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**The effect of general adiposity and central body fat distribution on the
circulating metabolome: a multi-cohort non-targeted metabolomics
observational and Mendelian randomization study**

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ABSTRACT

Obesity is associated with adverse health outcomes, but the metabolic effects have not yet been fully elucidated. We aimed to investigate the association between adiposity with circulating metabolites and to address causality with Mendelian randomization (MR). Metabolomics data was generated by non-targeted ultra-performance liquid-chromatography coupled to time-of-flight mass-spectrometry in plasma and serum from three population-based Swedish cohorts: ULSAM (N=1,135), PIVUS (N=970), and TwinGene (N=2,059). We assessed associations between general adiposity measured as body mass index (BMI) and central body fat distribution measured as waist-to-hip ratio adjusted for BMI (WHRadjBMI) with 210 annotated metabolites. We employed MR analysis to assess causal effects. Lastly, we attempted to replicate the MR findings in the KORA and TwinsUK cohorts (N=7,373), the CHARGE consortium (N=8,631), the Framingham Heart Study (N=2,076) and the DIRECT consortium (N=3,029). BMI was associated with 77 metabolites, while WHRadjBMI was associated with 11 and 3 metabolites in women and men, respectively. The MR analyses in the Swedish cohorts suggested a causal association (p -value <0.05) of increased general adiposity and reduced levels of arachidonic acid, dodecanedioic acid and lysophosphatidylcholine (P -16:0) as well as with increased creatine levels. The replication effort provided support for a causal association of adiposity on reduced levels of arachidonic acid (p -value 0.03). Adiposity is associated with variation of large parts of the circulating metabolome, however causality needs further investigation in well-powered cohorts.

INTRODUCTION

The increasing prevalence of obesity is a major health problem, as epidemiological as well as clinical evidence state that both general and central obesity constitute strong risk factors for several adverse health outcomes including cardiovascular disease and type 2 diabetes^{1, 2}. The metabolic effects of increased adiposity are not yet fully understood. Metabolomics methods can detect and quantify small compounds such as sugars, amino acids, organic acids, nucleotides and lipid molecules (up to ~1,500 Da) in biological samples and can be used to assess metabolic effects of different exposures. Contemporary metabolomics approaches include gas chromatography (GC) and ultra-performance liquid chromatography (UPLC) coupled with mass spectrometry-based methods (MS) or nuclear magnetic resonance (NMR) spectroscopy methods³. MS approach may entail greater sensitivity than NMR and thus has the potential to discover a larger number of metabolites⁴. Previous metabolomics studies have shown that increased adiposity is associated with many different metabolites such as increased levels of circulating aromatic amino acids, branched-chain amino acids, and certain fatty acids⁵⁻⁷.

Mendelian randomization (MR) is a framework to study the causal effects of modifiable exposures on different phenotypes, where genetic variants are used as instrumental variables for the exposures of interest. As the random assortment of genetic variants occurs at the time of conception, these variants are not affected by confounding or reverse causation⁸. Previous MR studies examining the causal relationship between general adiposity, which was measured as body mass index (BMI), and metabolomics as measured by NMR^{9, 10}, have indicated that increased adiposity has an impact on the levels of multiple circulating metabolites, including lipoproteins, branched-chain and aromatic amino acids, and inflammation-related glycoprotein acetyls. A recent study in TwinsUK (mainly females)⁷ found that up to a third of metabolites measured by MS methods were associated with obesity. However, in their genetic analysis, the

BMI associated genetic risk score based upon 97 BMI predisposing variants was not found to be associated with any specific metabolite.

Another approach to investigate causal effects of adiposity on the metabolome is through repeated measurements of the metabolome in weight-loss trials. Two weight-loss intervention studies, where one studied calorie restriction only, while the other focused on either calorie restriction only, calorie restriction combined with physical activity or calorie restriction combined with a centrally acting serotonin-norepinephrine reuptake inhibitor approved for weight loss, found that weight loss was associated with increased plasma levels of medium- and long-chain acylcarnitines^{11, 12}. Another study of 57 women allocated to an intensive lifestyle weight-loss program or control group showed that three months of lifestyle intervention led to higher levels of 3-hydroxybutyrate (3-HB), formate, methylguanidine, myo-inositol, and phosphocreatine as well as lower levels of proline and trimethylamine¹³.

However, more studies of the effect of general adiposity and body fat distribution on the circulating metabolome are necessary to better understand the metabolic consequences of obesity. The aim of the current study was therefore twofold, 1) to identify circulating metabolites measured with MS associated with adiposity and central body fat distribution, and 2) to investigate if these associations were due to a causal effect of adiposity.

MATERIALS AND METHODS

Cohorts

Three population-based Swedish cohorts were utilized. The Uppsala Longitudinal Study of Adult Men (ULSAM) cohort enrolled male residents born between 1920 to 1924 in Uppsala, Sweden at age 50 (N=2,322) and includes several in-person assessments over nearly 50 years¹⁴. The current study uses information and metabolomics profiling from the 1,135 individuals participating in the investigation at age 70. The Prospective Investigation of Vasculature in Uppsala Seniors (PIVUS) study (N=970) enrolled a random sample of both women and men, at age 70, from the residents of Uppsala in 2001. Participants were invited for repeated tests at age 75 and 80 also, and metabolomics profiling was performed on the 970 samples from the age 70 assessment. The TwinGene cohort includes a total of 12,591 twins born in Sweden before 1958¹⁵. The current study used a sample (N=2,059) that underwent metabolomics profiling in samples from 2004-2008 and with complete information on covariates, average age 68.6 (SD 8.3)¹⁶. In the subsampling strategy only one twin per pair were prioritized and few complete twin pairs were therefore included¹⁶. Metabolite measurements underwent a log₂-transformation followed by SD transformation.

The Cooperative Health Research in the Region of Augsburg (Kooperative Gesundheitsforschung in der Region Augsburg, KORA) is a population-based cohort from southern Germany that includes prospectively measured health assessment and blood samples collections between 2006-2008¹⁷. The current study (KORA F4) is based upon 1,768 participants and mean age 60.8 ± 8.8 years for whom the metabolomics assessment were performed. TwinsUK is a mainly (93%) women cohort of twins recruited from the UK. The current study is based upon 6,056 participants with a mean age of 53.4 ± 14.0 years who were analyzed for the metabolite profiling¹⁷. Summary GWAS data from meta-analysis of KORA

and TwinsUK¹⁷ were obtained from public repositories and were expressed as the per-allele log₁₀-unit change in metabolite levels.

The CHARGE consortium GWAS for plasma phospholipid fatty acid fractions was based on samples from 8,631 individuals (age range 45.8 to 72.0 years, 55% women) from the five epidemiological cohorts¹⁸. In CHARGE consortium, arachidonic acid was expressed as percentage of total fatty acids.

The Framingham Heart Study (FHS) is a population-based cohort of European ancestry participants (age range 45.0 to 65.0 years, 51% women) from the United States. Samples from 2,076 FHS study participants underwent plasma metabolite and GWAS profiling. In the FHS cohort, GWAS analysis were conducted using normalized residuals of metabolites levels employing linear mixed effect models accounting for age and sex¹⁹.

The DIRECT (Diabetes Research on Patient Stratification) consortium (N= 3,029) includes the participants with pre-diabetes (age range 56.0 to 68.0 years, 24% women) and type 2 diabetes (age range 56.0 to 68.1 years, 42% women) from the population based cohorts across Europe²⁰. In DIRECT, metabolite levels were expressed as residuals from a linear mixed model accounting for technical variables.

All participants provided written informed consent prior to inclusion in the respective cohort study and the research was approved by the Ethics Committees of Uppsala University (ULSAM, PIVUS) and Karolinska Institutet (TwinGene), or the respective Institutional Review Boards for the replication cohorts. The study was conducted according to the principles of the Declaration of Helsinki.

Anthropometric measurements

Across all three Swedish cohorts, height was objectively measured to the nearest cm and body weight to the nearest 0.1 kg. BMI was calculated as the ratio between weight (kg) to height

(m²), and used as a proxy for general adiposity. The waist was measured as a midway between the lowest rib and the iliac crest, while the hip circumference were measured over the widest part. Waist-to-hip ratio (WHR) was calculated as the ratio between waist (cm) to hip (cm), and used as a proxy for central body fat distribution.

Measurement of metabolites

In all Swedish cohorts, samples for metabolite assessment were taken on the same day as the anthropometric measurements. Blood was drawn from the study participants with overnight fasting in all cohorts.

Swedish cohorts

The samples were treated with methanol for protein precipitation. Non-targeted metabolite profiling was carried out using ultra-performance liquid chromatography (Acquity Ultra-Performance Liquid Chromatography) (UPLC) equipped with an Acquity UPLC BEH C8 analytical column (1.7 μ m, 2.1 mm x 100 mm) coupled to a time-of-flight mass spectrometer (Waters Corporation, Milford, MA, USA) with an electrospray source operated through positive ion mode. For quality control (QC), prior to each batch of two 96-well plates of samples, instrument maintenance (cone cleaning, mass calibration, and detector gain calibration) was performed, and an external QC standard mix was injected containing 2 μ g mL⁻¹ each of caffeine, terfenadine, sulfadimethoxime, and reserpine. The QC standards were evaluated for retention time (\pm 0.05 min), signal intensity ($< 25\%$ relative standard deviation), and mass accuracy (< 3 ppm). All samples were randomized prior to instrumental analysis. Since internal standards were not available at the time of analysis, randomized duplicate injections were performed to mitigate potential within-sample variation originating from the instrumental analysis. Average peak areas of the duplicate injections were then used for the relative quantitation.

Details of the metabolomic measurement procedures across these cohorts are available in the Supplement, and have also been described previously¹⁶. Several approaches for the annotation of metabolomic features were employed using both MS and MS/MS (MSE) information in accordance with the metabolomics standard initiative (MSI)¹⁶. After peak detection and data processing, metabolomic features were matched between cohorts and metabolites were annotated based on retention time, accurate mass, and MS/MS spectra as standards. After data processing and adjustment for technical factors, intensities were scaled to SD units prior to statistical analyses. The entire processing pipeline for metabolomics measurement has been described previously²¹. Annotation of metabolites were performed using the in-house spectral library of authenticated standards and several publicly available spectral databases. The level of confidence to which a positive metabolite annotation has been performed was categorized as: (level 1) match with accurate mass (± 5 ppm), fragmentation pattern, and retention time with the in-house spectral library containing > 930 authentic standards collected under the same experimental conditions; (level 2) match based on accurate mass and fragmentation pattern using available mass spectra in public data bases without retention time information; (level 3) match based on a combination of mass spectra and fragmentation pattern knowledge, accurate mass, and retention time window to assign the metabolite to a chemical class/formula. In total, 106 out of 220 metabolites were assigned at MSI Level 1; 98 out of 220 were assigned MSI Level 2; and 4 out 220 metabolites were assigned MSI Level 3.

Replication cohorts

Metabolites were assessed in serum or plasma using liquid chromatography-tandem mass spectrometry (LC-MS) applying the Metabolon platform in KORA, TwinsUK and DIRECT¹⁷. In the CHARGE consortium, arachidonic acid was measured in plasma phospholipids through thin layer gas chromatography (except in the InCHIANTI study where arachidonic acid was

directly measured by gas chromatography)¹⁸ and expressed as percentage of total plasma fatty acids. In the FHS cohort, plasma creatine metabolite was quantified through LC-MS method using triple quadrupole mass spectrometer (Applied Biosystems/Sciex)¹⁹.

Statistical Analyses

Observational analyses of the association of general adiposity with metabolites

The analysis plan and cohorts used are described in **Figure 1**. We used a series of linear regression models to assess the association between general adiposity measured as BMI and the annotated metabolites from the ULSAM and PIVUS cohorts. In PIVUS, models were adjusted for age and sex, while for the ULSAM cohort, the models were adjusted for age only; ULSAM contains only men.

The beta coefficients from the two cohorts were meta-analyzed using the DerSimonian-Laird random effects model, details are found in Supplemental Text 2²². The random effects model was chosen as initial analysis indicated considerable heterogeneity in effect estimates between cohorts.

A 5% false discovery rate (FDR) using the Benjamini–Hochberg procedure²³ was used to account for multiple testing. Metabolites that passed the FDR threshold were assessed in TwinGene. A similar model was used, but with cluster-robust standard errors to account for dependency within twins. Out of 2,059 individuals, 75 complete twin-pairs were included. Metabolites were considered replicated in TwinGene if the estimates were directionally similar and showed a p -value of <0.05 .

Observational analyses of the association of central body fat distribution with metabolites

Similarly, we used a series of linear regression models to assess the association between body fat distribution measured as WHR and the annotated metabolites in the ULSAM and PIVUS

cohorts. All models were also adjusted for age and BMI, and run separately in men and women as the distribution of WHR is sex-specific. This model is hereafter referred to as “WHRadjBMI”. For men, estimates from ULSAM and PIVUS men samples were pooled and meta-analyzed using the DerSimonian-Laird random effects model. For women, only PIVUS samples for WHR-metabolites association analyses were used in the first step as ULSAM is men-only. The Benjamini–Hochberg procedure²³ at a 5% FDR was again used to account for multiple testing. Metabolites that passed the FDR threshold were assessed in TwinGene. A similar model was used, but cluster-robust standard errors were used to account for dependency within twins. Out of 2,059 individuals, 75 twin-pairs were included. Metabolites were considered replicated in TwinGene if estimates were in the same direction and showed a p -value of <0.05 . As a sensitivity analysis, we used waist circumference (WC), hip circumference (HC) and unadjusted waist-hip ratio as alternative measures for body fat distribution. We assessed the association of WC and HC with metabolites identified in main analysis adjusting for weight, height and age using the same sex-stratified strategy as for WHRadjBMI while WHR was only adjusted for age.

Mendelian Randomization

Metabolites associated with BMI in the observational analyses were taken forward to Mendelian randomization analyses. We performed a two-sample MR analysis where the association between the genetic instrument and adiposity was based on previous large GWAS studies^{24, 25} and the association between the genetic instrument and each metabolite was assessed in ULSAM, PIVUS, TwinGene, KORA and TwinsUK.

We created three genetic instruments, one for BMI (sex-combined) and two sex-specific instruments for WHRadjBMI (male and female) based on two large GWAS studies^{24, 25}. BMI and WHRadjBMI was expressed as Z-scores in these GWAS, derived from inverse normal transformation of residuals from a regression adjusted for age, age-squared, study-specific

covariates if necessary. The WHR-phenotype was additionally adjusted for BMI. For the BMI-instrument, we included independent SNPs showing association (p -value $<5 \times 10^{-8}$) with BMI in sex-combined analysis²⁴. For each of the two sex-specific WHRadjBMI instruments, we included SNPs that showed association with WHRadjBMI if they a) had p -value $<5 \times 10^{-8}$ in sex-combined and p -value $<2.5 \times 10^{-4}$ in sex-specific analysis or b) p -value $<5 \times 10^{-8}$ in sex-specific analysis. The BMI instrument thus included 97 SNPs, the female WHRadjBMI instrument included 47 SNPs and the male WHRadjBMI instrument included 22 SNPs. Both for SNP-BMI²⁴ (estimates were taken from Locke *et al*²⁴ Table 1, Extended Table 2 and S4 Euro Sex Combined) and SNP-WHRadjBMI²⁵ (estimates were taken from Shungin *et al*²⁵ Table 1 and Supplementary Table 4) estimates were taken based upon the results from the European ancestry sample. A complete list of included genetic variants along with allele frequencies regarding BMI among sex combined while WHRadjBMI traits among females and males in the ULSAM, PIVUS and TwinGene cohorts are reported in **Tables S1, S2 and S3**, respectively. Variants were extracted using PLINK 2.0 (<http://pngu.mgh.harvard.edu/~purcell/plink/>) from ULSAM, PIVUS and TwinGene. Alleles were aligned to the reported obesity-increasing allele. We used both directly genotyped SNPs and imputed SNPs using Hap Map imputation (ULSAM and PIVUS cohorts) or 1000 genome imputation panel (TwinGene) variants. All SNPs had minor allele frequency $>1\%$, Hardy-Weinberg p -value > 0.01 and MACH2 imputation metric >0.95 .

We investigated the association of each SNP with each metabolite using linear regression models in each Swedish cohort separately, with subsequent random-effects meta-analysis across all three Swedish cohorts. The genetic effects were assumed to be additive. All analyses were adjusted for age, sex and the first four genetic principal components from each of three cohorts. Cluster-robust standard errors (twin-pair as cluster) were used in the TwinGene cohort. For WHRadjBMI, analyses were performed separately among women and men.

The multiplicative random effects inverse variance weighted²⁶ (IVW) method was used to estimate causal effects as the main MR analysis. Mendelian randomization-Egger (MR-Egger) and weighted median regression (WMM) methods were used as sensitivity analyses²⁶. The MR-Egger method can be used to detect and adjust for directional pleiotropy, while the robust WMM method provides consistent estimates as long as at least 50% of the weights are based upon valid (non-pleiotropic) instrumental variables^{26,27}. MR analyses were performed using the MendelianRandomization package in R studio (R version 3.6.0 (<https://www.r-project.org/>)). In MR analyses, we considered p -values <0.05 as statistically significant.

Pathway enrichment analysis

As an extended analysis, we performed enrichment analysis using the “fast gene set enrichment analysis” tool to identify groups of metabolites enriched for genetic associations of obesity variants with single metabolites. We followed the same MR pipeline as previously, except that no observational analysis was used for filtering out non-significant metabolites. The p -values from the MR analysis from the Swedish cohorts were then carried forward to fast gene set enrichment analysis (FGSEA) as implemented in the R-package *fgsea*. At least 10 metabolites had to be present in a metabolite class for the FGSEA analysis to be performed.

Replication

We attempted replication of MR results for metabolites with p -value <0.05 in the Swedish cohorts. Each replication cohort only had a few of the metabolites of interest available. The SNP-metabolite associations from replication cohorts were aligned to the BMI-increasing allele. Causal MR estimates were obtained using IVW while the MR sensitivity analyses were performed through MR-Egger and WMM methods. We applied the Stouffer p -value-based meta-analysis²⁸ to pool Z-scores from each replication cohort derived from one-sided p -values, accounting for directionality of the individual cohort estimate.

Statistical analyses were performed using Stata 15.0 (Stata, College Station, TX, USA) and R studio (R version 3.6.0) (<https://www.r-project.org/>), unless otherwise noted.

Data and Resource Availability

The data that support the findings of this study from ULSAM, PIVUS and TwinGene are available but restrictions apply to the availability of these data, which were used under license for the current study and therefore are not publicly available. Data are however available from the authors upon reasonable request and with permission of ULSAM, PIVUS and TwinGene steering committees and with permission of the Swedish Ethical Review Authority. Data from KORA and TwinsUK are available here: <http://metabolomics.helmholtz-muenchen.de/gwas/>. Data from the CHARGE consortium is publically available for download http://www.msi.umn.edu/~wguan/CHARGE_N6GWAS/. Summary data for the association of SNPs with creatine was retrieved from the FHS¹⁹. GWAS-metabolites data from the DIRECT consortium is publically available at DOI: 10.5281/zenodo.4475681²⁰.

RESULTS

Baseline characteristics of the study participants are reported in **Table 1**.

Observational analyses of the association of general adiposity with metabolites

In the meta-analysis of PIVUS and ULSAM, we found that BMI was associated with 109 out of 210 tested metabolites (**Table S4**), whereof 77 metabolites were replicated in TwinGene (**Table S5**). Of these 77 metabolites, BMI was inversely associated with 13/15 lysophosphatidylcholines, and positively associated with 8/8 glycerolipids, 6/7 unsaturated fatty acids, 4/6 amino acids, peptides, and derivatives, 3/3 carnitines and acyl carnitines, and 5/5 bile acids.

Observational analyses of the association of central body fat distribution with metabolites

In the meta-analysis of men from PIVUS (N=483) and ULSAM (N=1,112), WHRadjBMI was associated with 32 metabolites (**Table S6**), whereof only three metabolites were replicated in TwinGene (N=1,167) (**Table S7**). In women from PIVUS (N=487), WHRadjBMI was associated with 47 metabolites. Eleven of those metabolites were replicated in the female sample of TwinGene (N=879). Of these, positive associations were found for 7/7 glycerolipids (**Table S7**).

In the sensitivity analyses, most associations were comparable for the alternate exposures WC and unadjusted WHR with the exception of sphingomyelin(32:2). The association of sphingomyelin(32:2) with WHRadjBMI seems driven by association with HC adjusted for weight and height rather than WC (**Table S8**).

Mendelian Randomization – general adiposity

In the meta-analysis of estimates in the ULSAM, PIVUS, and TwinGene cohorts, we found results consistent with a causal effect of general adiposity reducing levels of arachidonic acid,

dodecanedioic acid and lysophosphatidylcholine(P-16:0) (**Table 2**). We also observed evidence consistent with a causal effect of general adiposity on increased creatine levels (**Table 2**). We observed that dodecanedioic acid and lysophosphatidylcholine (P-16:0) had a strong negative correlation with BMI while arachidonic acid and creatine were positively correlated (**Table S11**).

Mendelian Randomization – body fat distribution

We found evidence of a causal effect of central body fat distribution on decreased levels of sphingomyelin(32:2) in women (**Table 2**). No findings were present in men.

Pathway enrichment analysis

We were unable to identify any enrichment for metabolite class-wise associations for BMI or WHRadjBMI in the Mendelian Randomization (**Table S12**).

Replication

Next, we attempted to replicate MR results in four independent studies with data for three of the metabolites with $p < 0.05$ in the Swedish cohorts. Genetic association results were available for arachidonic acid results and creatine in three cohorts, and for dodecanedioic acid in one cohort. We observed directionally consistent results for all tested metabolites in all cohorts compared to the Swedish cohorts except for creatine in FHS (**Table 2**). Meta-analysis provided support of replication for causal effect of adiposity on lowering arachidonic acid (p -value 0.03) but not for creatine (p -value 0.25).

Sensitivity analyses

The sensitivity analysis using MR-Egger and WMM yielded wide confidence intervals but with point estimates in general agreeing with the IVW method. For arachidonic acid, we observed directionally similar beta coefficients for WMM and MR-Egger methods in the Swedish cohorts

(**Table S9**), KORA/TwinsUK, the CHARGE but not in the DIRECT consortium compared to IVW estimates (**Table S10**). For creatine, we observed directionally consistent beta coefficients for WMM and MR-Egger methods in the Swedish cohorts (**Table S9**), KORA/TwinsUK, the DIRECT (not for MR-Egger) and the FHS (not for WMM) compared to IVW estimates (**Table S10**). **Supplemental Figure 1A-D** show scatter plots of the ratio estimates for genetic associations between SNP-BMI and SNP-metabolites among the Swedish and the replication cohorts when available, which allow visualization of the causal effect estimates. **Supplemental Figure 2A** represent scatter plots of the ratio estimates for genetic associations between SNP-WHRadjBMI and SNP-metabolites (sphingomyelin(32:2)) among the Swedish cohorts.

DISCUSSION

In this study of middle-aged and elderly men and women, we found associations of BMI with large parts of the measured metabolome. We identified 77 BMI-metabolite associations, 11 WHRadjBMI-metabolite associations in women, and 3 WHRadjBMI-metabolite associations in men. We noted that the direction of association was similar within chemical classes of metabolites. Of the 77 BMI-associated metabolites, BMI was inversely associated with 13/15 lysophosphatidylcholines, and positively associated with 8/8 glycerolipids, 6/7 unsaturated fatty acids, 4/6 amino acids, peptides, and derivatives, 3/3 carnitines and acyl carnitines, and 5/5 bile acids. Our findings further provided some evidence of causal association of general adiposity on lower levels of arachidonic acid.

Comparison with literature

A previous²⁹ study comparing metabolites measured with MS to different measures of adiposity found that metabolites showed similar relationships with BMI and waist circumference adjusted for hip circumference. Their main findings included positive associations with four amino acids and two sphingomyelin while negative correlations with LysoPCs, and mixed directions for phosphatidylcholines. In our study, we adjusted all WHR analyses for BMI, which may explain that few metabolites overlapped between the phenotypes.

Unsaturated Fatty acids

In observational analysis, we found positive associations between BMI with six out of seven unsaturated fatty acids including arachidonic acid. Arachidonic acid, a derivative of linoleic acid, is an omega-6 polyunsaturated fatty acid generally present in the human cell membrane, and the majority of arachidonic acid-related metabolites are pro-inflammatory³⁰.

However, our MR analyses suggested an inverse association of adiposity on arachidonic acid. In the Swedish cohorts, and in KORA/TwinsUK as well as the DIRECT study, the levels of

arachidonic acid represent free circulating arachidonic acid, while in CHARGE the % of arachidonic acid were measured in phospholipids (PL). The discrepancies between observational analysis and MR directions as found in our study, might be explained by bidirectional effects or in the case of confounding of the observational estimate. Previously published literature about the relationship between obesity and circulating arachidonic acid is inconclusive. Some studies report higher plasma phospholipid arachidonic acid in obese children³¹ and adults³² and other have reported lower erythrocyte phospholipid arachidonic acid in obese³³. The potential biological mechanisms for how genetic predisposition to higher BMI would lower circulating arachidonic acid indicated in our MR analyses are unclear. However, we speculate that it might involve lowered amounts of the enzyme delta-5-desaturase (D5D), which is encoded by the *FADS1* gene and converts the precursor dihomo-gamma-linolenic acid into arachidonic acid. Surrogate measures of D5D activity were indeed found negatively correlated with BMI when assessed in circulating PL^{34, 35}. Other regulators of arachidonic acid levels are the amount of linoleic acid, the enzymes delta-6-desaturase, encoded by the *FADS2* gene, and elongases.

We found some evidence of a causal effect of general adiposity on reduced dodecanedioic acid levels in the Swedish data, but the results were not replicated in external data. Reduced levels of dodecanedioic acid has been observed in obese children compared with non-obese controls⁵. Dodecanedioic acid is a water soluble even-numbered dicarboxylic acid and structurally similar to medium chain free fatty acids. The metabolic pathway of these fatty acids substances are intermediate to lipids and carbohydrates³⁶.

Amino acids, Peptides, and Derivatives

In observational analyses, BMI was positively associated with four of the amino acids, peptides, and derivatives while negatively associated with two of the metabolites from this class. In observational and MR analyses BMI was positively associated with creatine in the Swedish

cohort, but these MR results were not replicated in external data. Creatine is an important metabolite for cellular energetics within skeletal muscle and plays a critical role in muscle catabolism during different physiological processes through increasing the ability of muscles to generate more ATP from ADP to match energy demands³⁷. Increasing body fat percentage, BMI, and waist circumference have previously been associated with higher creatine levels³⁸, likely due to larger muscle mass. Similarly, in a lifestyle intervention study, weight loss was associated with decreased creatine¹³. Hence, our observational and MR results in the Swedish data align well with previous reports, but MR results need to be confirmed.

Sphingomyelins

Sphingomyelin is one of the major lipids in the mammalian plasma membrane, and changes in membrane lipid composition not only changes structure but also membrane function and receptor reorganization³⁹. In a previous study of the sphingomyelin profile of Korean middle-aged men with early stages of diabetes, most species of sphingomyelins were increased in subjects with abdominal obesity compared to those who were lean⁴⁰. In the current study, we observed that BMI was associated with reduced levels of two sphingomyelins. Similarly, in the current study we found preliminary evidence for increased central body fat distribution lowering sphingomyelin 32:2 in women but no external replication data was available. However, sensitivity analysis using alternative measures of body fat distribution indicated that the identified association with WHRadjBMI was driven by increased hip circumference rather than reduced waist circumference. We are not aware of any similar studies in women and our findings need to be validated before drawing further conclusions.

Lysophosphatidylcholines

Lysophosphatidylcholines (LysoPC) are the major components of oxidized low-density lipoprotein cholesterol. In the current study, we found inverse associations between BMI and

thirteen LysoPC and positive association with two. In MR analyses in the Swedish data, we found evidence that increased adiposity was causally associated with reduced levels of LysoPC (P-16:0). However, no replication data was identified, and these results need confirmation. Most observational studies have reported inverse associations between adiposity and LysoPC. Reduced LysoPC levels have been observed after weight loss⁴¹ while other studies observed non-significant differences in LysoPC species following weight reduction¹¹. LysoPC (P-16:0) levels has positively been associated with neonatal birth weight⁴².

Strengths and limitations

The strengths of the study include the combination of cross-sectional analysis with Mendelian randomization analyses and objectively assessed anthropometric measures. However, a few potential limitations deserve mentioning. First, the study size only yielded moderate statistical power to detect causal effects of obesity on metabolites, and also the non-targeted metabolomics approach only captured a limited set of metabolites. Replication data availability was limited. Second, all analyses were conducted in Europeans who were of older age and therefore our results may have limited generalizability to other ethnicities and age groups. The limited number of annotated metabolites are due to the single liquid chromatography setting and by somewhat limited availability of in-house standards. Another limitation is related to the measurements of central body fat distribution. We explored alternative measures of body fat distribution for our top findings from the main analysis. We observed comparable results for most metabolites with the alternative waist-related measures but few associations with hip circumference supporting waist-related adiposity as the main link to these metabolites. Sphingomyelin (32:2) was exception to this pattern where the inverse associations with WHRadjBMI were driven by increased hip circumference. Furthermore, although the causal associations between adiposity and arachidonic acid were mostly consistent across different MR methods, our findings require validation using physiological models, particularly to rule

out any potential biases related to unmeasured horizontal pleiotropy and canalization. Another potential limitation is that, the non-targeted metabolomics platform was biased towards detection of metabolites that can be separated with reverse-phase liquid chromatography and that are readily detected in positive electrospray ionization mode, i.e., various phospholipids, fatty acids, acyl-carnitines, non-polar amino acids and their derivatives as well as several other semi-polar metabolites such as for example imidazopyrimidines (caffeine and theobromine) and indoles (indolelactic acid, 3-indolepropionic acid). Another potential limitation is that the external validation data set DIRECT consortium includes participants with prediabetes and patients having newly diagnosed type 2 diabetes, while the participants of all other cohorts were predominantly disease free. Since metabolites were measured on a relative scale and with different normalization procedures in Swedish (ULSAM, PIVUS and TwinGene), KORA/TwinsUK cohorts, CHARGE and DIRECT consortia, effect size comparison between the Swedish cohorts, the KORA/TwinsUK, CHARGE and DIRECT consortia are not possible, hence, *p*-value based meta-analysis was performed. The effect estimates were in general larger for WHRadjBMI compared to BMI. This is due to the difference in the unit of measurement for BMI and WHR. For SNP-BMI and WHRadjBMI association analysis z-score transformation was used. Some metabolite measurements may be affected by difference in matrix (plasma or serum), which may have limited the power of the study. In our previous studies, we have, however, been able to replicate a large proportion of associations with disease phenotypes across the three Swedish cohorts^{43, 44}. Lastly, although we aimed to assess the causality between adiposity and annotated metabolites, we did not assess reverse causality, mainly because no specific genetic variants are available for the majority of studied metabolites.

Conclusions

In summary, we confirmed previous findings that adiposity are associated with large parts of the circulating metabolome. In the MR analysis, our findings suggested a causal association of

increased general adiposity and reduced levels of arachidonic acid. Our findings regarding arachidonic acid need to be verified in larger sample, and investigated in relation to cardiometabolic disease to understand whether they represent pathways of obesity causing diabetes and cardiovascular disease. Circulating arachidonic acid was not associated with future risk of CVD in a large observational study⁴⁵. However, plasma phospholipid arachidonic acid has shown some evidence for increasing risk of ischaemic heart disease, ischaemic stroke, and peripheral artery disease in an Mendelian randomization analysis, but our results do not support that obesity aggravates this pathway as our results indicate that obesity lower circulating arachidonic acid⁴⁶. Although we observed robust observational associations of BMI with a large number of metabolites, the explained variance of these circulating metabolites was in general low. In summary, larger studies including genetic data, metabolomics, anthropometric measurements and follow-up for incident diabetes and cardiovascular events are needed to tease out whether metabolites mediate some of the increased risk of cardiometabolic disease in obesity.

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Author Contributions: SA contributed to the study design, conducted statistical analysis writing first draft of the manuscript. UH contributed to the study design and conducted statistical analysis. SS assisted in the data analysis, metabolomics annotation and contributed to the writing of manuscript. LL, JS, UR and PKEM provided data and reviewed, revised, and approved the manuscript. UH, BK, AG, JÄ, CB and SCL reviewed, revised, and approved the manuscript. TF contributed to the study design, provided data, wrote, reviewed, revised and approved the manuscript. SA and TF are the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of Statement: J.Ä has served on advisory boards for AstraZeneca and Boehringer Ingelheim and have received lecturing fees from AstraZeneca and Novartis, all unrelated to the present project. No other potential conflicts of interest relevant to this article were reported.

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Figure 1. Schematic flow and cohorts used in the current analysis

Table 1 Clinical Characteristics of the ULSAM, PIVUS and TwinGene Swedish Cohorts

	ULSAM	PIVUS	TwinGene
N total	1135	970	2059
% men	100	50	58
Age, years	70.9 (0.6)	70.2 (0.2)	68.6 (8.3)
BMI, kg/m ²	26.3 (3.4)	27.1 (4.3)	26.3 (4.0)
WHR	0.95 (0.05)	0.90 (0.7)	0.91 (0.1)
Waist (cm)	94.9 (9.6)	90.1 (15.7)	93.6 (11.9)
Diastolic blood pressure (mmHg)	79.6 (8.6)	78.6 (10.2)	82.0 (10.6)
Systolic blood pressure (mmHg)	140.0 (16.3)	149.7 (22.6)	142.7 (20.2)
HDL-cholesterol (mmol/l)	1.3 (0.3)	1.5 (0.4)	1.4 (0.4)
LDL-cholesterol (mmol/l)	3.9 (0.9)	3.4 (0.9)	3.7 (1.0)
Triglycerides (mmol/l)	1.5 (0.8)	1.3 (0.6)	1.4 (0.8)
Total cholesterol (mmol/l)	5.8 (1.0)	5.4 (1.0)	5.7 (1.2)

All values (except N total and % men) were represented as mean (SD).

Table 2. Observational and causal associations between BMI and metabolites across the combined sample (N=3,610) of ULSAM, PIVUS and TwinGene and causal WHRadjBMI and metabolomics signatures among women in the combined sample (N=1,158) of PIVUS and TwinGene. Validation of BMI-associated metabolites in the KORA/TwinsUK cohorts (N=7,373), the CHARGE consortium (N= 8,631), the DIRECT consortium (N= 3,029) and FHS cohort (N=2,076).

Observational analysis					Mendelian randomization									
Metabolites	ULSAM and PIVUS		TwinGene		Swedish cohorts		KORA/TwinsUK		CHARGE consortium		DIRECT consortium		FHS cohort	
	Beta (95% CIs)	P	Beta (95% CIs)	P	Beta (95% CIs)	P	Beta (95% CIs)	P	Beta (95% CIs)	P	Beta (95% CIs)	P	Beta (95% CIs)	P
Dodecanedioic acid	-0.04 (-0.05, -0.02)	4.1E-10	-0.02 (-0.04, -0.01)	3.8E-06	-0.35 (-0.57, -0.12)	2.0E-03	-0.03 (-0.06, 0.004)	0.25	NA		NA		NA	
Lysophosphatidylcholine(P-16:0)	-0.05 (-0.07, -0.04)	3.2E-09	-0.04 (-0.05, -0.03)	3.4E-11	-0.29 (-0.52, -0.05)	0.02	NA		NA		NA		NA	
Arachidonic acid	0.02 (0.01, 0.03)	4.4E-03	0.02 (0.01, 0.04)	1.3E-04	-0.26 (-0.48, -0.04)	0.02	-0.01 (-0.03, 0.01)	0.48	-0.25 (-0.51, 0.003)	0.05	-0.03 (-0.16, 0.20)	0.60	NA	
Creatine	0.02 (0.01, 0.03)	6.0E-03	0.02 (0.01, 0.03)	5.9E-03	0.25 (0.01, 0.50)	0.04	0.02 (-0.01, 0.05)	0.13	NA		0.07 (-0.06, 0.09)	0.30	-0.21 (-0.49, 0.08)	0.16
WHRadjBMI associated Metabolites (Females)	Beta (95% CIs)	P	Beta (95% CIs)	P	Beta (95% CIs)	P								
Sphingomyelin(32:2)	-2.46 (-3.78, -1.14)	3.0E-04	-0.71 (-1.30, -0.12)	0.02	-0.51 (-0.90, -0.13)	8.0E-03	NA		NA		NA		NA	

Mendelian randomization estimates were retrieved using the inverse variance weighted method. Units of metabolites are not similar in discovery and replication cohorts. For the SNP-metabolites association analysis, in the Swedish cohorts log2 and then SD transformation was applied. In the KORA/TwinsUK cohorts for GWAS-metabolites analysis, log10-unit change in metabolite levels were used. In the CHARGE consortium, GWAS results for SNP association with arachidonic acid expressed as percentage of total fatty acids. In the DIRECT consortium, GWAS-metabolites association analysis were residualised after removing technical covariates using a linear mixed model. In the FHS cohort, GWAS-creatine analyses were performed using normalized residuals of creatine levels, adjusted for age and sex. NA – not available.