2</sup>Reasons for exclusion in SMCC: existing T2D, got diabetes treatment (using insulin homologs, diabetes medication, dietary advice, or combination of those), impaired fasting glucose at baseline (fasting plasma glucose >7 mmol/L), participants with implausible energy intake (<500 or >3500 kcal/d), and participants with missing/incomplete metabolome data. T2D, type 2 diabetes.

Among these participants, individuals with existing T2D diagnosis prior to visiting between 2003 and 2008, as indicated by crosslink with disease registry, self-reported diabetes treatment (insulin homologs, diabetes medication, dietary advice, or their combination), or fasting plasma glucose concentrations of  $\geq 7.0$  mmol/L according to the guidelines from WHO [27] were excluded. Additional exclusions were made based on self-reported energy intake of <500 or >3500 kcal/d [38,39], participants with missing metabolomics or metadata, or missing matched case or control pair. Eventually, data from 4457 participants of SMCC Uppsala were included in the replication study (Supplemental Table 1), consisting of 236 participants who developed T2D and 4221 participants who remained free of T2D by the last follow-up date (December 31, 2021).

## Dietary assessment

### Discovery study in VIPs

Dietary data from the VIP were derived from FFQs capturing habitual intake in the past year [30,31]. Estimates of energy and nutrient intakes were derived by multiplying the number of intakes per

day by the energy or nutrient content from a food composition database from the Swedish Food Agency [40].

Meat intake was calculated based on 9 food items in the FFQ (Table 1). Blood-based food, liver, kidney, and smoked meat/fish were not included. For common meat-containing dishes, meat content was calculated using the national food database provided by the Swedish Food Agency, using, for example, 43% for minced meat dishes, 25% for meat stew, 70% for sausage, and 37% for hamburgers as dishes [41]. Unprocessed red meat comprised minced meat dishes, meat stew, and steak/chop. Processed red meat included sausage, liver pâté and meat on bread, and bacon and sausage as dishes. The consumed amounts of unprocessed and processed red meat intake were then summed up to total red meat intake. White meat (poultry) was assessed separately (Table 1).

### Replication in SMCC.

FFQ used in SMCC contained 15 items regarding meat intake (Table 1). Processed red meat contained bacon, sausages [lean sausage, *falukorv* (Swedish emulsion type) sausage, other sausage, cold cut sausages], cold cut meat, sandwich filling of meat (meat/

**TABLE 1**  
Food items in the FFQ in VIP and SMCC composing each meat group

| FFQ meat variables |                                |  |             |                |
|--------------------|--------------------------------|--|-------------|----------------|
|                    | VIP                            | SMCC   | Meat groups |                |
| Included           | Sausage or liver pâté on bread | Meat/sausage on sandwich; liver pâté; low-fat liver pâté; unspecified liver pâté | Processed   | Total red meat |
|                    | Meat on bread                  | Cold cut meat  |             |                |
|                    | Bacon                          | Bacon  |             |                |
|                    | Sausage as dish                | Lean sausage; Falukorv; sausage; other sausage; cold cut sausage                 | Unprocessed |                |
|                    | Hamburger                      |  |             |                |
|                    | Minced meat dishes             | Minced meat (meatballs, hamburger, minced meat sauce)                            |             |                |
|                    | Meat stew                      | Pork (steak/casserole)   |             |                |
|                    | Steak, chop, etc               | Beef/veal (steak/casserole)  |             |                |
|                    | White meat (poultry)           | Chicken/other poultry  |             |                |
| Excluded           | Blood-based food               | Blood pudding/sausage  |             |                |
|                    | Smoked fish/meat               |  |             |                |
|                    | Liver, kidney                  | Liver/kidney   |             |                |

Four meat groups were analyzed in this study: processed, unprocessed, total red meat, and poultry. The second column on the left shows all meat products in the FFQ in VIP. The blood-based food and liver/kidney were only included in the 84-item FFQ and not in the 64-item FFQ used in the later part of VIP and, therefore, excluded in the study. The smoked fish/meat were excluded because of the fish inclusion in the variable. The third column shows all meat products in the 123-item FFQ in SMCC. Hamburger was included in the minced meat and, hence, was included as unprocessed meat. Total red meat group comprising both processed and unprocessed meat. White meat (poultry) was assessed separately.

Abbreviations: FFQ, food frequency questionnaire; SMCC, Clinical subcohort of Swedish Mammography Cohort; VIP, Västerbotten Intervention Program.

sausage on sandwich), and liver pâté (liver pâté, low-fat liver pâté, and unspecified liver pâté). Unprocessed red meat included minced meat (meatballs, hamburger, and minced meat sauce), pork (steak/casserole), and beef/veal (steak/casserole). Similar to VIP, total meat content for total sausage and minced meat was set to 70% and 43%, respectively, whereas hamburger was included in the minced meat category in this data set. Total red meat was calculated as the sum of processed and unprocessed red meat, with chicken and other poultry considered as a separate “poultry” category (Table 1). In SMCC, 125 participants did not report the consumption of poultry, wherefore replication of poultry candidate biomarkers was performed on 4096 instead of 4221 controls.

### Untargeted metabolomics analysis

A detailed description of sample preparation and analysis for liquid chromatography coupled with mass spectrometry (LC-MS)-based metabolomics has been described in detail elsewhere [35]. In brief, plasma samples for both VIP and SMCC underwent a protein precipitation procedure with acetonitrile. The protein-free filtrate was then collected and stored at 4°C before untargeted LC-MS analysis.

Untargeted metabolomics analysis for VIP was performed at the LC-MS facility at Biocenter Kuopio, University of Eastern Finland, as previously described [35]. In brief, 4 µL sample was injected to both hydrophilic interaction chromatography (HILIC) and reversed-phase (RP) columns in both positive (ESI+) and negative electrospray ionization (ESI-) modes (Supplemental Method).

The LC-MS system for untargeted metabolomics analysis of SMCC samples consisted of an Agilent 6550 iFunnel qTOF and a 1290 II UHPLC system. Analyses were performed only in RP column (Waters Acquity UPLC HSS T3 column; 100 × 2.1 mm, 1.8 µm), for both positive and negative ESI as previously described [42]. The sample injection volume was 4 µL for ESI- and 2 µL for ESI+.

The performance of the LC-MS analysis was monitored using 2 types of quality control (QC) samples: within-batch and between batch

QC samples [35]. The batch-specific QC samples were used for correcting the drift within each batch, and the long-term reference samples were used to aid in feature alignment and correct for differences in signal intensity between batches.

### Data analysis

All data processing and statistical computing were conducted in R version 4.0.0 at the UPPMAX High Performance Computation facility for sensitive data (Bianca).

### Preprocessing of metabolomics data

The raw VIP LC-MS data were converted to .mzML using Proteowizard [43] for further preprocessing using XCMS for peak-picking and alignment [44] and batchCorr [35] for batch correction, as has been described in previous publication [22], followed by imputation of missing values (Supplemental Method). After stringent preprocessing of the raw data, 24,759 features with a unique mass-to-charge ratio ( $m/z$ ) and RT remained for further downstream analysis [22,35].

The preprocessing of metabolomics data for SMCC was performed in a similar manner as the metabolomics data from VIP using R packages xcms for peak-picking and alignment [44], batchCorr [35] for batch correction, and ramclustR [45] for clustering of metabolite features, as previously described [42,46]. These preprocessing procedures yielded 4882 features from 4883 samples. After removal of mismatched or missing participants' metabolomics or metadata, information from 4457 participants was retained, consisting of 236 participants who developed T2D and 4221 participants who remained free of T2D by the last follow-up date (December 31, 2021).

### Participants' characteristics

Characteristics of VIP participants were summarized for the 25% highest (last quartile) and 25% (first quartile) lowest meat consumers of total red meat, processed meat, unprocessed meat, and poultry among controls (Table 2) [33], whereas SMCC participants were summarized

**TABLE 2**Baseline characteristics of the participants of discovery study (VIP) who remained free of T2D (controls) in the first and last quartiles of each meat group<sup>1</sup>

|   | Processed meat                        |                                      |          | Unprocessed meat                      |                                       |          | Total red meat                        |                                      |          | Poultry                               |                                      |          |
|---|---------------------------------------|--------------------------------------|----------|---------------------------------------|---------------------------------------|----------|---------------------------------------|--------------------------------------|----------|---------------------------------------|--------------------------------------|----------|
|   | Q1 ( <i>n</i> = 111),<br>median (IQR) | Q4 ( <i>n</i> = 85),<br>median (IQR) | <i>P</i> | Q1 ( <i>n</i> = 106),<br>median (IQR) | Q4 ( <i>n</i> = 102),<br>median (IQR) | <i>P</i> | Q1 ( <i>n</i> = 109),<br>median (IQR) | Q4 ( <i>n</i> = 97),<br>median (IQR) | <i>P</i> | Q1 ( <i>n</i> = 108),<br>median (IQR) | Q4 ( <i>n</i> = 78),<br>median (IQR) | <i>P</i> |
| Meat intake (g/d)                                       | 9.9 (6.7–12.5)                        | 46.4<br>(42.5–62.2)                  | <0.001   | 22.0<br>(18.8–26.5)                   | 80.0<br>(63.0–97.7)                   | <0.001   | 35.8<br>(29.2–43.9)                   | 116.2<br>(106.1–148.1)               | <0.001   | 0.4 (0.3–0.5)                         | 21.0<br>(15.4–24.0)                  | <0.001   |
| Age (y)   | 50.2<br>(49.8–59.9)                   | 49.9<br>(40.2–50.5)                  | 0.003    | 50.2<br>(49.8–59.8)                   | 50.0<br>(40.4–57.3)                   | 0.056    | 50.2<br>(49.8–59.8)                   | 49.9 (40.2–50.5)                     | 0.016    | 50.3<br>(49.8–59.9)                   | 50.0<br>(49.8–57.6)                  | 0.095    |
| Total energy intake<br>(kcal/d)                         | 1409<br>(1124–1710)                   | 2133<br>(1734–2534)                  | <0.001   | 1323<br>(1050–1568)                   | 2212<br>(1768–2520)                   | <0.001   | 1306<br>(1032–1533)                   | 2270<br>(1825–2535)                  | <0.001   | 1559<br>(1249–1865)                   | 1828<br>(1524–2365)                  | <0.001   |
| BMI (kg/m <sup>2</sup> )                                | 25.0<br>(23.0–27.5)                   | 25.4<br>(23.4–28.4)                  | 0.228    | 24.6<br>(22.6–27.3)                   | 24.8<br>(23.1–28.1)                   | 0.324    | 24.8<br>(22.6–27.4)                   | 25.3 (23.4–28.4)                     | 0.158    | 24.9<br>(22.2–27.8)                   | 25.3<br>(23.2–28.4)                  | 0.150    |
| Alcohol intake (as g/<br>d ethanol)                     | 1.89<br>(0.15–4.36)                   | 3.92<br>(0.46–6.55)                  | 0.007    | 1.84<br>(0.16–3.40)                   | 3.85<br>(0.65–6.87)                   | 0.001    | 1.89<br>(0.16–3.94)                   | 4.17 (0.50–7.40)                     | 0.002    | 0.78<br>(0.06–3.34)                   | 4.34<br>(1.42–7.33)                  | <0.001   |
| Alcohol consumers <sup>2</sup>                          |                                       |                                      | 0.319    |                                       |                                       | 0.547    |                                       |                                      | 0.679    |                                       |                                      | 0.004    |
| Nonconsumers  | 17                                    | 7                                    |          | 13                                    | 9                                     |          | 13                                    | 8                                    |          | 18                                    | 6                                    |          |
| Below median  | 36                                    | 31                                   |          | 41                                    | 36                                    |          | 38                                    | 36                                   |          | 47                                    | 22                                   |          |
| Above median  | 58                                    | 47                                   |          | 52                                    | 57                                    |          | 58                                    | 53                                   |          | 43                                    | 50                                   |          |
| Sex   |                                       |                                      | <0.001   |                                       |                                       | <0.001   |                                       |                                      | <0.001   |                                       |                                      | 0.002    |
| Male  | 32                                    | 62                                   |          | 28                                    | 74                                    |          | 29                                    | 77                                   |          | 40                                    | 48                                   |          |
| Female  | 79                                    | 23                                   |          | 78                                    | 28                                    |          | 80                                    | 20                                   |          | 68                                    | 30                                   |          |
| Smoking status<br>(Occasional)<br>smokers               | 21                                    | 19                                   | 0.095    | 25                                    | 26                                    | 0.407    | 22                                    | 23                                   | 0.278    | 23                                    | 13                                   | 0.713    |
| Former smokers  | 30                                    | 33                                   |          | 28                                    | 34                                    |          | 30                                    | 34                                   |          | 35                                    | 28                                   |          |
| Nonsmokers  | 60                                    | 33                                   |          | 53                                    | 42                                    |          | 57                                    | 40                                   |          | 50                                    | 37                                   |          |
| Work and leisure-time<br>physical activity <sup>3</sup> |                                       |                                      | 0.544    |                                       |                                       | 0.227    |                                       |                                      | 0.775    |                                       |                                      | 0.540    |
| Inactive  | 22                                    | 14                                   |          | 24                                    | 12                                    |          | 22                                    | 15                                   |          | 18                                    | 8                                    |          |
| Moderately<br>inactive                                  | 44                                    | 28                                   |          | 33                                    | 35                                    |          | 39                                    | 35                                   |          | 31                                    | 28                                   |          |
| Moderately active                                       | 25                                    | 22                                   |          | 28                                    | 32                                    |          | 29                                    | 26                                   |          | 32                                    | 24                                   |          |
| Active  | 20                                    | 21                                   |          | 21                                    | 23                                    |          | 19                                    | 21                                   |          | 27                                    | 18                                   |          |

Abbreviations: T2D, type 2 diabetes; VIP, Västerbotten Intervention Program.

<sup>1</sup> Differences between the first and last quartiles within meat intake group were tested using Wilcoxon rank-sum test for continuous variables and  $\chi^2$  test for categorical variables. *P* < 0.05 was considered significant.<sup>2</sup> Data are presented as median (IQR). Median intake for alcohol was sex specific. The median level for males and females were 4.338 and 0.984 g/d, respectively.<sup>3</sup> Physical activity was assessed based on a validated measure of Cambridge index of physical activity [33].

for prospective T2D cases and controls (Supplemental Table 1). The differences between groups were reported as median (IQR) and analyzed using Wilcoxon rank-sum test for continuous variables and  $\chi^2$  test for categorical variables.

### Feature selection in VIP

Selection of metabolite features reflecting self-reported meat intake was performed in the control group ( $n = 403$  participants) to avoid potential artifacts relating to T2D progression affecting dietary intake and/or metabolome [47]. To select features discriminating lowest compared with highest self-reported intake quartiles, we used the R package MUVr [48], which performs random forest modeling within a repeated double crossvalidation to reduce overfitting. The MUVr algorithm also performs a minimally biased variable selection by incorporating recursive variable elimination within the nested cross-validation procedure [48], being used with the following key parameters:  $nRep = 50$ ,  $varRatio = 0.90$ , and  $nOuter = 8$ . Permutation analyses ( $n = 100$ ) were performed to ascertain that results were not due to overfitting [49]. After MUVr-random forest analysis, metabolite features were log transformed (pseudocount of  $10^{-3} \times$  lowest nonzero value added), centered, and scaled to unit variance.

Features selected using random forest (max model) were subjected to partial Spearman correlation analysis with meat intake, adjusting for age, sex, and energy intake, according to the presumed relationship visualized using a directed acyclic graph (Supplemental Figure 1A). Adjustment of energy intake was performed using “the standard multivariate” model (adding energy intake as covariate) [50]. As a sensitivity analysis, energy adjustment was also performed using the “residual method” [51], which gave similar results. In addition, a second model with additional adjustment for BMI was performed to investigate the unresolved contribution of BMI as a mediator or as a confounder in the association between meat intake and metabolite profile. Additional adjustments for education were performed as a sensitivity analysis, which did not qualitatively change the estimates of the associations (data not shown). We decided a priori to consider metabolite features from model 1 with a false discovery rate (FDR) of  $<0.01$  as significant. However, for unprocessed and total red meat intake, no metabolite associated at that cutoff in the discovery study. Therefore, an a posteriori cutoff limit of FDR of  $<0.1$  was used for unprocessed and total red meat. Overlap of the metabolites selected by random forest to represent different meat intakes are shown in Supplemental Figure 2A, and those further refined by the partial Spearman correlation analysis are shown in Supplemental Figure 2B.

### Metabolite annotation in the discovery study (VIP)

Features correlated with the consumption of  $\geq 1$  meat group in VIP and were subjected to metabolite annotation according to the reporting guidelines from Metabolomics Standard Initiative [52] (Supplemental Method). For features with high quality and symmetric chromatographic peaks, but no MS2 data, samples with high abundance were subjected to additional, targeted MS2 analysis with previously described protocol [53]. After removal of noise features and redundant entries owing to in-source fragmentation or multiple adducts, metabolites associated with  $\geq 1$  meat group were annotated (Supplemental Table 2).

### Replication of metabolites previously associated with meat intake in VIP

A list of 43 metabolites previously reported to associate with meat intake [13,21,54–60], (Supplemental Table 3) were searched in both

cohorts. A feature was considered to match the metabolite of interest if it fell within  $\pm 10$  ppm tolerance from the reported  $m/z$ -value and was further supported by MS2 spectra. Associations with meat intake were analyzed using partial Spearman correlation as described earlier.

### Replicated associations in SMCC

Retention time differences between the 2 data sets were matched using a locally estimated scatterplot smoothing based on 40 unequivocally identified features in RP ESI+ and 43 in ESI- (Supplemental Table 4). The fitted locally estimated scatterplot smoothing model was then used to predict the retention time of the features of interest in SMCC. Features of interest were considered to match if they fell within  $m/z$ -value tolerance of  $\pm 10$  ppm, retention time tolerance of  $\pm 0.75$  min, and additional confirmation from MS1 peak shape and MS2 fragmentation spectra. After this procedure, discovered metabolites in VIP cohort and metabolites previously associated with meat intake in the literature that could be found in SMCC were then subjected to partial Spearman correlation with meat intake. The same set of covariates as in the discovery set was applied, except for sex, since SMC is a cohort of females only.

### Performance of meat composite score built based on replicated metabolites

Among meat-associated metabolites that were found in SMCC, metabolites that were significantly associated with  $\geq 1$  meat category in both VIP and SMCC were then used to compose an elastic net model with optimized  $\alpha$  and  $\lambda$  (Supplemental Table 5) to predict meat intake of all participants (Supplemental Method). Prediction performance was evaluated in both cohorts by root mean square error and Pearson correlation with intake (following log transformation, centering, and scaling). Q2 was calculated as  $1 - (\text{PRESS}/\text{TSS})$ , with PRESS being prediction error sums of squares and TSS total sums of squares. Owing to differences in habitual meat consumption in cases and controls (Supplemental Table 1) and different sex proportion in high-meat-intake and low-meat-intake groups (Table 2) [33], the population was stratified based on cases and controls status, along with additional sex-based stratification for VIP to separately assess the performance metrics.

### Assessment of associations with T2D risk

In VIP, the odds of developing T2D was calculated per metabolite by conditional logistic regression using the clogit function from the survival package [61] with the normalized values of self-reported meat intake, selected metabolites, or meat intake score (from elastic net) modeled as the independent variable and T2D incidence (1 for cases and 0 for controls) as the dependent variable. Based on the presumed assumptions of causality between blood metabolome and the risk of T2D visualized using directed acyclic graph (Supplemental Figure 1B), adjustment for age, sex, smoking (smokers, former smokers, and nonsmokers), physical activity (Cambridge physical activity index; 1 = inactive, 2 = moderately inactive, 3 = moderately active, and 4 = active), total energy, and alcohol (zero, below or above sex-specific median) intake was performed. In addition, further adjusting for BMI was performed in the second model to account for the unresolved potential role of BMI as a confounder or a mediator. In a sensitivity analysis, additional adjustment for education as a proxy of social classes was performed, with negligible changes of the estimates (data not shown).

In SMCC, the association with T2D risk was assessed using Cox regression (survival package [61]), with the number of days until the T2D diagnosis or the last follow-up date as time and T2D status (0 for controls and 1 for cases) as the event using the efron method. The same

set of covariates as in VIP was included, except for sex and that the physical activity was stated as metabolic equivalent of task hours per week instead of categorical Cambridge index.

## Results

### Baseline characteristics

In the discovery cohort (VIP), participants with no T2D incidence at the end of follow-up (controls) who consumed more meat also had a higher energy intake and higher alcohol intake. Hence, we performed Wilcoxon rank-sum test for continuous variables and  $\chi^2$  test for categorical variables to investigate whether these differences were potentially systematic. This association seemed consistent across the different investigated meat intakes (Table 2) [33]. In addition, those with higher consumption levels of processed and total red meat were also younger than those with a lower intake (Table 2) [33]. Compared with those who developed T2D (cases), controls had lower BMI ( $P < 0.001$ ) and higher alcohol consumption ( $P = 0.003$ ) and lower intake of processed meat ( $P = 0.009$ ). In SMCC, those who remained free of T2D had higher physical activity than those who developed T2D, which was not observed in VIP (Supplemental Table 1).

### Metabolites associated with consumption of various meat groups

In total, 882 features were selected by random forest to be associated with processed, unprocessed, total meat intake, or poultry (Figure 2). The random forest modeling performed the best for total meat [classification rate (CR) = 0.75 and  $P = 3.4 \times 10^6$ ], followed by processed meat (CR = 0.72,  $P = 8.5 \times 10^6$ ), unprocessed meat (CR = 0.66,  $P = 6.8 \times 10^4$ ), and finally poultry (CR = 0.62 and  $P = 0.029$ ) (Supplemental Figure 3).

After partial correlation, 173 features correlating with the consumption of  $\geq 1$  meat group in VIP were subjected to metabolite annotation, which resulted in 76 annotated metabolites (Supplemental Table 2). Among these, 37 metabolites associated with processed meat, including acylcarnitine 8:1 ( $r = 0.32$ ), acylcarnitine 10:3 ( $r = 0.24$ ), piperine glucuronide ( $r = 0.22$ ), putatively annotated acylcarnitine 8:2 ( $r = 0.25$ ), and methylproline ( $r = -0.18$ ). Piperine glucuronide and 4 unidentified metabolites were also found to associate with poultry intake.

The consumption of unprocessed and total red meat was associated with 11 and 12 metabolites (FDR  $< 0.1$ ), respectively (Supplemental Table 2). Among them, phosphatidylcholine (PC) 38:7 had negative association ( $r = -0.15$ ), whereas lysophosphatidylethanolamine (LPE) O-16:1 ( $r = 0.14$ ) had positive associations with total red meat intake. Further adjustment for BMI attenuated correlation estimates mildly toward the null, such that 6 of the associations (PC 38:7 and 5 other unidentified metabolites) with total red meat intake were no longer significant (FDR  $> 0.1$ ). PS 39:6 was negatively associated with processed meat intake ( $r = -0.19$  and  $-0.18$ ), with and without further adjustment for BMI. The positive associations of LPE O-18:1 with processed ( $r = 0.13$ ; FDR = 0.021) and total red meat intake ( $r = 0.15$ ; FDR = 0.085), however, were significant only after additional adjustment for BMI.

Among the metabolites associated with poultry intake, an unidentified metabolite with  $m/z$  176.1282 eluting at 4.08 min in HILIC-positive mode showed the strongest positive association ( $r = 0.20$ ), followed by a fatty acid derivative with  $m/z$  230.1750 eluting at 6.79 min in RP-positive mode ( $r = 0.18$ ). 2-Hydroxyvaleric acid showed positive association ( $r = 0.17$ ). LPC 17:1 and PC 15:0/18:2 showed the strongest negative associations ( $r = -0.20$  and  $r = -0.18$ , respectively). Across all meat groups, further adjustment for BMI did not influence the results significantly (Supplemental Table 2).

### Metabolites previously associated with meat intake

Of the 43 metabolites previously associated with meat intake in the literature, 20 could be detected in VIP (Supplemental Table 3). Among these 20 metabolites, however, only 4 metabolites were associated with  $\geq 1$  meat intake group: acylcarnitine 4:0, creatine, piperettine, and piperine glucuronide (Supplemental Table 6).

### Replication in SMCC

Among 76 metabolites discovered in VIP, 15 could be found in SMCC (Table 3), of which all but 2 were also associated with the corresponding meat intake, that is, LPE 16:0, which showed significant associations in VIP and an unknown lipophilic molecule with  $m/z$  794.5749 eluting at 12.72 mins at RP positive (RP\_794.5749@763.28) in SMCC. Because SMCC used only RP chromatography, 16 biomarker candidates discovered in VIP with hydrophilic properties, shown by elution in HILIC column, naturally could not be replicated in

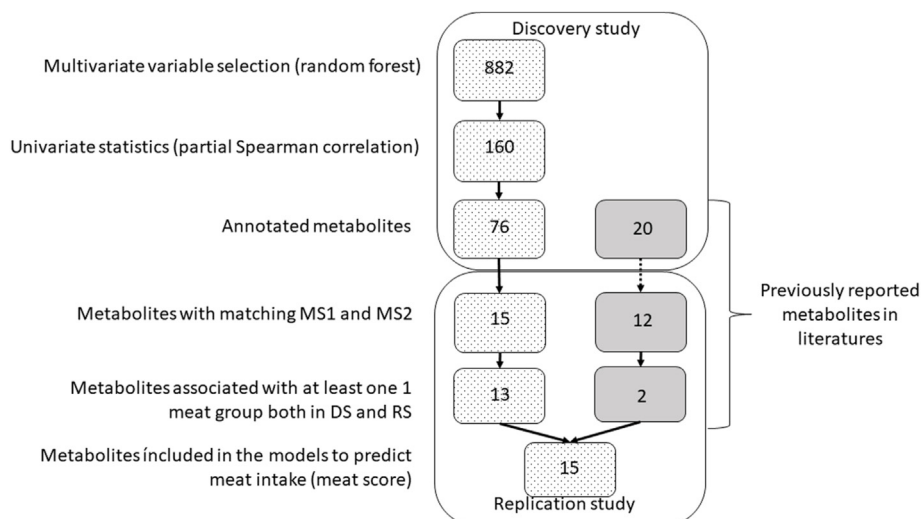


FIGURE 2. Number of metabolites surviving each step.

**TABLE 3**  
Associations between meat intake and 15 metabolites selected in discovery study (VIP) that were also found in replication study (SMCC)<sup>1</sup>

| Metabolites           | VIP            |                       |         |                       |         |       |                  |       |         |       |                |       |         |         |         |  |
|-----------------------|----------------|-----------------------|---------|-----------------------|---------|-------|------------------|-------|---------|-------|----------------|-------|---------|---------|---------|--|
|                       | Processed meat |                       |         |                       |         |       | Unprocessed meat |       |         |       | Total red meat |       |         |         | Poultry |  |
|                       | Model 1        |                       | Model 2 |                       | Model 1 |       | Model 2          |       | Model 1 |       | Model 2        |       | Model 1 | Model 2 |         |  |
|                       | r              | FDR                   | r       | FDR                   | r       | FDR   | r                | FDR   | r       | FDR   | r              | FDR   | r       | FDR     |         |  |
| 2-Hydroxyvaleric acid | 0.02           | 0.673                 | 0.00    | 0.944                 | -0.03   | 0.715 | -0.03            | 0.763 | 0.01    | 0.832 | 0.00           | 0.946 | 0.17    | 0.004   | 0.17    |  |
| Carnitine 8:1         | 0.32           | 1.3 × 10 <sup>9</sup> | 0.30    | 1.7 × 10 <sup>8</sup> | 0.03    | 0.715 | 0.02             | 0.944 | 0.16    | 0.011 | 0.14           | 0.017 | 0.09    | 0.160   | 0.06    |  |
| Carnitine 8:2         | 0.25           | 3.8 × 10 <sup>6</sup> | 0.23    | 2.7 × 10 <sup>5</sup> | -0.03   | 0.715 | -0.03            | 0.763 | 0.08    | 0.132 | 0.07           | 0.200 | 0.08    | 0.237   | 0.06    |  |
| Carnitine 10:3        | 0.24           | 3.8 × 10 <sup>6</sup> | 0.22    | 3.4 × 10 <sup>5</sup> | 0.00    | 0.925 | -0.01            | 0.944 | 0.10    | 0.067 | 0.09           | 0.145 | 0.07    | 0.253   | 0.06    |  |
| LPC 17:1              | -0.20          | 1.2 × 10 <sup>4</sup> | -0.17   | 1.4 × 10 <sup>3</sup> | -0.05   | 0.658 | -0.03            | 0.763 | -0.10   | 0.067 | -0.08          | 0.199 | -0.20   | 0.001   | -0.18   |  |
| LPE O-16:1            | 0.09           | 0.076                 | 0.10    | 0.057                 | 0.10    | 0.278 | 0.10             | 0.199 | 0.14    | 0.017 | 0.15           | 0.016 | 0.09    | 0.160   | 0.10    |  |
| PC 15:0/18:2          | -0.14          | 0.007                 | -0.12   | 0.023                 | -0.07   | 0.443 | -0.05            | 0.630 | -0.11   | 0.060 | -0.09          | 0.170 | -0.18   | 0.003   | -0.17   |  |
| RN_222.114@408.52     | 0.24           | 3.8 × 10 <sup>6</sup> | 0.23    | 2.7 × 10 <sup>5</sup> | -0.04   | 0.715 | -0.04            | 0.719 | 0.08    | 0.162 | 0.07           | 0.229 | 0.01    | 0.852   | 0.01    |  |
| RN_394.3337@642.16    | 0.17           | 1.0 × 10 <sup>3</sup> | 0.14    | 0.008                 | 0.01    | 0.867 | 0.00             | 0.991 | 0.09    | 0.128 | 0.07           | 0.244 | 0.04    | 0.525   | 0.02    |  |
| RN_488.194@420.53     | 0.18           | 5.1 × 10 <sup>4</sup> | 0.16    | 0.003                 | 0.07    | 0.443 | 0.06             | 0.630 | 0.13    | 0.024 | 0.11           | 0.082 | 0.16    | 0.008   | 0.15    |  |
| RN_864.5774@743.37    | -0.16          | 0.002                 | -0.14   | 0.009                 | 0.04    | 0.715 | 0.05             | 0.719 | -0.05   | 0.300 | -0.04          | 0.512 | 0.05    | 0.429   | 0.07    |  |
| RP_215.0225@216.66    | 0.13           | 0.014                 | 0.12    | 0.023                 | 0.13    | 0.118 | 0.13             | 0.130 | 0.15    | 0.011 | 0.15           | 0.016 | 0.05    | 0.428   | 0.05    |  |
| RP_284.1282@505.51    | 0.20           | 1.9 × 10 <sup>4</sup> | 0.19    | 3.6 × 10 <sup>4</sup> | 0.06    | 0.545 | 0.05             | 0.630 | 0.15    | 0.014 | 0.14           | 0.017 | 0.11    | 0.086   | 0.11    |  |
| RP_332.262@539.74     | 0.15           | 0.005                 | 0.14    | 0.011                 | 0.02    | 0.782 | 0.01             | 0.944 | 0.11    | 0.060 | 0.10           | 0.128 | 0.01    | 0.854   | 0.00    |  |
| RP_794.5749@763.28    | 0.02           | 0.713                 | 0.02    | 0.697                 | -0.13   | 0.118 | -0.13            | 0.130 | -0.06   | 0.279 | -0.06          | 0.316 | -0.03   | 0.579   | -0.03   |  |

| VIP   | SMCC    |                        |         |                        |         |                        |                  |                        |         |                        |                |                        |         |                       |         |                       |
|-------|---------|------------------------|---------|------------------------|---------|------------------------|------------------|------------------------|---------|------------------------|----------------|------------------------|---------|-----------------------|---------|-----------------------|
|       | Poultry |                        |         |                        |         |                        | Unprocessed meat |                        |         |                        | Total red meat |                        |         |                       | Poultry |                       |
|       | Model 2 |                        | Model 1 |                        | Model 2 |                        | Model 1          |                        | Model 2 |                        | Model 1        |                        | Model 2 |                       | Model 1 | Model 2               |
|       | r       | FDR                    | r       | FDR                    | r       | FDR                    | r                | FDR                    | r       | FDR                    | r              | FDR                    | r       | FDR                   | r       | FDR                   |
| 0.005 | 0.05    | 0.003                  | 0.04    | 0.005                  | 0.06    | 2.1 × 10 <sup>4</sup>  | 0.06             | 6.5 × 10 <sup>4</sup>  | 0.07    | 1.5 × 10 <sup>5</sup>  | 0.06           | 7.2 × 10 <sup>5</sup>  | 0.03    | 0.089                 | 0.03    | 0.109                 |
| 0.401 | 0.09    | 8.0 × 10 <sup>8</sup>  | 0.07    | 1.9 × 10 <sup>5</sup>  | 0.02    | 0.187                  | 0.01             | 0.526                  | 0.06    | 8.3 × 10 <sup>5</sup>  | 0.05           | 0.004                  | -0.03   | 0.051                 | -0.04   | 0.013                 |
| 0.412 | 0.07    | 1.2 × 10 <sup>5</sup>  | 0.05    | 0.001                  | 0.01    | 0.491                  | 0.00             | 0.838                  | 0.05    | 0.003                  | 0.03           | 0.039                  | -0.04   | 0.020                 | -0.05   | 0.005                 |
| 0.401 | 0.08    | 8.8 × 10 <sup>7</sup>  | 0.07    | 4.6 × 10 <sup>5</sup>  | 0.01    | 0.491                  | 0.00             | 0.838                  | 0.05    | 0.002                  | 0.04           | 0.020                  | -0.06   | 0.002                 | -0.06   | 3.7 × 10 <sup>4</sup> |
| 0.002 | -0.06   | 3.7 × 10 <sup>4</sup>  | -0.05   | 0.001                  | 0.02    | 0.205                  | 0.03             | 0.143                  | -0.02   | 0.333                  | -0.01          | 0.472                  | -0.02   | 0.385                 | -0.01   | 0.490                 |
| 0.102 | 0.00    | 0.870                  | 0.01    | 0.570                  | -0.02   | 0.351                  | -0.01            | 0.561                  | -0.01   | 0.651                  | 0.00           | 0.953                  | 0.01    | 0.466                 | 0.02    | 0.373                 |
| 0.005 | -0.08   | 3.6 × 10 <sup>7</sup>  | -0.07   | 9.5 × 10 <sup>6</sup>  | -0.09   | 2.8 × 10 <sup>8</sup>  | -0.08            | 4.4 × 10 <sup>7</sup>  | -0.10   | 1.6 × 10 <sup>10</sup> | -0.09          | 1.1 × 10 <sup>8</sup>  | -0.04   | 0.022                 | -0.03   | 0.057                 |
| 0.928 | 0.07    | 7.2 × 10 <sup>6</sup>  | 0.06    | 4.8 × 10 <sup>4</sup>  | 0.05    | 0.006                  | 0.04             | 0.034                  | 0.07    | 3.1 × 10 <sup>5</sup>  | 0.05           | 1.3 × 10 <sup>3</sup>  | -0.02   | 0.313                 | -0.03   | 0.142                 |
| 0.737 | 0.10    | 2.9 × 10 <sup>9</sup>  | 0.08    | 2.7 × 10 <sup>7</sup>  | 0.06    | 4.1 × 10 <sup>4</sup>  | 0.05             | 0.002                  | 0.10    | 2.5 × 10 <sup>9</sup>  | 0.08           | 1.5 × 10 <sup>7</sup>  | 0.06    | 2.0 × 10 <sup>4</sup> | 0.06    | 5.4 × 10 <sup>4</sup> |
| 0.012 | 0.12    | 3.2 × 10 <sup>13</sup> | 0.11    | 1.3 × 10 <sup>11</sup> | 0.10    | 2.8 × 10 <sup>10</sup> | 0.09             | 6.6 × 10 <sup>9</sup>  | 0.13    | 5.2 × 10 <sup>17</sup> | 0.12           | 8.1 × 10 <sup>15</sup> | 0.07    | 1.1 × 10 <sup>4</sup> | 0.07    | 1.9 × 10 <sup>4</sup> |
| 0.401 | -0.05   | 0.004                  | -0.03   | 0.059                  | -0.05   | 0.002                  | -0.04            | 0.029                  | -0.06   | 2.0 × 10 <sup>4</sup>  | -0.04          | 0.007                  | 0.05    | 0.010                 | 0.06    | 8.1 × 10 <sup>4</sup> |
| 0.412 | 0.05    | 0.002                  | 0.05    | 0.005                  | 0.04    | 0.011                  | 0.04             | 0.031                  | 0.06    | 1.2 × 10 <sup>4</sup>  | 0.06           | 6.9 × 10 <sup>4</sup>  | 0.01    | 0.623                 | 0.01    | 0.752                 |
| 0.083 | 0.08    | 1.2 × 10 <sup>6</sup>  | 0.08    | 3.5 × 10 <sup>7</sup>  | 0.06    | 2.1 × 10 <sup>4</sup>  | 0.07             | 8.9 × 10 <sup>5</sup>  | 0.08    | 4.0 × 10 <sup>7</sup>  | 0.09           | 9.5 × 10 <sup>8</sup>  | 0.07    | 8.6 × 10 <sup>5</sup> | 0.07    | 3.1 × 10 <sup>5</sup> |
| 0.977 | 0.13    | 3.5 × 10 <sup>17</sup> | 0.13    | 2.8 × 10 <sup>16</sup> | 0.10    | 2.8 × 10 <sup>10</sup> | 0.10             | 5.5 × 10 <sup>10</sup> | 0.14    | 1.3 × 10 <sup>19</sup> | 0.14           | 1.4 × 10 <sup>18</sup> | 0.05    | 0.008                 | 0.05    | 0.007                 |
| 0.644 | -0.09   | 8.0 × 10 <sup>8</sup>  | -0.08   | 3.5 × 10 <sup>7</sup>  | -0.05   | 1.3 × 10 <sup>3</sup>  | -0.05            | 0.002                  | -0.08   | 6.9 × 10 <sup>7</sup>  | -0.08          | 2.3 × 10 <sup>6</sup>  | -0.04   | 0.047                 | -0.03   | 0.081                 |

Abbreviations: FDR, false discovery rate; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; RN, reversed-phase chromatography negative polarity; SMCC, Clinical subcohort of Swedish Mammography Cohort; VIP, Västerbotten Intervention Program.

<sup>1</sup> Association assessed by Spearman correlation adjusted for age, energy intake (kcal/d), and sex (only in VIP), with an additional adjustment for BMI in the second model. Adjustment for multiple comparisons was achieved by the false discovery rate (FDR) method. FDR < 0.05 was considered significant.

SMCC. Among the previously reported metabolites, 12 could be found in SMCC (Supplemental Table 6), of which only 2 metabolites had consistent associations in both VIP and SMCC (Figure 3, Supplemental Table 6). Those were acylcarnitine 4:0, which associated with total red meat, and piperettine, which associated with both processed and total red meat (Supplemental Table 6), making up 15 metabolites to compose multimarker panels to predict meat intake. These 15 metabolites are shown in Figure 3, in relation to their associated meat intake group. Notably, 2-hydroxyvaleric acid with inconsistent associations with poultry intake in VIP but with other meat groups in SMCC was excluded from the graph.

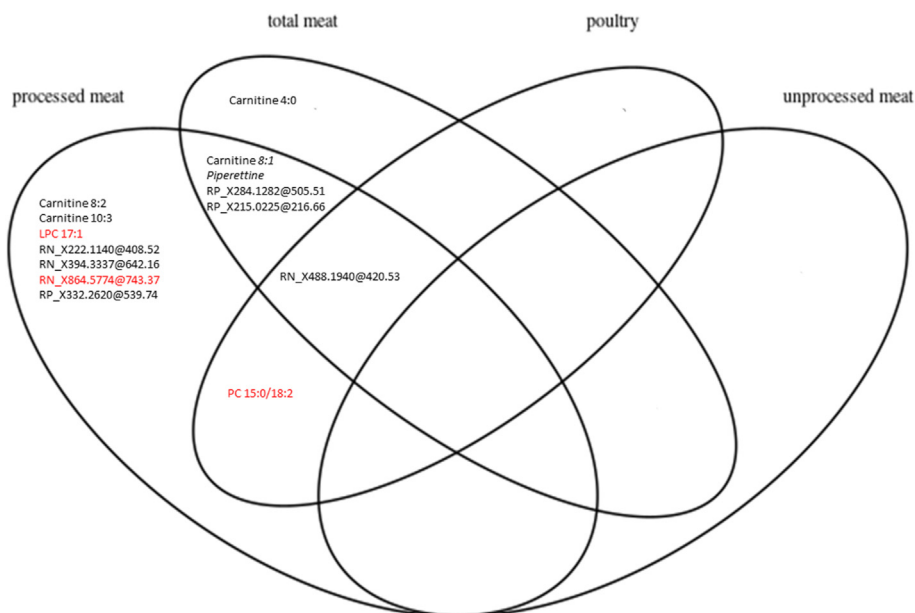
### Performance of metabolite panels associated with meat intake

The meat composite scores built from the panels of metabolites selected for each meat exposure did not associate strongly to meat intake (Q2 < 0.1). The highest correlation between reported intake and composite score was obtained for processed meat (r = 0.29; FDR = 2.1 × 10<sup>15</sup>) (Supplemental Table 7), with consistent performance across stratified analyses. Stronger associations were observed in VIP than those in SMCC (Supplemental Table 7).

Consistent correlations between poultry intake and composite score based on the poultry-associated metabolites were observed across all stratified populations in both VIP and SMCC, except for males in VIP (r = 0.06; FDR = 0.294). Stronger correlations were observed in controls (r = 0.23; FDR = 2.2 × 10<sup>6</sup>) and females (r = 0.23; FDR = 9.8 × 10<sup>7</sup>) in VIP and cases in SMCC (r = 0.18; FDR = 0.007). By contrast, correlations for total red meat were quite consistent in total populations and after stratification for the case/control status but not after stratification for sex. The associations were stronger for total population and cases in VIP and for both total and controls in SMCC (Supplemental Table 7).

### Association between discovered metabolites and the risk of T2D

Predicted processed meat intake from the composite score from the panel of 15 replicated metabolites was consistently associated with higher risk of T2D in both VIP (OR: 1.08; 95% CI: 1.05, 1.11; FDR = 3.1 × 10<sup>6</sup>) and SMCC (OR: 1.29; 95% CI: 1.13, 1.47; FDR = 9.2 × 10<sup>4</sup>) (Table 4), although the association was attenuated after additional adjustment for BMI. Predicted total red meat intake from the composite score associated with higher T2D risk only in VIP and was attenuated



**FIGURE 3.** Mapping of meat intake-related metabolites and their associated meat intake variables in both VIP and SMCC. Piperettine was previously associated with meat intake in the literatures. Metabolites in red indicate inverse associations. (L)PC, (lyso)phosphatidylcholine; RN, reversed-phase chromatography negative polarity; RP, reversed-phase chromatography positive polarity; VIP, Västerbotten Intervention Program.

after additional adjustment for BMI. Interestingly, predicted poultry intake was associated with higher T2D risk in VIP but with lower risk in SMCC.

When looking at individual metabolites, acylcarnitines 4:0, 8:1, 8:2, and 10:3 and an unidentified metabolite with  $m/z$  222.114 eluting at 6.81 mins in RP-negative mode (RN\_222.114@408.52) were associated with higher T2D risk in both data sets. The associations remained significant after additional adjustment for BMI in SMCC but not in VIP. Conversely, LPC 17:1 and an unidentified lipophilic metabolite with  $m/z$  864.5774 eluting after 12.39 mins at RP negative (RN\_864.5774@743.37) associated with lower risk in both data sets, but the association was attenuated after additional adjustment for BMI, except for RN\_864.5774@743.37 in VIP (Table 4). Piperettine and an unidentified metabolite with  $m/z$  284.1282 eluting after 8.43 mins at RP positive (RP\_284.1282@505.51) were the only metabolites with no association with T2D risk in both data sets (Table 4). Further analysis showed that most of these metabolites were correlated with BMI in both data sets ( $|r| \geq 0.05$ ; FDR < 0.01), except for 2 unknown metabolites in VIP (Supplemental Table 8).

## Discussion

We discovered 76 and replicated 4 previously reported putative biomarkers of processed, unprocessed, total red meat, and poultry intake in a case-control study nested in a Swedish population-based cohort. Among those associations, 15 (13 discovered and 2 previously reported) were replicated in another independent cohort. We further combined the biomarkers into a panel score for quantitative predictions of meat intake and explored their associations with T2D risk. We found evidence of associations between higher consumption of processed meat, reflected by either individual candidate biomarkers or as a combined score, with higher T2D risk. The associations were attenuated after adjusting for BMI, indicating that BMI may play a causal role in these associations as either a mediator or a confounder.

After replication of biomarkers from the discovery cohort, a final number of 15 metabolites showed potential to indicate meat intakes (Figure 2). HILIC was not available in the SMCC used for replication, which limited the possibility for replication and, consequently, the number of potential biomarkers. In addition, several metabolites could not be observed at all in SMCC. This low number of replicated candidate biomarkers effectively highlights that limiting biomarker discovery to single studies may result in high risk of false discovery that may prove difficult to replicate.

Among the 15 metabolites associated with  $\geq 1$  meat group in both discovery and replication studies, we found piperettine and RP\_284.1282@505.51 as the only metabolites with no association with T2D risk in either of the study. Because piperettine was reported to originate from pepper [62], its association with processed meat intake in previous [55] and this study may highlight potential confounding from other dietary components that are consumed along with meat intake, the component-of-interest. Although we have adjusted for known confounders related to meat intake, confounding by other metabolites reflecting dietary components or lifestyle factors related to meat intake cannot be ruled out.

We confirmed previously reported associations between acylcarnitines and phospholipids of various chain lengths with meat intake in this study, and some of them could be replicated in SMCC. In addition to previously reported associations, novel associations of PC 15:0/18:2 and acylcarnitines 8:1, 8:2, and 10:3 with processed, total red meat, and poultry intakes were also established in both discovery and replication cohorts. Acylcarnitines have previously been suggested as a generic intake biomarker of food of animal-origin and hence not suitable as individual marker to quantitatively indicate red meat intakes [13]. Combining multiple biomarker candidates into biomarker panel may hence be promising to give a better specificity than single biomarkers [54] because it gives different weights to each biomarker candidate, as performed in this study. Among the elastic net-derived multimarker panel scores, the strongest performance was observed for processed meat. However, even this showed only

**TABLE 4**  
Associations of self-reported and predicted meat intake with the risk of developing type 2 diabetes (T2D)<sup>1</sup>

|                       | VIP               |                       |                   |       |          | SMCC          |                   |                       |          |               |                   |       |
|-----------------------|-------------------|-----------------------|-------------------|-------|----------|---------------|-------------------|-----------------------|----------|---------------|-------------------|-------|
|                       | Model 1           |                       | Model 2           |       | <i>n</i> | No. of events | Model 1           |                       | <i>n</i> | No. of events | Model 2           |       |
|                       | OR (95% CI)       | FDR                   | OR (95% CI)       | FDR   |          |               | HR (95% CI)       | FDR                   |          |               | HR (95% CI)       | FDR   |
| Processed meat        |                   |                       |                   |       |          |               |                   |                       |          |               |                   |       |
| Actual                | 1.01 (1.00, 1.02) | 0.009                 | 1.01 (1.00, 1.02) | 0.353 | 3145     | 164           | 1.01 (1.00, 1.03) | 0.055                 | 3137     | 164           | 1.01 (1.00, 1.02) | 0.176 |
| Predicted             | 1.08 (1.05, 1.11) | 3.1 × 10 <sup>6</sup> | 1.04 (1.00, 1.08) | 0.139 | 3145     | 164           | 1.29 (1.13, 1.47) | 9.2 × 10 <sup>4</sup> | 3137     | 164           | 1.17 (1.02, 1.33) | 0.065 |
| Unprocessed meat      |                   |                       |                   |       |          |               |                   |                       |          |               |                   |       |
| Actual                | 1.00 (1.00, 1.01) | 0.451                 | 1.00 (1.00, 1.01) | 0.744 | 3145     | 164           | 1.00 (0.99, 1.01) | 0.777                 | 3137     | 164           | 0.99 (0.98, 1.01) | 0.426 |
| Predicted             | 0.99 (0.95, 1.04) | 0.808                 | 0.99 (0.93, 1.04) | 0.700 | 3145     | 164           | 1.10 (1.00, 1.22) | 0.083                 | 3137     | 164           | 1.02 (0.92, 1.13) | 0.870 |
| Total red meat        |                   |                       |                   |       |          |               |                   |                       |          |               |                   |       |
| Actual                | 1.00 (1.00, 1.01) | 0.053                 | 1.00 (1.00, 1.01) | 0.484 | 3145     | 164           | 1.00 (1.00, 1.01) | 0.363                 | 3137     | 164           | 1.00 (0.99, 1.01) | 0.922 |
| Predicted             | 1.03 (1.01, 1.05) | 9.2 × 10 <sup>4</sup> | 1.02 (0.99, 1.04) | 0.342 | 3145     | 164           | 1.07 (1.00, 1.14) | 0.070                 | 3137     | 164           | 1.02 (0.95, 1.09) | 0.801 |
| Poultry               |                   |                       |                   |       |          |               |                   |                       |          |               |                   |       |
| Actual                | 1.02 (1.00, 1.03) | 0.089                 | 1.01 (0.99, 1.03) | 0.497 | 3080     | 160           | 0.99 (0.97, 1.00) | 0.069                 | 3072     | 160           | 0.98 (0.97, 1.00) | 0.061 |
| Predicted             | 1.24 (1.12, 1.37) | 1.2 × 10 <sup>4</sup> | 1.07 (0.95, 1.22) | 0.450 | 3080     | 160           | 0.84 (0.72, 0.97) | 0.037                 | 3072     | 160           | 0.87 (0.75, 1.01) | 0.155 |
| Metabolites           |                   |                       |                   |       |          |               |                   |                       |          |               |                   |       |
| 2-Hydroxyvaleric acid | 1.60 (1.34, 1.92) | 2.3 × 10 <sup>6</sup> | 1.47 (1.19, 1.82) | 0.005 | 3145     | 164           | 1.09 (0.93, 1.27) | 0.363                 | 3137     | 164           | 1.06 (0.90, 1.25) | 0.740 |
| Carnitine 4:0         | 1.29 (1.11, 1.49) | 1.4 × 10 <sup>3</sup> | 1.18 (0.99, 1.41) | 0.230 | 3145     | 164           | 1.28 (1.10, 1.49) | 0.004                 | 3137     | 164           | 1.21 (1.04, 1.42) | 0.052 |
| Carnitine 8:1         | 1.41 (1.21, 1.65) | 6.8 × 10 <sup>5</sup> | 1.09 (0.90, 1.31) | 0.503 | 3145     | 164           | 1.60 (1.35, 1.89) | 1.1 × 10 <sup>6</sup> | 3137     | 164           | 1.37 (1.15, 1.64) | 0.004 |
| Carnitine 8:2         | 1.23 (1.05, 1.44) | 0.018                 | 1.01 (0.84, 1.23) | 0.885 | 3145     | 164           | 1.55 (1.31, 1.84) | 4.5 × 10 <sup>6</sup> | 3137     | 164           | 1.37 (1.15, 1.63) | 0.004 |
| Carnitine 10:3        | 1.37 (1.17, 1.61) | 2.4 × 10 <sup>4</sup> | 1.23 (1.02, 1.49) | 0.139 | 3145     | 164           | 1.43 (1.20, 1.70) | 3.4 × 10 <sup>4</sup> | 3137     | 164           | 1.27 (1.07, 1.51) | 0.044 |
| LPC 17:1              | 0.71 (0.61, 0.83) | 8.3 × 10 <sup>5</sup> | 0.88 (0.73, 1.06) | 0.353 | 3145     | 164           | 0.82 (0.72, 0.93) | 0.010                 | 3137     | 164           | 0.85 (0.74, 0.97) | 0.052 |
| PC 15:0/18:2          | 0.73 (0.62, 0.85) | 2.4 × 10 <sup>4</sup> | 0.82 (0.68, 0.99) | 0.164 | 3145     | 164           | 0.93 (0.80, 1.08) | 0.418                 | 3137     | 164           | 1.01 (0.87, 1.18) | 0.922 |
| Piperettine           | 1.13 (0.97, 1.32) | 0.124                 | 1.05 (0.87, 1.27) | 0.700 | 3145     | 164           | 1.10 (0.93, 1.29) | 0.356                 | 3137     | 164           | 1.03 (0.87, 1.21) | 0.894 |
| RN_222.114@408.52     | 1.46 (1.24, 1.72) | 2.6 × 10 <sup>5</sup> | 1.38 (1.13, 1.68) | 0.011 | 3145     | 164           | 1.39 (1.19, 1.63) | 3.2 × 10 <sup>4</sup> | 3137     | 164           | 1.27 (1.08, 1.50) | 0.025 |
| RN_394.3337@642.16    | 1.39 (1.18, 1.64) | 2.5 × 10 <sup>4</sup> | 1.18 (0.97, 1.45) | 0.288 | 3145     | 164           | 1.17 (1.01, 1.36) | 0.063                 | 3137     | 164           | 1.09 (0.93, 1.26) | 0.451 |
| RN_488.194@420.53     | 1.14 (0.97, 1.33) | 0.124                 | 0.92 (0.76, 1.12) | 0.503 | 3145     | 164           | 1.22 (1.03, 1.43) | 0.037                 | 3137     | 164           | 1.11 (0.94, 1.32) | 0.381 |
| RN_864.5774@743.37    | 0.63 (0.53, 0.74) | 8.7 × 10 <sup>7</sup> | 0.69 (0.56, 0.83) | 0.003 | 3145     | 164           | 0.75 (0.65, 0.88) | 1.2 × 10 <sup>3</sup> | 3137     | 164           | 0.86 (0.73, 1.01) | 0.141 |
| RP_215.0225@216.66    | 1.16 (1.00, 1.35) | 0.069                 | 1.16 (0.97, 1.39) | 0.288 | 3145     | 164           | 1.27 (1.08, 1.49) | 0.011                 | 3137     | 164           | 1.22 (1.04, 1.44) | 0.052 |
| RP_284.1282@505.51    | 0.98 (0.85, 1.14) | 0.825                 | 0.99 (0.82, 1.18) | 0.885 | 3145     | 164           | 0.99 (0.85, 1.16) | 0.920                 | 3137     | 164           | 1.02 (0.87, 1.19) | 0.922 |
| RP_332.262@539.74     | 1.25 (1.07, 1.47) | 0.011                 | 1.11 (0.92, 1.35) | 0.450 | 3145     | 164           | 1.07 (0.91, 1.26) | 0.457                 | 3137     | 164           | 1.00 (0.86, 1.18) | 0.960 |

HR, hazard ratio; (L)PC, (lyso)phosphatidylcholine; OR, odds ratio; RN, reversed-phase chromatography negative polarity; RP, reversed-phase chromatography positive polarity; SMCC, Clinical subcohort of Swedish Mammography Cohort; VIP, Västerbotten Intervention Program.

<sup>1</sup> Predicted meat intake was based on elastic net models comprising 15 metabolites with significant associations with meat intake in both VIP and SMCC. Associations between metabolites and the risk of T2D were assessed using conditional logistic regression in discovery study (VIP) and Cox regression in replication study (SMCC) with number of event referring to the number of T2D incidences during follow-up. Model 1 was adjusted for age, smoking, physical activity, energy, and alcohol intake, with additional adjustment for sex in VIP. Model 2 include further adjustment for BMI. False discovery rate (FDR) < 0.05 was considered significant.

modest predictive power ( $Q2 < 0.1$ ). Further attempts to refine intake biomarkers, to validate the prediction models for total meat and poultry in other cohorts, to assess the application of the multimarker panel in controlled intake (eg, in randomized controlled trials), and to discover intake biomarkers of unprocessed meat intake are hence warranted.

Acylcarnitines 4:0, 8:1, 8:2, and 10:3 and an unidentified metabolite (RN\_222.114@408.52) were consistently associated with higher T2D risk, whereas LPC 17:1 and PC 15:0/18:2 were consistently associated with lower T2D risk in both data sets prior to adjustment for BMI, except for PC 15:0/18:2, which was only significant in VIP. Although causality cannot be inferred in this study, the consistent associations between the acylcarnitines positively with processed meat intake and higher T2D risk and with the phospholipids containing odd-chain fatty acids negatively with processed meat intake and with lower T2D risk may suggest lipid metabolism as a potential mediating pathway between processed red meat intake and higher T2D risk. Processed meat may impact the metabolite profile differently from unprocessed meat for several reasons: First, processed meat may contain compounds added or formed during curing and heating processes with potentially adverse effects on health, such as salt, N-nitroso compounds, heterocyclic aromatic amines, polycyclic aromatic hydrocarbons, and their derivatives [63]. Moreover, consumption of processed meat may coexist with unhealthy behaviors and lifestyle [64], which may influence gut microbiota, host–microbiota crosstalk, inflammation, and lipid metabolism in several ways, for example, due to fat intake, fat quality, or fat sources, and eventually, metabolite profiles. Noteworthy, most of these associations were attenuated after further adjustment for BMI, which can be explained by the correlations between most metabolites and BMI in both cohorts. These correlations may at least partially explain the disappearing associations of the metabolites with T2D risk after adjustment for BMI, although we cannot ascertain the causal role of BMI as either a mediator or a confounder in this study. Further mechanistic investigations would, however, be necessary to establish causal relationship between the consumption of processed meat, these metabolites, BMI, and T2D risk.

This study has several strengths. First, both the discovery and replication cohorts were prospective in a free-living population, with extensive information on habitual diet at baseline and follow-up of T2D incidence, hence providing temporality for the association of diet and metabolites with risk of developing disease. The observed associations between both meat intake and T2D risk in the discovery cohort were replicated in another independent cohort, which effectively reduced the likelihood of false discovery. The untargeted metabolomics approach applied in this study was performed with a stringent QC procedure. The variable selection was performed using both multivariate and univariate approaches, which enabled a robust selection of important metabolites associated with both meat intakes and T2D risk. This procedure led to the discovery of novel metabolites with no previously established association with meat consumption, for example, 2-hydroxyvaleric acid. Noteworthy, the 2-hydroxyvaleric acid was associated with poultry in the discovery cohort and with other meat groups in replication cohort, which deserves further attention.

This study also had limitations. Cross-sectional assessment of meat intake and metabolites may be subject to spurious association and potential false discovery. The dietary assessment using FFQ may be prone to recall bias and misestimation of eating frequencies and portion sizes, which may dilute the observed associations. Similarly, hamburgers were included as processed meat in VIP and unprocessed meat

in SMC, which might have weakened the observed associations. Despite the efforts to adjust for potential covariates, associations among meat intake, plasma metabolome, and T2D risk may still be affected by unmeasured confounding. Consequently, we cannot rule out the possibility that discovered metabolites could more accurately reflect other dietary or lifestyle factors coexisting with meat intake instead of meat intake per se. The replication study had metabolomics data from RP chromatography only, which skewed the replication attempt toward amphiphilic compounds and effectively limited the possibility to replicate potential biomarker candidates from HILIC. In addition, the replication cohort included only females, imposing limits on the generalizability of the findings. Moreover, a controlled intervention study in concert with the use of targeted methods toward these novel biomarkers would be required to further validate the findings. Despite the rapid progression of spectral libraries and annotation strategies, metabolite annotation remains a major bottleneck in untargeted metabolomics studies. Hence, the unidentified metabolites were reported with all known information to enable further elucidation of their identities and structures.

## Conclusion

In this study, 15 metabolites, 13 newly discovered and 2 replicated from literature, were found to reflect meat consumption in 2 independent Swedish population-based cohorts. Most of these metabolites reflected the consumption of processed meat, with less consistent associations for poultry, total, and unprocessed red meat. Processed meat intake was associated positively with short-chained and medium-chained acylcarnitines and negatively with certain phospholipids. The acylcarnitines were also associated with higher T2D risk, whereas the phospholipids were associated with a lower risk, although the associations were attenuated after additional adjustment for BMI. These findings suggest that lipid metabolism may underpin the associations between processed red meat intake and a higher risk of T2D, with potential involvement of BMI in these associations. The low number of biomarker candidates that could be replicated highlights the challenges in achieving replicable intake biomarkers, which need to be considered in future studies. More efforts to replicate the findings and to identify especially the unidentified metabolites are necessary to establish a multimarker panel of meat intake biomarkers with improved specificity before its application in further studies aimed to elucidate the complex association between diet and health.

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## Author contributions

The authors' responsibilities were as follows – RL, CB: designed the study; IJ: processed dietary data for VIP; ML, KH: managed the LC-MS analysis; AJ, LS: conducted initial data analysis under supervision of CB and RL; SN: conducted the study including the final data analysis; SN, ML: performed the annotation of metabolite features; SN: wrote the manuscript; and all authors: read, provided scientific input, and approved the final version of the manuscript.

## Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Ethics statement

The study protocol for VIP was approved by the Regional Ethics Committee in Uppsala with registration number 2014/011. The study protocol for SMCC was approved by the Regional Ethics Committee in Stockholm with registration number 2006/1490-31/1. The biobank and responsibility for SMCC were then moved to Uppsala under registration number 2017/232 and 2018/261. All participants provided written informed consent prior to participation.

## Data availability

According to the general data protection regulation (GDPR), participants' information and metabolomics data cannot be made available for public. However, data are available for researchers by submitting electronic application through SIMPLER infrastructure ([www.simpler4health.se](http://www.simpler4health.se)) for SMCC data. R packages used in this study are publicly available.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ajcnut.2024.02.012>.

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