A Mendelian randomization study of metabolite profiles, fasting glucose and type 2 diabetes

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Abstract

Mendelian randomization (MR) provides us the opportunity to investigate the causal paths of metabolites in type 2 diabetes and glucose homeostasis. We developed and tested an MR approach based on genetic risk scoring for plasma metabolite levels, utilizing a pathway-based sensitivity analysis to control for non-specific effects. We focused on 124 circulating metabolites which correlate with fasting glucose in the Erasmus Rucphen Family study (n = 2,564) and tested the possible causal effect of each metabolite with glucose and type 2 diabetes and vice versa. We detected fourteen paths with potential causal effects by MR, following pathway based sensitivity analysis. Our results suggest that elevated plasma triglycerides might be partially responsible for increased glucose level and type 2 diabetes risk, which is consistent with previous reports. Additionally, elevated high-density lipoprotein (HDL) components i.e. S-HDL-triglycerides might have a causal role of elevating glucose levels. In contrast, large (L) and extra-large (XL) HDL lipid components i.e. XL-HDL-cholesterol, XL-HDL-free cholesterol, XL-HDL-phospholipids, L-HDL-cholesterol and L-HDL-free cholesterol as well as HDL-cholesterol seem to be protective against increasing fasting glucose, but not against type 2 diabetes. Finally, we demonstrate that genetic predisposition to type 2 diabetes associates with increased levels of alanine, and decreased levels of phosphatidylcholine alkyl-acyl C42:5 and phosphatidylcholine alkyl-acyl C44:4. Our MR results provide novel insight into promising causal paths to and from glucose and type 2 diabetes and underline the value of additional information from high resolution metabolomics over classical biochemistry.

Introduction

Type 2 diabetes is a progressive metabolic disease characterized by hyperglycemia, initially as a result of insulin resistance and in later stages also as a result of insulin insufficiency. Type 2 diabetes is also associated with dyslipidemia, including higher circulating concentrations of triglycerides and lower concentrations of high-density lipoprotein (HDL) cholesterol. In addition, several circulating molecules have previously been shown to be dysregulated in type 2 diabetes, including phospholipids, branched-chain amino acids, keto-acid metabolites and other metabolites such as acyl-carnitines (1-3). However, the causal paths between these metabolites and glucose/type 2 diabetes in human remain unclear from observational studies, and require randomized controlled trials that are difficult to conduct.

As an alternative, Mendelian randomization (MR) is an instrumental variable method that has gained in popularity over the last decade, to investigate causal effects of traits using genetic predictors. MR uses the principle that the allocation of genetic variants, that affect a specific trait, from parents to offspring is random and unrelated to factors other than the trait (4). Furthermore, associations between the genotype and the outcome will not be affected by reverse causation because disease will occur after the meiosis. MR has previously been used to determine whether metabolic markers such as classical blood lipids are causally involved in type 2 diabetes (5-11) and has yielded contradicting results. One reason for this could be that these studies are affected by the heterogeneous nature of the metabolic markers chosen, such as in the example of total HDL cholesterol, which in reality consists of a collection of different sized HDL particles possibly with different functions. This may dilute the causal effects of SNPs when only combined (total) HDL is

considered. However, false signals may also be due to pleiotropic effects of the chosen genetic variants leading to possibly invalid instrumental variables. As high-throughput analyses techniques improve, the quantification of circulating molecules is becoming ever more detailed and precise. For instance, instead of LDL-cholesterol, HDL-cholesterol and total triglycerides (TG) determined by routine clinical biochemistry, lipoprotein particle size distribution and content as well as tens of biochemical components can now be measured using Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS) based approaches (12; 13). These additional measures offer an opportunity to gain novel insight into the pathogenesis of diseases like type 2 diabetes. With the knowledge of genetic determinants of metabolites gained from genome-wide association studies (GWAS) (14-16), one can use MR for causal inference given the specific conditions encoded in Figure 1. In the present study, with the aim of unraveling potentially causal metabolic paths that underlie the observed associations, we used genetic predictors from published metabolite GWAS, guided by pathway-based evidence to select instrumental variables, and performed MR between selected metabolic markers and glucose/type 2 diabetes.

Materials and Methods

Study population

The observational associations between metabolites and fasting glucose/type 2 diabetes were tested in the Erasmus Rucphen Family (ERF) study which is a prospective family based study with 3,465 individuals in the Southwest of the Netherlands. The study protocol for ERF was approved by the medical ethics board of the Erasmus Medical Center Rotterdam, the Netherlands (17). The baseline demographic data and

measurements of the ERF participants were collected between 2002 and 2006. Venous blood samples were collected after at least eight hours fasting. The detailed description of the ERF study and related measurements were reported previously (17). Type 2 diabetes was defined according to a fasting plasma glucose ≥ 7·0 mmol/L and/or anti-diabetic treatment. The analytical sample included 2,564 non-diabetic and 212 diabetic participants.

Metabolite measurements

Metabolic markers were measured by five different metabolomics platforms using the methods which have been described in earlier publications (15; 16; 18; 19). In total 562 metabolic markers including sub-fractions of lipoproteins, triglycerides, phospholipids, ceramides, amino acids, acyl-carnitines and small intermediate compounds, which throughout this article will be referred as "*metabolites*", were measured either by NMR spectrometry or by MS. The platforms used in this research are: (1) Liquid Chromatography-MS (LC-MS, 116 positively charged lipids, comprising of 39 triglycerides, 47 phosphatidylcholines, 8 phosphatidylethanolamines, 20 sphingolipids, and 2 ceramides, available in up to 2,638 participants) measured in the Netherlands Metabolomics Center, Leiden using the method described before (18); (2) Electrospray-Ionization MS (ESI-MS, in total 148 phospholipids and sphingolipids comprising of 16 plasmologens, 72 phosphatidylcholines, 27 phosphatidylethanolamines, 24 sphingolipids, 9 ceramides, available in up to 878 participants), measured in the Institute for Clinical Chemistry and Laboratory Medicine, University Hospital Regensburg, Germany using the method described previously (14); (3) Small molecular compounds window based NMR spectroscopy (NMR-COMP, 41 molecules comprising of 29 low-molecular weight

molecules and 12 amino acids available in up to 2,639 participants) measured in the Center for Proteomics and Metabolomics, Leiden University Medical Center (19; 20); (4) Lipoprotein window-based NMR spectroscopy (NMR-LIPO, 104 lipoprotein particles size sub-fractions comprising of 28 VLDL components, 30 HDL components, 35 LDL components, 5 IDL components and 6 plasma totals, available in up to 2,609 participants) measured in the Center for Proteomics and Metabolomics, Leiden University Medical Center and lipoprotein sub-fraction concentrations were determined by the Bruker algorithm (Bruker BioSpin GmbH, Germany) as detailed in Kettunen *et al* (16); (5) AbsoluteIDQTM p150 Kit of Biocrates Life Sciences AG (153 molecules comprising of 14 amino acids, 91 phospholipids, 14 sphingolipids, 33 acyl-carnitines and hexose available in up to 989 participants) measured as detailed in publication from Draisma *et al* (15) and the experiments were carried out at the Metabolomics Platform of the Genome Analysis Center at the Helmholtz Zentrum München, Germany as per the manufacturer's instructions. The laboratories had no access to phenotype information.

Statistical methods

We assessed the pairwise partial correlation between each metabolite and each glycemic trait (i.e. fasting glucose, fasting insulin, HOMA-IR, BMI and WHR) in the non-diabetic participants group. We included age, sex and lipid lowering medication as covariates. Bonferroni correction was applied based on the number of independent vectors in the data. By the Matrix Spectral Decomposition (MSD) method (21), we estimated 191 independent vectors using the pairwise bivariate correlation matrix of the 562 metabolites. A P-value $< 5.24 \times 10^{-5}$ (0.05/191/5) adjusted by number of independent vectors and number of outcomes was used as the threshold for metabolome-wide significance. The

metabolites associated with glucose in the ERF study were taken forward (n = 124) as candidates for MR. In this set of 124 metabolites, we also tested the association with type 2 diabetes using logistic regression.

Mendelian randomization

For each metabolite associated with glucose, we performed two-sample bi-directional MR. The same method on two-sample MR has been performed in the previous MR studies on type 2 diabetes (6; 9; 22). We tested if genetically varying levels of a particular metabolite affect the risk for elevated glucose and type 2 diabetes (we call this the *forward* approach) and if genetically increased risk of type 2 diabetes or elevated glucose is associated with circulating levels of a particular metabolite (we call this the *backward* approach). The associations between the instrumental variables and the exposure and the outcome are estimated from different studies, either the metabolite GWAS (14-16; 19) or fasting glucose/type 2 diabetes GWAS published by MAGIC and DIAGRAM consortium (23; 24), using the genetic risk score method. The effect of the genetic risk score was constructed by summing up the weighted effects of genome wide significant SNPs on the exposure variable, in relation to their effects on the outcome, as detailed in a previous publication (6). This was performed using summary statistics level data utilizing the method described by Dastani *et al* (25) and implemented in the R-package "*gtx*". Figure 2 shows the overview of the instrumental variable construction. All SNPs were mapped to human genome build hg19. Given that MR assumes no pleiotropic effect beyond that on the risk factor of interest (i.e. exposure), we excluded the top SNPs from previously published body-mass index (BMI) and WHR GWAS (26; 27), and any SNPs within a 1 Mbp window distance of these, from the genetic score. We additionally excluded the

genetic loci (1 Mbp window) of glucose, type 2 diabetes, insulin and HOMA-IR extracted from previous publications (23; 24; 28) in the *forward* MR and the genetic loci (1 Mbp window) of the particular metabolite of interest, using the published GWAS information in the *backwards* MR (see Supplemental Table 1 for list of genetic loci excluded at this stage). We restricted the SNP lists to a set of independent SNPs in low linkage disequilibrium (pairwise $R^2 < 0.05$) for each test (29) based on the genotype data in ERF. SNPs with disproportionate effects in the risk score were excluded to reduce pleiotropy (see Supplemental Table 2 for list of SNPs excluded at this stage). Genetic risk scores comprising > 5 SNPs which explain > 1% of variance in exposure were taken forward. This effort yielded 20 metabolite-glucose/type 2 diabetes sets in the *forward* MR and 76 glucose-metabolite sets and 79 type 2 diabetes-metabolite sets in the *backward* MR. A false discovery rate (FDR) of 0.05 was used as the significance threshold for the four series (i.e. metabolite-glucose, metabolite-type 2 diabetes, glucose-metabolite and type 2 diabetes-metabolite series).

Pathway-based sensitivity analysis

Although we applied several restrictions on the SNPs in the genetic risk scores as explained above, the instrumental variable assumption that the locus is associated with the outcome only via the association with the exposure (Figure 1) is still hard to justify in practice. We harnessed the extensive background biological knowledge available to make the additional semi parametric assumption to get the MR estimates of the causal effect. That is, for each set, we evaluated whether we could identify the gene in proximity to the locus that could explain the change in exposure levels. If a gene codes for an enzyme that catalyzes the exposure or a related compound, or if it is present in a signaling cascade

that affects the exposure, we assumed that the link between the instrumental variable and the exposure was direct and not mediated by the outcome. For the *forward* approach, we checked the biological link between the locus and the target metabolite and for the *backward* approach the link with glucose. As the pathway in the disease type 2 diabetes is complex, we did not check the biological link with type 2 diabetes in the *backward* approach. If the gene directly links to the exposure, the related SNPs are taken forward to calculate the genetic risk score. Then, MR is performed for any genetic risk score (comprising > 5 SNPs) which explains > 1% of variance in exposure. To explore potential mechanistic links between the locus and the exposure, we used an automated workflow that was developed in house to gather gene-specific knowledge of all genes in proximity to each locus. In detail, we downloaded a number of online databases from the respective ftp servers and integrated them offline in Matlab®. Subsequently, for each SNP we selected genes within a window of 100kbp, with coordinates based on the dbSNP (30), and NCBI Gene (http://www.ncbi.nlm.nih.gov/gene; GRCh37), and genes whose expression is affected by the locus (GTEx-eQTL). Then, for each gene we gathered protein-related knowledge from UniProtKB (31) and affected pathways from ConsensusPathDB (32). Finally, for each protein we investigated metabolic activity by checking if it concerned a transporter protein in TCDB (33), or enzyme in ExPASy (34), and, if so, what the catalyzed metabolic reaction was in the KEGG database (35). For the KEGG database the last freely available version was used. The database integration pipeline generated one HTML file for each locus, containing gene-specific knowledge and hyperlinks to the original database entries, which was then inspected for finding a mechanistic link with the exposure. The strength of this approach is that it identifies loci

for which the instrumental variable assumption can be validated using genetic and biochemical evidence from online databases. We have successfully applied this workflow in earlier studies (19; 36-38). Heemskerk *et al* gives the best example of the power of our method (37), where we re-analyzed published results of a GWAS on metabolite levels (39) and confirmed the annotation by an *in vitro* experiment.

Results

Observed associations

Characteristics of the present study population are given in Table 1. Participants with type 2 diabetes were older, tended to be more often male, and more likely to be on lipid-lowering medication. They had higher BMI, WHR, systolic blood pressure, triglycerides, fasting glucose, insulin, HOMA-IR, and lower HDL-cholesterol, adiponectin and LDL-cholesterol.

We identified 124 metabolites that observationally associate with fasting glucose in the control population with P-value $< 5.24 \times 10^{-5}$ (Figure 3). These consisted of 36 phospholipids (Figure 3A), 20 triglycerides (Figure 3B), 24 small molecular compounds (Figure 3C) and 44 lipoprotein particle sub-fractions (Figure 3D). Correlation coefficients, P-values as well as the overlap with previous research for all 124 metabolites are given in Supplemental Table 3. A clustered heatmap of correlation structure in-between the 124 selected metabolites are shown in Supplemental Figure 1. Among the 124, 112 of them also associated with type 2 diabetes ($P < 0.05$), and their associations with type 2 diabetes and glucose were in the same direction. In addition to that, their associations with BMI, WHR, fasting insulin and HOMA-IR were in line with the direction of their associations with glucose. Out of the 124 metabolites, 90 of them correlated positively and 34

correlated negatively with fasting glucose. We observed negative correlation between glucose and alkyl-acyl and diacyl phosphatidylcholines, mostly of the poly-unsaturated type, lysophosphatidylcholines, mostly of the saturated type and parts of the lipoprotein sub-fractions from LDL and HDL. These lipoprotein sub-fractions particularly consisted of lipid components of extra-large (XL) and large (L) LDL particles, XL-HDL and L-HDL particles as well as total HDL measurements. The second cluster of metabolites which we observed to correlate positively with glucose included several phospholipids; phosphatidylethanolamines, and lysophosphatidylcholines. Amino acids and low-molecular weight compounds also correlated positively with glucose, in addition to lipid side-groups, and triglycerides. Finally, from the lipoprotein sub-fractions, small (S), extra-small (XS), medium (M) and XL-VLDL particles, as well as the total VLDL components, followed by IDL and LDL-triglycerides, XS-LDL to M-LDL particle components, as well as the ApoA1 and triglyceride content of S-HDL particles were correlated positively with fasting glucose in the non-diabetic population.

Mendelian randomization

Table 2 shows the significant results from the association of the relevant metabolites with fasting glucose using MR. Among the 20 eligible metabolite-glucose/type 2 diabetes sets, genetically decreased levels of eight metabolites associated significantly with fasting glucose (FDR < 0.05). These include XL-HDL-cholesterol (FDR = 0.03), XL-HDL-phospholipids (FDR = 2.76×10^{-3}), XS-VLDL-phospholipids (FDR = 0.04), XL-HDL-free cholesterol (FDR = 0.01), L-HDL-cholesterol (FDR = 0.01), L-HDL-free cholesterol (FDR = 2.76×10^{-3}), HDL-cholesterol (FDR = 0.04), and IDL-phospholipids (FDR = 0.04). After the pathway-based subset analysis, a causal role for IDL-phospholipids was

not supported (FDR = 0.17). At the same time, pathway-based sensitivity analysis revealed possibly causal roles for three additional metabolic markers, including S-VLDL-triglycerides (FDR = 0.04), S-HDL-triglycerides (FDR = 0.04), and plasma-triglycerides (FDR = 0.04). Table 3 shows the suggested causal effects of metabolites on type 2 diabetes, i.e. XS-VLDL-phospholipids, IDL-phospholipids and plasma-triglycerides. Interestingly, the statistical significance for both XS-VLDL-phospholipids and IDL-phospholipids in the initial results are filtered out after the sensitivity analysis (FDR: XS-VLDL-phospholipids 0.03 *vs* 0.31; IDL-phospholipids 0.01 *vs* 0.24), while plasma-triglycerides shift to being borderline significant (FDR = 0.07 *vs* 0.046). The results from the full lists of performed *forward* MR tests are given in Supplemental Table 4 and the SNPs included in the all the genetic risk scores are given in Supplemental Table 5.

The significant results of the *backward* MR are shown in Table 4. We found that genetic predisposition to type 2 diabetes is associated with lower levels of phosphatidylcholine alkyl-acyl 42:5 (FDR = 0.02) and phosphatidylcholine alkyl-acyl 44:4 (FDR = 0.02) and higher levels of alanine (FDR = 0.02). The details of all the tested SNP sets are shown in Supplemental Table 6 and Supplemental Table 7. No possible causal role for glucose was supported. As the genetic risk scores of the glucose explained less than 1% of variance, the *backward* MR with pathway analysis is not performed. Figure 4 displays the suggested paths discovered by the MR approach after the pathway-based sensitivity analysis. Overall, the associations estimated by MR were in the consistent direction with the observed associations in ERF.

Discussion

We selected 124 metabolites that are correlated with glucose in the non-diabetic

population and, using MR, we tested if this metabolic profile points to any causal paths involved in glucose level or type 2 diabetes. Combining metabolomics and MR, we detected fourteen candidate causal associations; ten metabolites influencing fasting glucose, one influencing type 2 diabetes and three influenced by type 2 diabetes.

Our initial observational association tests yielded correlation estimates within the expected range of power calculations for the 124 glucose-associated metabolites. To our knowledge, 35 of these metabolites were previously published to be associated with glucose or type 2 diabetes, including 31 concordant and 4 discordant results (Supplemental Table 3) in studies with very limited sample size (40; 41). Our significant results on subfractions of lipoproteins yielded resolution on the established association of dyslipidemia, especially for the HDL subfractions.

One of our main findings is that genetically increased cholesterol, free cholesterol and phospholipid content of circulating XL-HDL and L-HDL particles together with XS-VLDL-phospholipids associate with decreased glucose level. Our second finding is that triglyceride content of S-HDL and S-VLDL particles as well as total plasma triglycerides seem to have a glucose increasing causal effect and considering the total triglycerides, this effect has been extended to the outcome type 2 diabetes. Finally, we showed that genetic predisposition to type 2 diabetes associates with lower levels of two alkyl-acyl phosphatidylcholines and higher level of alanine. Our report is the first one using higher resolution (metabolomics driven) lipoprotein based exposure variables. Hence no other study exists for comparison except for HDL-cholesterol, LDL-cholesterol and total triglycerides which, from routine biochemistry, have been previously studied as exposure variables for MR to understand their causal effects on type 2 diabetes and glucose (An

overview is given in Table 5). Our method is similar to the method of White *et al* (9) and Fall *et al* (6) in terms of the application of the genetic risk score function utilizing the DIAGRAM/MAGIC datasets. White *et al* (9) showed that high levels of all three blood lipids (HDL-cholesterol, LDL-cholesterol and plasma triglycerides) were genetically associated with a lower risk of diabetes, although the results for triglycerides were inconsistent. However, the study did not consider the genetic variants that might be involved in the confounding phenotypes such as BMI, WHR, nor did they exclude the SNPs that are involved in type 2 diabetes directly. Fall *et al* (6) showed that the association between total HDL-cholesterol risk score and low fasting glucose was attenuated when adjusted for the effects of SNPs on LDL-cholesterol, triglycerides and surrogates of adiposity. Different from the two studies mentioned above, current MR was done in a broad spectrum of metabolites included a detailed sub-classification of lipoproteins that have not been tested before. Using such high resolution phenotypes, we demonstrate that decreasing effect of HDL-cholesterol on fasting glucose is more specific to the L-HDL or XL-HDL subclasses, whereas for S-HDL-triglycerides, an increasing effect exists. These results advocate that a higher resolution of high density in lipoproteins may reveal the observed epidemiological associations or biological functions of HDL-cholesterol more accurately and will uncover the mystery of complex lipids such as HDL. Certain HDL sub-fractions and characteristics of these sub-fractions may have independent associations with glucose, particularly for the small vs large size particles. Such a different role for HDL-triglycerides and HDL-large fractions occurred upon sleeve gastrectomy of obese patients and was associated with reduced insulin resistance and HDL remodeling (42). In addition, as experimentally shown, HDL indeed may

mediate glucose regulation in the pathophysiology of T2DM (43). Suggested mechanisms include (44): (i) Insulin secretion from pancreatic beta cells combating cellular lipid accumulation and lipotoxicity (45), endoplasmic reticulum stress and apoptosis (46; 47); (ii) Insulin-independent glucose uptake by muscle via the AMP-activated protein kinase(48), calcium/calmodulin activated protein kinase(49); (iii) and insulin sensitivity (50). The ILLUMINATE trial (51) demonstrated that in a subgroup of diabetic participants statin treatment led to increased glucose levels, while this effect was not observed in participants treated with combination of statin and CETP-inhibitor torcetrapib, suggesting that CETP inhibition and consequent HDL cholesterol elevation may improve glycemic control in diabetic patients. It is of note that *CETP* gene is a major determinant of XL-HDL and was included in our MR experiment.

We have detected three associations potentially pointing out an influence of type 2 diabetes over the metabolome. The first two are long chain polyunsaturated alkyl-acyl phosphatidylcholines which are decreased in type 2 diabetes. This is interesting considering our previous report which showed that three shorter chain polyunsaturated alkyl-acyl phosphatidylcholines are increased in type 2 diabetes patients and decreased in patients using the glucose lowering drug metformin (52). The other molecule affected by diabetes was alanine, which is a non-essential amino acid and can be synthesized in the body from pyruvate and branched chain amino acids such as valine, leucine, and isoleucine. Alanine has been previously implicated in glucose response (53). The enzyme alanine aminotransferase (ALT) catalyzes the conversion of alanine to pyruvate and glutamate and high levels of ALT indicate liver damage.

Our study differs from previous reports in three ways. Firstly, we used a bidirectional

approach and included a wide range of molecular markers to be tested, using high resolution phenotypes, measured by MS or NMR. Secondly, we exploited pathway knowledge that was gathered through an automated workflow to perform subset analysis in MR. Statistical methods that deal with pleiotropy in MR analyses, such as the Egger regression method (9; 54; 55), exist but are still being refined. They all rely on additional strong assumptions about the unobserved pleiotropy, such as the InSIDE assumption, and are sensitive to violations of these assumptions. They also suffer from a lack of power. However, one can harness the available genetic and biological knowledge in online databases in order to maximize the uniqueness of the genetic risk score for the exposure variable for this purpose and to validate the chosen instruments. It has to be mentioned that although powerful for most metabolites, our approach with the genetic and biological knowledge is also firstly conservative because it ultimately relies on the comprehensiveness of the content of the databases that are included. As a consequence, all loci for which no strong evidence is present that a nearby gene directly affects the exposure, e.g. because the involved gene is affected through a yet unknown regulatory mechanism, are excluded. Considering glucose for which the instrumental strength was initially lower compared to the others, the pathway approach yielded lower explained variance in exposure (R^2 < 1%). While one can argue that this would lead to lack of power, it may also reflect the fact that such polygenic traits like glucose may not be the most suitable exposure variables for an MR analysis. To limit this, we utilized the large-population-based GWAS of broad-spectrum metabolites and fasting glucose/type 2 diabetes with the combined instrument MR approach (25). We want to point out that although we controlled the pleiotropic effects between the outcome and exposure by (1)

excluding the known predictors, (2) heterogeneity tests and (3) finally by pathway analysis, we cannot exclude a correlation between the genetic instruments tested, especially for the HDL subfractions, for which the genes coding overlap. Whilst effect alleles were weighted by their original effects estimates from each GWAS (of exposure variables), there was strong overlap in the SNPs used for different lipid subfractions, meaning the genetic instruments were not highly specific to these subfractions.

In conclusion, using MR, the present study provide evidence for potentially causal metabolic paths of glucose homeostasis and type 2 diabetes. Our results indicate that increase of large HDL particles might have decreasing effect on glucose, while increase of small HDL particles have increasing effect, refining earlier MR findings suggesting a possible causal effect of HDL on glucose levels, as well as pointing these particles out as targets for glucose management. We further found evidence that type 2 diabetes may alter levels of alkyl-acyl phosphatidylcholines and alanine which also here can be translated into prevention of disease complications and prognosis.

Author contributions

Designed the study: CMvD and AD. Generated the metabolomics data: AV, ACH and TH. Analyzed the data: JL and JBvK. Explained the results and wrote the manuscript: JL, JBvK, SS, AV, ACH, TH, ES, KWvD, NAS, CMvD, and AD.

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1 **Table 1** Characteristics of the study population

	Controls n=2,564	Cases n=212	P-value	P-value*
Male [n (%)]	1132 (44.1)	108 (50.9)	0.059	0.20
Age (years)	48.2 ± 14.3	59.8 ± 11.8	6.4×10^{-32}	2.1×10^{-12}
Body mass index (kg/m2)	26.7 ± 4.6	30.0 ± 5.9	3.4×10^{-13}	3.7×10^{-12}
Waist-to-hip ratio	0.86 ± 0.10	0.95 ± 0.10	9.5×10^{-27}	2.6×10^{-17}
Systolic blood pressure (mmHg)	139 ± 20	154 ± 21	7.3×10^{-19}	8.2×10^{-6}
Diastolic blood pressure (mmHg)	80.3 ± 10.0	82.9 ± 9.9	4.5×10^{-4}	0.11
LDL-cholesterol (mmol/l)	3.8 ± 1.0	3.2 ± 1.0	4.8×10^{-15}	1.0×10^{-9}
Triglycerides (mmol/l)	1.2 (0.8, 1.6)	1.6 (1.1, 1.9)	2.0×10^{-10}	5.1×10^{-6}
HDL-cholesterol (mmol/l)	1.3 ± 0.4	1.1 ± 0.3	2.7×10^{-11}	5.6×10^{-8}
Fasting glucose (mmol/l)	4.5 ± 0.7	7.4 ± 2.2	9.4×10^{-44}	1.5×10^{-54}
Fasting insulin (mU/L)	11 (8, 15)	16 (11, 22)	1.2×10^{-7}	9.0×10^{-7}
HOMA-IR	2.3 (1.6, 3.1)	5.0 (3.7, 7.4)	1.5×10^{-23}	2.5×10^{-24}

Lipid lowering medication [n (%)]	265 (10.3)	99 (46.7)	7.2×10^{-20}	1.5×10^{-22}

Data are means ± standard deviations (SD), medians (inter-quartile range) or percentages. Triglycerides, fasting insulin, adiponectin, and HOMA-IR were natural logarithm transformed prior to analysis. P-value: T-test and Chi-squire test were used in continuous variables and categorical variables, respectively. P-value*: Logistic regression was used with adjusting age, sex and lipid lowering medication.

Table 2 Mendelian randomization of metabolites (exposure) on fasting glucose (outcome)

		Outcome							
		Fasting glucose				Fasting glucose*			
		R^2 (%)	n	β	FDR	R^2 (%)	n	β	FDR
Exposure	S-VLDL-triglycerides	4.80	13	0.06	0.08	3.92	10	0.08	0.04
	XS-VLDL-phospholipids	7.97	23	-0.06	0.04	6.30	15	-0.07	0.04
	IDL-phospholipids	7.16	26	-0.06	0.04	4.84	15	-0.05	0.17
	XL-HDL-cholesterol	4.25	10	-0.09	0.03	4.25	10	-0.09	0.03
	XL-HDL-free cholesterol	6.48	16	-0.09	0.01†	6.48	16	-0.09	0.01†
	XL-HDL-phospholipids	10.21	22	-0.08	2.76×10^{-3}†	9.61	19	-0.09	1.72×10^{-3}†
	L-HDL-cholesterol	7.58	17	-0.08	0.01†	7.41	16	-0.08	0.01†
	L-HDL-free cholesterol	7.58	18	-0.09	2.76×10^{-3}†	7.27	16	-0.10	1.72×10^{-3}†
	HDL-cholesterol	4.84	10	-0.07	0.04	4.67	9	-0.07	0.04
	S-HDL-triglycerides	3.97	11	0.07	0.08	3.52	9	0.09	0.04
	Plasma-triglycerides	3.93	11	0.07	0.08	2.78	7	0.10	0.04

The Mendelian randomization sets with FDR < 0.05 with respect to either outcome is shown in this table. R^2 (%): the percentage of explained variance in the exposure by genetic risk score. n: the number of SNPs in the genetic risk score. β: the weighted effect of the

genetic risk score of exposure on outcome FDR: A false discovery rate on the number of Mendelian randomization sets adjusted P-value. * results of pathway-based analysis. † the Mendelian randomization sets with P-value < Bonferroni P-value 2.5×10^{-3} (0.05/20).

Table 3 Mendelian randomization of metabolites (exposure) on type 2 diabetes (outcome)

		Outcome							
		Type 2 diabetes				Type 2 diabetes*			
		R^2 (%)	n	β	FDR	R^2 (%)	n	β	FDR
Exposure	XS-VLDL-phospholipids	8.02	23	-0.08	0.03	6.34	15	-0.06	0.31
	IDL-phospholipids	7.18	26	-0.09	0.01	4.86	15	-0.07	0.24
	Plasma-triglycerides	4.21	12	0.08	0.07	3.16	8	0.12	0.046†

The Mendelian randomization sets with either FDR < 0.05 are shown in this table. R^2 (%): the percentage of explained variance in the exposure by genetic risk score. n: the number of SNPs in the genetic risk score. β: the weighted effect of the genetic risk score of exposure on outcome. FDR: A false discovery rate on the number of Mendelian randomization sets adjusted P-value. * results of pathway-based analysis. † the Mendelian randomization sets with P-value < Bonferroni P-value 2.5×10^{-3} (0.05/20).

Table 4 Mendelian randomization of fasting glucose/type 2 diabetes (exposure) on metabolites (outcome)

		Exposure							
		Fasting glucose				Type 2 diabetes			
		R^2 (%)	n	β	FDR	R^2 (%)	n	β	FDR
Outcome	PC alkyl-acyl C42:5	0.83	13	NP	NP	1.51	32	-0.08	0.02†
	PC alkyl-acyl C44:4	1.10	15	0.02	0.95	1.51	32	-0.08	0.02
	Alanine	1.06	14	0.06	0.48	1.48	31	0.08	0.02

The Mendelian randomization sets with either FDR < 0.05 are shown in this table. PC: Phosphatidylcholine. R^2 (%): the percentage of explained variance in the exposure by genetic risk score. n: the number of SNPs in the genetic risk score. β: the weighted effect of the genetic risk score of exposure on outcome. FDR: A false discovery rate on the number of Mendelian randomization sets adjusted P-value. NP: Not performed. † the Mendelian randomization sets with P-value < Bonferroni P-value 6.33×10^{-4} (0.05/79).

23 **Table 5** Review of the previous MR in metabolites or lipids and type 2 diabetes (T2D) or glucose

Study	Methods	Exposure	Outcome	OR/β (95%CI)	P-value	Instrumental variables and pleiotropy control
Luca LA(22)	Two-sample MR	Isoleucine	T2D (n=315,571)	1.44 (1.22, 1.17)	2.0×10^{-5}	1) Independent SNPs from GWAS meta-analysis. 2)Control for pleiotropy.
		Leucine		1.73 (1.28, 2.34)	3.4×10^{-4}	
		Valine		1.45 (1.18, 1.77)	3.4×10^{-5}	
Marott SC, 2016(8)	Two-stage least-squares regression	HDL-C	T2D (n=93,097)	0.86 (0.43, 1.72)	0.68	3 variants from *ABCA1, CETP*
		TG	T2D (n=97,199)	1.05 (0.88, 1.24)	0.60	4 variants from *TRIB1, APOA5, LPL*.
White J, 2016 (9)	Conventional two-sample MR; Multivariate MR;	LDL-C	T2D (DIAGRAM)	0.79 (0.71, 0.88)	P < 0.05	1) Independent SNPs from GLGC GWAS.
		HDL-C		0.83 (0.76, 0.90)	P < 0.05	
		TG		0.83 (0.72, 0.95)*	P < 0.05	

	MR-Egger					2) *gtx* package with pleiotropic control.
Haase CL, 2015(5)	Two-stage least-squares regression	HDL-C	T2D (n=47,627)	0.93 (0.78, 1.11)	0.42	9 variants from *ABCA1, CETP, LCAT, LIPC, APOA1*.
Fall T, 2015(6)	Two-sample MR	LDL-C	T2D (DIAGRAM)	-0.03 (-0.19, 0.12)*	0.67	1) Independent SNPs with large effect on the lipid and smaller effect on other lipid fractions from GLGC GWAS. 2) *gtx* package with pleiotropic control.
			FG (MAGIC)	0 (-0.03, 0.03)*	0.85	
		HDL-C	T2D (DIAGRAM)	-0.19 (-0.38, -0.01)*	0.04	
			FG (MAGIC)	-0.02 (-0.06, 0.01)*	0.24	
Andersson C, 2015(10)	Two-stage least-squares regression	LDL-C	Incident T2D	0.85 (0.76, 0.96)	0.009	*GRS* from 37 LDL-C related SNPs without any pleiotropic control.

Islam M, 2012(11)	Two-stage least-squares regression	TG	T2D (n=2,111)	0.04 (0.014, 0.072)*	0.004	Included 10 independent SNPs from previous studies (excluded *FADS1* and *GCKR*).
De Silva NM, 2011(7)	Two-stage least-squares regression	TG	T2D (n=8,335)	0.99 (0.97, 1.01)	0.26	Included 10 independent SNPs from previous studies (excluded *FADS1* and *GCKR*).
			FG (n=8,271)	0 (-0.01, 0.01)*	0.88	

* β (95%CI).

Figure 1 Overview of the Mendelian randomization process

Figure 2 Data handling, quality checks and exclusions during Mendelian randomization

*MAGIC and DIAGRAM sets are imputed based on HapMap2 and therefore do not include indels.

Figure 3 Metabolites correlated with markers of T2DM and anthropometric risk factors

A: Phosphatidylcholines; B: Triglycerides; C: small molecules and amino acids; D: lipoproteins. The associations between metabolites and continuous variables were performed by partial correlation in the non-diabetic population. The color in the figure displays the value of correlation coefficient. The associations between metabolites and type 2 diabetes status were performed by logistic regression. The color in the figure displays the standardized effect of metabolites on type 2 diabetes. Age, sex and lipid lowering medication are considered as covariates. FG: fasting glucose. FI: fasting insulin. HOMA-IR: homeostasis model assessment of insulin resistance. BMI: body mass index. WHR: waist-to-hip ratio. *: $0.05 < \text{P-value} < 5.24 \times 10^{-5}$ (0.05/191/5). •: P-value < 0.05 & P-value $\geq 5.24 \times 10^{-5}$. (B): Selected measurement is from the Biocrates platform when the same metabolite is also captured by the LC-MS/NMR-COMP/NMR-LIPO platform. (E): Selected measurement is from the ESI-MS platform when the same metabolite is also captured by the LC-MS platform.

Figure 4 Suggested causal paths for glucose homeostasis and type 2 diabetes after pathway-based sensitivity analysis.

FG: fasting glucose; TG: triglycerides; C: cholesterol; FC: free cholesterol; P: phospholipids; PCae: phosphatidylcholine alkyl-acyl. The gene names above the metabolite names indicate the loci where the SNPs used in the genetic risk score are located.

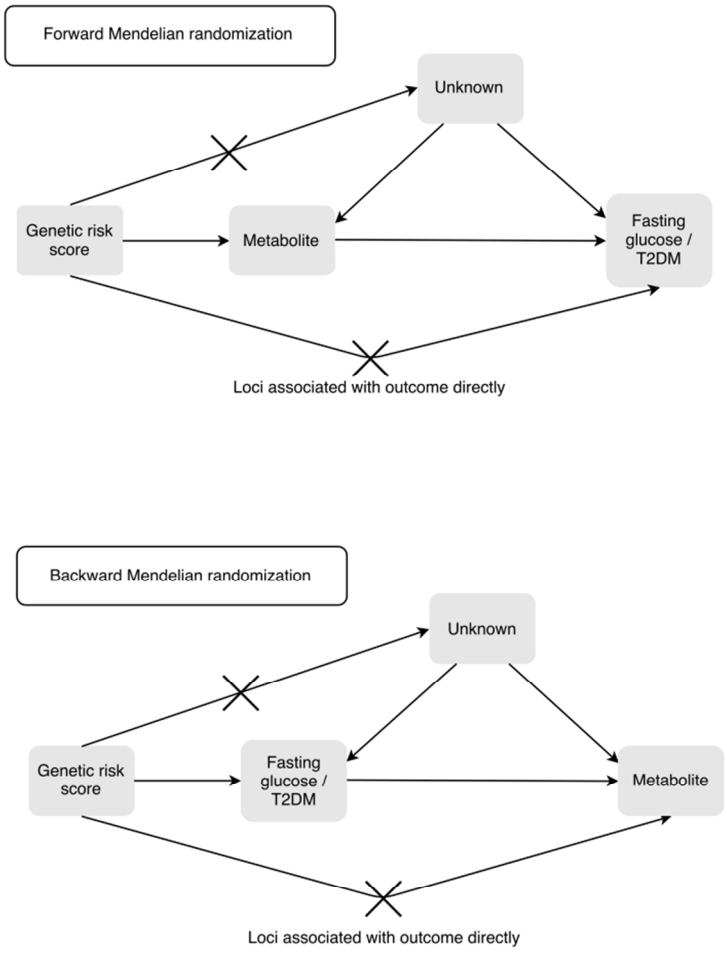

Figure 1 Overview of the Mendelian randomization process

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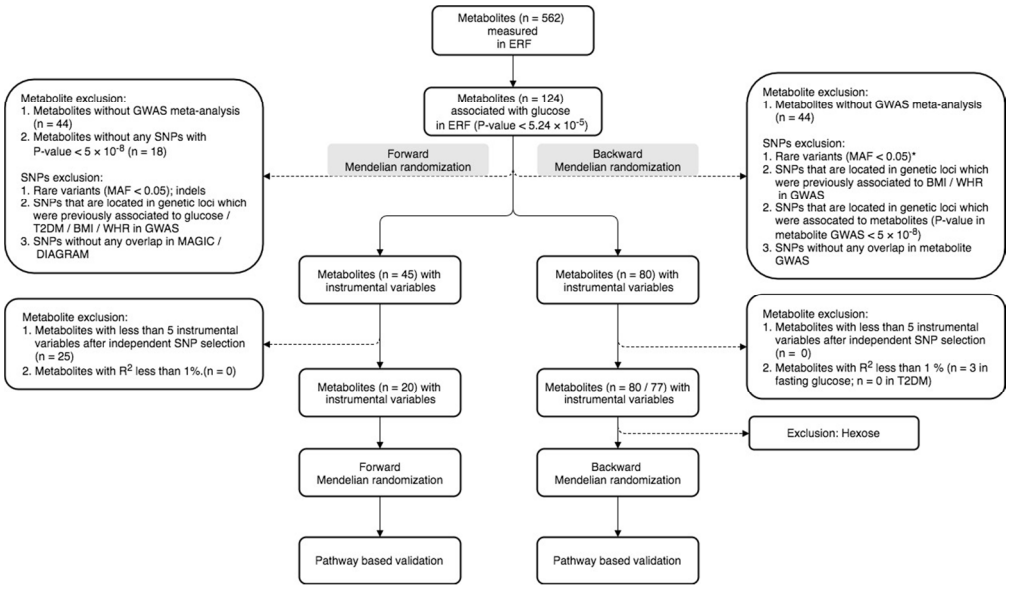


Figure 2 Data handling, quality checks and exclusions during Mendelian randomization‼ ┼ ‼ ┼ .
*MAGIC and DIAGRAM sets are imputed based on HapMap2 and therefore do not include indels.

438x253mm (72 x 72 DPI)

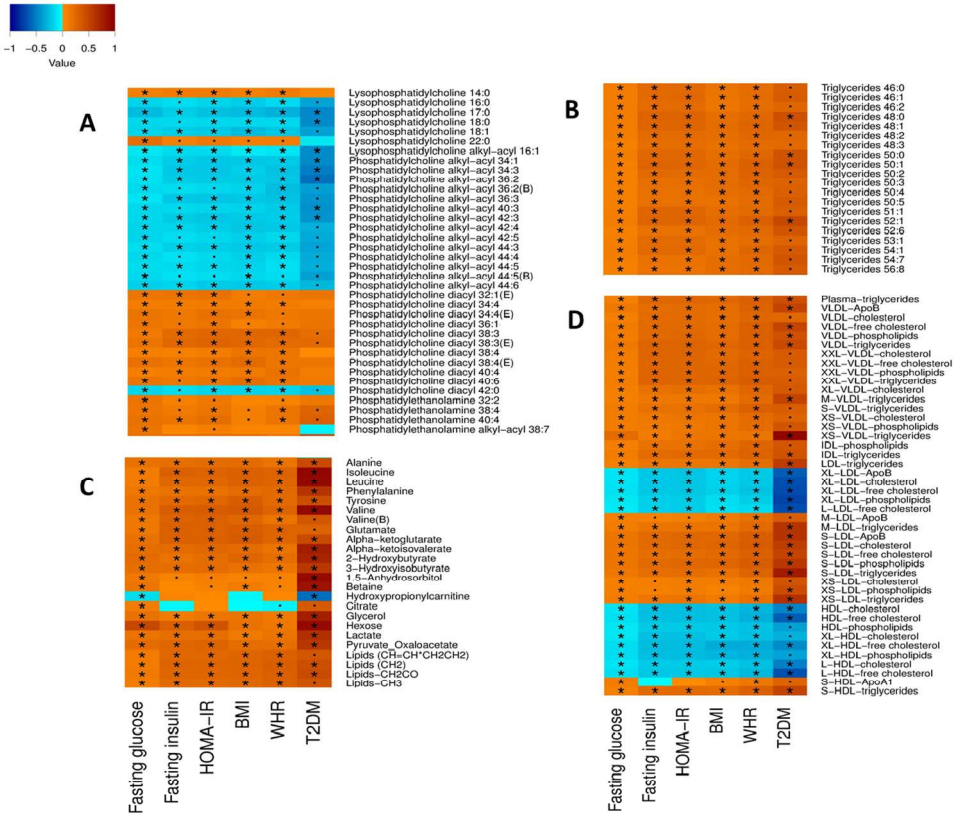


Figure 3 Metabolites correlated with markers of T2DM and anthropometric risk factors

A: Phosphatidylcholines; B: Triglycerides; C: small molecules and amino acids; D: lipoproteins. The associations between metabolites and continuous variables were performed by partial correlation in the non-diabetic population. The color in the figure displays the value of correlation coefficient. The associations between metabolites and type 2 diabetes status were performed by logistic regression. The color in the figure displays the standardized effect of metabolites on type 2 diabetes. Age, sex and lipid lowering medication are considered as covariates. FG: fasting glucose. FI: fasting insulin. HOMA-IR: homeostasis model assessment of insulin resistance. BMI: body mass index. WHR: waist-to-hip ratio. *: 0.05 < P-value < 5.24 × 10-5 (0.05/191/5). : P-value < 0.05 & P-value 5.24 × 10-5. (B): Selected measurement is from the Biocrates platform when the same metabolite is also captured by the LC-MS/NMR-COMP/NMR-LIPO platform. (E): Selected measurement is from the ESI-MS platform when the same metabolite is also captured by the LC-MS platform.

364x329mm (96 x 96 DPI)

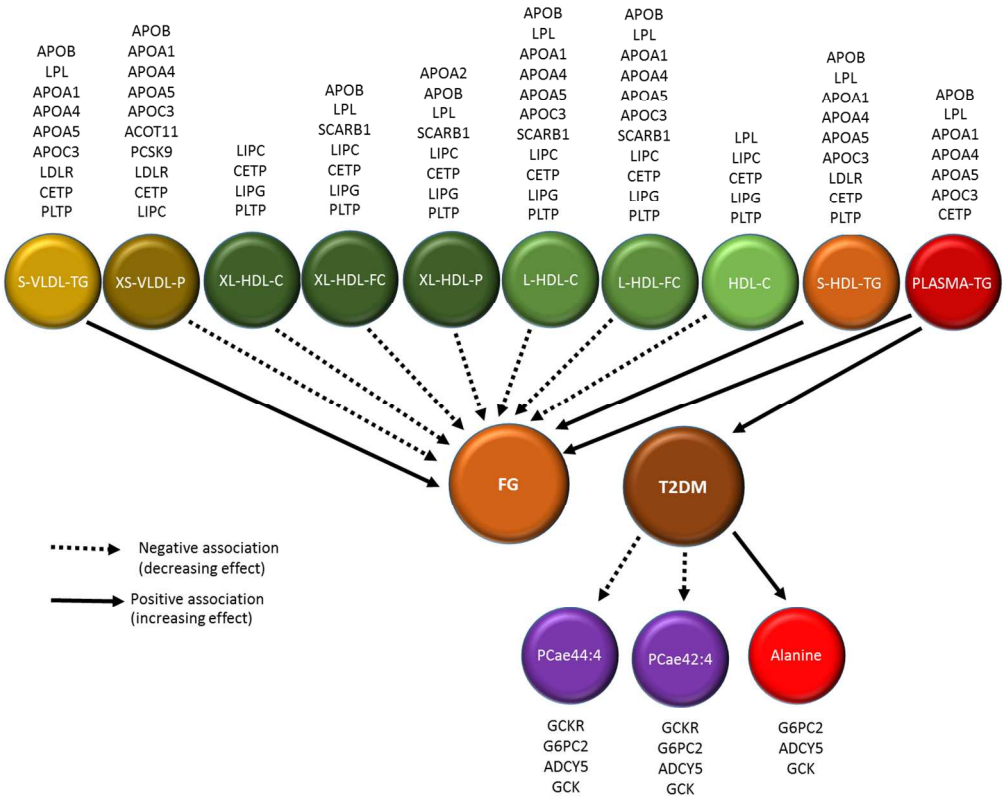
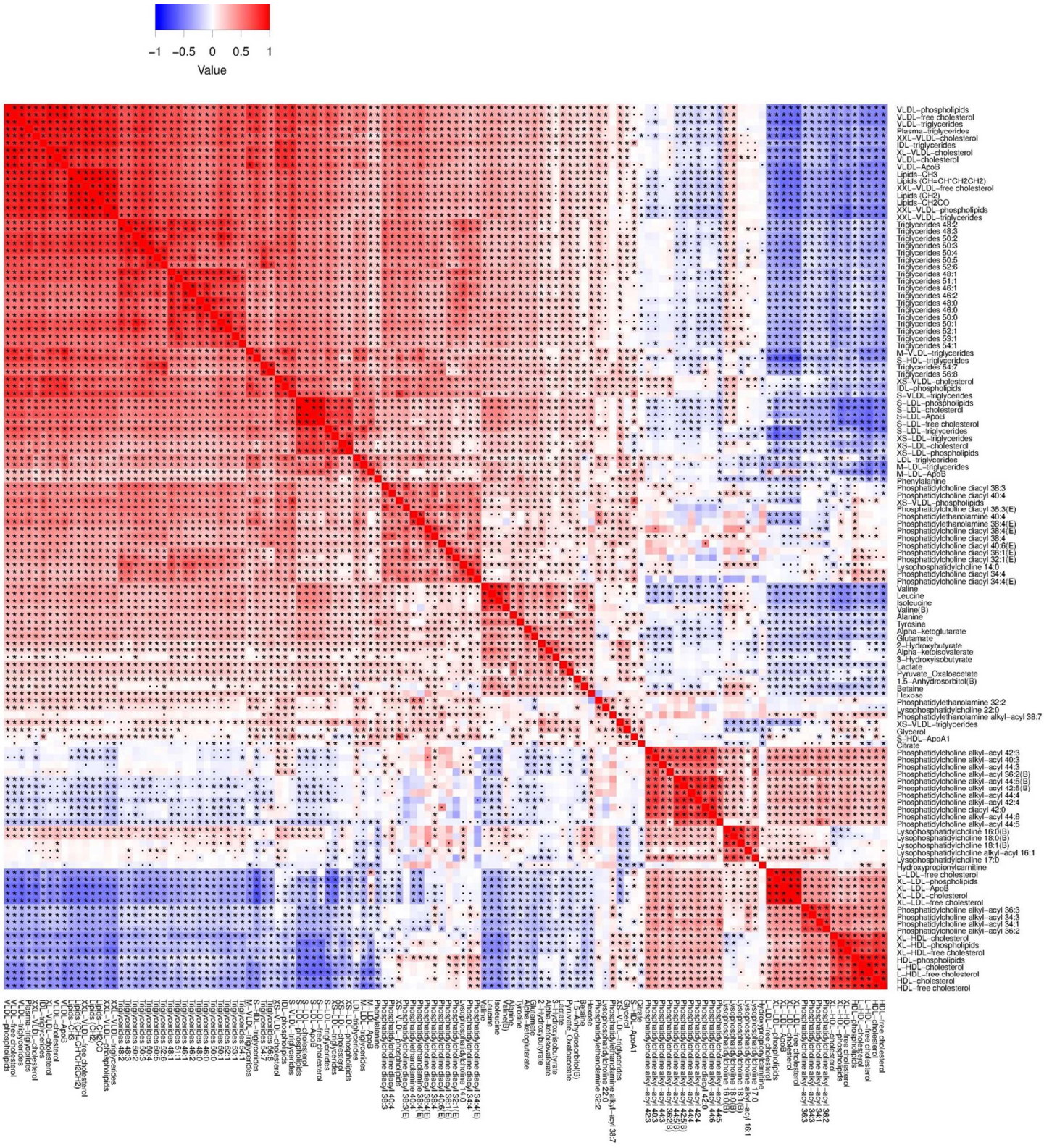


Figure 4 Suggested causal paths for glucose homeostasis and type 2 diabetes after pathway-based sensitivity analysis.

FG: fasting glucose; TG: triglycerides; C: cholesterol; FC: free cholesterol; P: phospholipids; PCae: phosphatidylcholine alkyl-acyl. The gene names above the metabolite names indicate the loci where the SNPs used in the genetic risk score are located.

393x319mm (96 x 96 DPI)

Supplemental Figure 1 Clustered correlation between the glucose-related metabolites.



The correlations between the glucose-related metabolites are performed in the non-diabetic population. The color in the figure displays the value of correlation coefficient. *: P-value < 0.001 (0.05/50), 50 is the independent equivalents from the 124 metabolites; •: correlation

P-value < 0.05 & P-value ≥ 0.001. (B): Selected measurement is from the Biocrates platform when the same metabolite is also captured by the LC-MS / NMR-COMP/ NMR-LIPO platform. (E): Selected measurement is from the ESI-MS platform when the same metabolite is also captured by the LC-MS platform.

Supplemental table 1 SNPs that are excluded from the genetic risk score because of their major association to outcome phenotypes or possible confounders BMI and WHR

BMI: body mass index. WHR: waist-to-hip ratio. T2DM: type 2 diabetes.

Please find the table (Suppl_t_1) in the link
https://www.dropbox.com/s/cd0xp33gube4rky/Supplementary_tables_LIU_J_revised.xlsx?dl=0

Supplemental table 2 SNPs that are excluded from the genetic risk score because of disproportionate weights in the genetic risk score, as detected by the *gtx* function

T2DM: type 2 diabetes. R2 (%): the percentage of explained variance in the exposure by genetic risk score.

Please find the table (Suppl_t_2) in the link
https://www.dropbox.com/s/cd0xp33gube4rky/Supplementary_tables_LIU_J_revised.xlsx?dl=0

Supplemental table 3 All 124 metabolites' correlation coefficients, p-values as well as the overlap with previous research

R: correlated coefficient. HOMA-IR: Homeostatic model assessment insulin resistance; BMI: body mass index; WHR: waist-to-hip ratio; T2DM: type 2 diabetes. ESI-MS: Electrospray Ionisation-Mass Spectrometry; LC-MS: Liquid Chromatography-Mass Spectrometry; NMR-COMP: Small Compound Nuclear Magnetic Resonance Spectroscopy; NMR-LIPO: Lipoprotein Nuclear Magnetic Resonance Spectroscopy. (P): positive association; (N): negative association.

Please find the table (Suppl_t_3) in the link
https://www.dropbox.com/s/cd0xp33gube4rky/Supplementary_tables_LIU_J_revised.xlsx?dl=0

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Supplemental table 4 Results of Mendelian randomization of metabolites on fasting glucose/T2DM (forward approach)

® Pathway based sensitivity analysis. T2DM: type 2 diabetes. R2 (%): the percentage of explained variance in the exposure by genetic risk score. FDR: false discovery rate. NP: not performed.

Please find the table (Suppl_t_4) in the link
https://www.dropbox.com/s/cd0xp33gube4rky/Supplementary_tables_LIU_J_revised.xlsx?dl=0

Supplemental table 5 SNP lists included in exposure genetic risk score in Mendelian randomization of metabolites on fasting glucose/T2DM (forward approach)

T2DM: type 2 diabetes. MAF: minor allele frequency. Standardized effect is the t-statistic in the association analysis. (NA): no genes confirmed by the outcome in the pathway analysis. ? The gene in the pathway analysis is not confirmed. R2 (%): the percentage of explained variance in the exposure by genetic risk score. NP: not performed.

Please find the table (Suppl_t_5) in the link
https://www.dropbox.com/s/cd0xp33gube4rky/Supplementary_tables_LIU_J_revised.xlsx?dl=0

Supplemental table 6 Mendelian randomization of fasting glucose/T2DM on metabolites (backward approach)

® Pathway based sensitivity analysis. T2DM: type 2 diabetes. FDR: false discovery rate. NP: not performed. R2 (%): the percentage of explained variance in the exposure by genetic risk score. As the genetic risk scores of the glucose explained less than 1% of variance, the backward MR with pathway analysis is not performed.

Please find the table (Suppl_t_6) in the link
https://www.dropbox.com/s/cd0xp33gube4rky/Supplementary_tables_LIU_J_revised.xlsx?dl=0

Supplemental table 7 SNP lists included in exposure genetic risk score in Mendelian randomization of fasting glucose/T2DM on metabolites (backward approach)

T2DM: type 2 diabetes. MAF: minor allele frequency. Standardized effect is the t-statistic in the association analysis. (NA): no genes confirmed by the outcome in the pathway analysis. ? The gene in the pathway analysis is not confirmed. Only fasting glucose was done the pathway analysis. NP: not performed. R2 (%): the percentage of explained variance in the exposure by genetic risk score.

Please find the table (Suppl_t_7) in the link
https://www.dropbox.com/s/cd0xp33gube4rky/Supplementary_tables_LIU_J_revised.xlsx?dl=0