Multi-omics in research: epidemiology, methodology, and advanced data analysis

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Part II
Epidemiological Research and Advanced Data Analysis

Chapter 5

Analyses of metabolites and biochemical pathways associated with hepatic triglyceride content indicate extensive metabolite dysregulation

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Abbreviations
NAFLD, Non-alcoholic fatty liver disease; HTGC, hepatic triglyceride content; GMM, Gaussian graphical model; GSMM, genome scale metabolic model; BCAA, branched chain amino acids; IR, insulin resistance; T2D, type 2 diabetes; NEO, The Netherlands Epidemiology of Obesity Study; ^{1}H-MRS, Proton magnetic resonance spectroscopy, BMI, body mass index; MRI, magnetic resonance imaging; HOMA1-IR, homeostatic model assessment index for insulin resistance; CVD, cardiovascular disease; UHPLC-MS/MS, ultra-high-performance liquid chromatography mass spectrometry; HILIC, hydrophilic interaction liquid chromatography; HMR2, Human metabolic reactions database; EBIC, Extended Bayesian information criterium; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositol; GPC, Glycerophosphorylcholine; HCER, hexosyl/glucosylceramide ; LCER, lactosylceramide ; AKG, alpha ketoglutarate; BCKA, branched chain keto acids.

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Conflicts of Interest

R.L.-G. is a part-time clinical research consultant for Metabolon, Inc. P.A.S. is an associate director and G.M. is the science director at Metabolon, Inc. All other co- authors have no conflicts of interest to declare.

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1 ABSTRACT

Background & Aims

Non-alcoholic fatty liver disease (NAFLD) is characterized by the pathological accumulation of triglycerides in hepatocytes and is associated with insulin resistance, atherogenic dyslipidemia, and cardiometabolic diseases. Thus far, the extent of metabolic dysregulation associated with hepatic triglyceride accumulation has not been fully addressed. In this study we aimed to identify metabolites associated hepatic triglyceride content (HTGC) and map these associations using network analysis.

Methods

To gain insight in the spectrum of metabolites associated with hepatic triglyceride accumulation, we performed a comprehensive plasma metabolomics screening of 1,363 metabolites in apparently healthy middle aged (age 45-65) individuals (N=496) in whom HTGC was measured by proton magnetic resonance spectroscopy. An atlas of metabolite-HTGC associations, based on univariate results, was created using correlation-based Gaussian graphical model (GGM) and genome scale metabolic model (GSMM) network analyses.

Results

Our analyses revealed that 118 metabolites were univariately associated with HTGC (P-value $< 6.59\times10^{-5}$), including 106 endogenous, 1 xenobiotic, and 11 partially characterized/uncharacterized metabolites. These associations were mapped to several biological pathways including branched amino acids (BCAA), diglycerols, sphingomyelin, glucosyl- and lactosyl- ceramides. We also identified novel a possible HTGC related pathway connecting glutamate, metabolonic lactone sulfate, and X-15245 using the GGM network. The full interactive metabolite-HTGC atlas is provided online: https://tofaquih.github.io/AtlasLiver/.

Conclusions

The combined network and pathway analyses indicated extensive associations between BCAA and the lipids pathways with HTGC. Moreover, we report a novel pathway glutamate-metabolonic lactone sulfate-X-15245 with a potential strong association with HTGC. These findings can aid elucidating HTGC metabolomic profiles and provide insight in novel drug targets for treatment or monitoring of NAFLD.

2 INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a highly prevalent liver condition and a common cause of liver disease. It is estimated that NAFLD has a global prevalence of approximately 25% (95% CI: 22 – 28) (1, 2). NAFLD is considered a metabolic disease and is strongly associated with cardiovascular disease, insulin resistance (IR), type 2 diabetes (T2D), obesity, dyslipidemia, and hypertension. NAFLD is diagnosed when the accumulation of triglycerides in the liver exceeds 5%, in people without excessive alcohol intake and alternative causes for liver disease, such as hepatitis infection (1). Assessment of triglyceride content in the liver is commonly measured by ultrasonography, due to its low coast and wide availability, but quantitative assessment is mostly performed using proton magnetic resonance spectroscopy (^{1}H-MRS) (3). The term NAFLD covers a wide range of liver damage levels, including minor steatosis to major cirrhosis. Triglyceride deposition occurs depending on, amongst other factors, diet and fasting status (4). Pathological hepatic triglyceride accumulation is the consequence of an imbalance between hepatic uptake of endogenous triglycerides and fatty acids, hepatic triglyceride secretion, de-novo lipogenesis and fatty acid oxidation. Disturbances in these processes are strongly associated with insulin resistance and may also cause further progression of metabolic diseases such as T2D and NAFLD (4). The development of NAFLD as well as the progression to steatosis and cirrhosis varies greatly between individuals. This is due to the complex and multifactorial pathogenesis of fatty liver diseases (5). In addition to environmental acquired factors (6, 7), genetic factors also play an important role (8).

Several studies have been performed to gain insight into the complex etiology of NAFLD by applying metabolomics (9). Changes in circulating metabolites are thought to reflect the composite of both environmental, acquired, and genetic factors of an individual. Metabolomics can thus provide holistic insight to capture the complexity of multi-factorial diseases such as NAFLD (10, 11). Current high-throughput untargeted metabolomic platforms are capable of measuring and mapping over 1000 metabolites from an array of biological pathways from a single biological sample (e.g., blood, urine, or saliva). In addition to well-annotated endogenous metabolites, both xenobiotic metabolites derived from the diet and medications as well as uncharacterized metabolites are reported (12). Although metabolomic analysis has been previously performed in patients with NAFLD (13) most studies had limited sample sizes or focused on a specific subset of metabolites using targeted metabolomics methods (9).

Here we aimed to elucidate the HTGC metabolomic profile as assessed by proton magnetic resonance spectroscopy (^{1}H-MRS), in a middle-aged population (N=496) using an untargeted metabolomics platform (1,363 metabolites). We further created a comprehensive atlas of HTGC-associated metabolites and pathways from our results using two pathway analysis approaches that allows flexible and interactive examination of our results.

3 METHODS

3.1 Study Population

For our present study we included 599 participants from the Netherlands Epidemiology of Obesity (NEO) with available metabolomics data from the general Leiderdorp subpopulation. The Leiderdorp subpopulation was included based on postcode and age (45-65 years) only. We excluded 103 participants who did not undergo direct assessment of the hepatic triglyceride content (HTGC) by ^{1}H-MRS. Therefore, final number of participants included in the present study was 496. The characteristics of the included participants are presented in Table 1. Metabolites were measured using the Metabolon™ Discovery HD4 platform (Metabolon Inc., Durham, North Carolina, USA). In total, 1,363 serum metabolites were measured of which 840 metabolites were from various endogenous pathways (56% lipids, 31% amino acids, 13% other), 227 xenobiotic metabolites, and 296 metabolites that were uncharacterized (unknown chemical structure and biological properties). Further details regarding the study design, HTGC assessment procedure, and metabolite measurements are described in detail in previous works (14, 15) and in the Supplementary Materials.

The NEO study was approved by the Medical Ethics committee of the Leiden University Medical Centre under protocol P08.109. The study is also registered at clinicaltrials.gov under number NL21981.058.08 / P08.109. All participants gave written informed consent (14).

3.2 Statistical Analysis

For the analysis we used multiple linear regressions to test the associations between the metabolites from the untargeted platform with the outcome HTGC. We further adjusted for potential confounding by including sex, age, total body fat, alcohol intake, and lipid lowering medication in the models.

Natural log-transformation was applied to the outcome variable, HTGC, as it was heavily skewed. One individual had a measurement of 0 units of HTGC and was imputed to half the minimum (0.1) before the log-transformation. Missing values in the measured metabolites were imputed using multiple imputation by chained equations as described in our previous work (12). Details regarding the analysis, imputation and scaling of the metabolites are described in the Supplementary Methods.

Additionally, to examine the known sex differences in metabolites, we performed the analysis separately for men and women. We further stratified the women subgroup by menopausal status to examine the metabolomic profile after menopause. Menopausal status was defined as a binary variable based on a questionnaire (Supplementary Material) wherein postmenopausal women were coded as 1 and premenopausal and perimenopausal women were coded as 2. Finally, as sensitivity analysis, we adjusted for HOMA-IR to investigate if the associations of metabolites with HTGC were dependent on IR, particularly for those known to be associated with IR i.e., amino acids and carbohydrates.

3.3 Pathway Analysis

Significant metabolites from the main analysis and the sex stratified analysis were subsequently analyzed with two pathway/network analysis methods: Gaussian Graphical Model (GGM) and Genome Scale Metabolic Model (GSMM). GGM has been used and described in previous studies as a viable approach for the visualization and reconstruction of biological pathways from correlation data. This method is particularly useful for our study and other studies with large metabo-

lomic datasets from untargeted platforms (16). In contrast, GSMM methods are based on a priori defined and curated pathways and have also been used in metabolomic and non-metabolomic studies (17). In addition, we used an inhouse developed GSMM tool to construct the networks. Both methods are thus complementary in their basis, i.e., without and with prior pathway knowledge. Full details regarding the methodology used to create both networks are available in the Supplementary Methods. These interactive networks can be accessed on https://tofaquih.github.io/AtlasLiver/.

4 RESULTS

4.1 Association Analyses of Metabolites and HTGC

Univariate linear regression analyses were performed to examine the associations between the 1,365 metabolites and the outcome HTGC, adjusting for sex, age, total body fat, alcohol intake, and lipid-lowering medication. In total, and after considering multiple testing correction (P-value < 6.59×10^{-5}), 118 metabolites were associated with HTGC, of which 101 were associated with higher levels of HTGC. From these metabolites, many were from the lipids and amino acid classes, as well as a few from the vitamin, nucleotide, carbohydrate classes, uncharacterized metabolites, 1 partially characterized metabolite, and 1 xenobiotic (Supp Table 1). Excluding individuals with lipid lowering medications did not alter the results.

Additional analyses after stratification for sex and menopausal status were performed, which showed complete overlap in the directions of the effects between men and women, between men above and below the age of 60, and women before and after menopause. In general, a higher number of associations present and the effect estimates were larger in women (particularly post-menopause) compared to men, with 82 and 35 metabolites associated with HTGC respectively (Figure 1). As the direction of the effects was identical between men and women for all metabolites (Figure 2), all subsequent descriptions and analyses were performed in the full set of 118 associated metabolites.

4.2 Amino acid and Carbohydrate Metabolism

In total, 37 out of the 264 measured amino acids and peptides were associated with HTGC, of which 34 were associated with higher HTGC levels—particularly BCAA and their keto forms. The amino acids and derivatives with the strongest association were glutamate [β: 2.41 (95% CI 1.93; 2.9)], 3-methyl-2-oxovalerate [β: 2.36 (95% CI 1.75; 2.97)], isoleucine [β: 2.28 (95% CI 1.75; 2.82)], tyrosine [β: 2.03 (95% CI 1.5; 2.55)], and lactoylvaline [β: 1.97 (95% CI 1.5; 2.44)] in addition to the carbohydrates, glucose [β: 1.84 (95% CI 1.21; 2.47)] and pyruvate [β: 1.44 (95% CI 0.93; 1.95)]. Sensitivity analyses adjusting for HOMA- IR were also performed for the insulin resistance related subgroup of amino acid and carbohydrate metabolites (n=264). Accordingly, 33 metabolites were associated with HTGC in this model, of which 32 overlapped with the findings in the main model, with the exception of lysine. Moreover, 10 metabolites, including pyruvate and glucose, were not associated with HTGC in the sensitivity analysis. For the overlapping associations between the two models, the effect of the metabolites on HTGC was weaker after adjusting for HOMA-IR. The findings indicated that the associations of the amino acids and carbohydrates metabolites with the levels of HTGC were overall largely independent from IR. These results are detailed and discussed in the Supplementary Materials and Supplementary Figure 1.

4.3 Lipids

Out of the 475 measured lipid-related metabolites, 62 lipid metabolites belonging to 19 lipid subclasses were associated with HTGC, including phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), phosphatidylinositols (PIs), sphingomyelins, glycerolipids, corticosteroids, ceramides, and dihydroceramides.

Among these lipid metabolites. 52 were associated with higher HTGC levels. Some of these strongly associated lipids were: palmitoyl-oleoyl-glycerol (16:0/18:1) [β: 2.23 (95% CI 1.72; 2.73)], 1-palmitoyl-2-palmitoleoyl-glycerophosphorylcholine (16:0/16:1) [β: 2.16 (95% CI 1.63; 2.69)], myristoyl linoleoyl glycerol (14:0/18:2) [β: 2.12 (95% CI 1.61; 2.63)], and two isomers of diacylglycerol (14:0/18:1, 16:0/16:1) [β: 2.09 (95% CI 1.57; 2.6) and [β: 1.94 (95% CI 1.46; 2.42)]. All dihydrosphingomyelin species had positive associations with HTGC of which three metabolites had a P-value above the significance threshold, with the most salient being sphingomyelin (d18:0/18:0, d19:0/17:0) [β: 2.15 (95% CI 1.63; 2.66)].

Ten lipid metabolites were associated with lower levels of HTGC, amongst which were several ether-PC species and glycosylceramide (HCER) and lactosylceramide (LCER) species. Among those metabolites were two sphingomyelins with long fatty acyl chains—sphingomyelin (d18:2/24:1, d18:1/24:2) [β: -1.29 (95% CI -1.79; -0.78)] and sphingomyelin (d18:2/24:2) [β: -1.68 (95% CI -2.27; -1.09)]. For the HCER and LCER metabolites, the HCER glycosylceramide (d18:2/24:1, d18:1/24:2) [β: -1.31 (95% CI -1.87; -0.74)] and two LCER metabolites lactosyl-N-nervonoyl-sphingosine (d18:1/24:1) [β: -1.57 (95% CI -2.06; -1.08)] and lactosyl-N-palmitoyl-sphingosine (d18:1/16:0) [β: -1.17 (95% CI -1.68; -0.65)] reduced HTGC levels. Finally, glycerophosphorylcholine (GPC), a derivate of choline and a breakdown product of PCs, reduced HTGC levels as well [β: -1.13 (95% CI -1.68; -0.59)].

4.4 Other Metabolites

Metabolonic lactone sulfate, a partially characterized metabolite, was found to be strongly associated with higher HTGC levels [β: 2.18 (95% CI 1.73; 2.63)]. Only one xenobiotic metabolite, 4-ethylcatechol, was associated with HTGC and it was strongly associated with the reduction of HTGC levels [β: -1.63 (95% CI -2.43; -0.84]. Ten uncharacterized metabolites were associated with higher HTGC. Other metabolites included two nucleotides: xanthine and guanine. Xanthine was associated with the higher HTGC levels [β: 1.25 (95% CI 0.76; 1.75] while guanine had the opposite effect [β: -1.09 (95% CI -1.55; -0.63]. Finally, tocopherol metabolites including alpha-tocopherol [β: 1.27 (95% CI 0.77; 1.78] and gamma-tocopherol/beta-tocopherol [β: 1.18 (95% CI-0.62; 1.73] also associated with higher HTGC levels.

4.5 Correlation Based Network Analysis Using GGM

GGM networks were generated including all significantly (P-value < 6.59×10^{-5}) associated metabolites (N=118). This method identified two major clusters: amino acids and lipids (Figure 3).

The amino acids and related metabolites can be organized into 3 groups: 1) Primary amino acids e.g., glutamate; 2) Derivates of amino acids with either a carbon group (lactoylvaline), oxo- and methyl groups (e.g., 3-methyl-2-oxovalerate) or an acetyl group (e.g., N-acetyltryptophan); 3) Ketoacids, products of incomplete breakdown of amino acids (e.g., 4-methyl-2-oxopentanoate).

Overall, the network pattern shows the primary BCAA connecting to each other via the amino acids derivates and ketoacids. The common endpoint of these connections led to glutamate,

pyruvate, and glucose. Moreover, all uncharacterized metabolites related to BCAAs were intermediates for glucose, pyruvate, and alpha ketoglutarate (AKG). Some amino acids were correlated with metabolites in the lipid cluster such as the connection between leucine and N-palmitoylsphinganine (d18:0/16:0).

Regarding the lipids cluster, the GGM network showed strong interconnectivity between diacylglycerols, monoacylglycerols, as well as links to PIs, PCs, and PEs (https://tofaquih.github.io/AtlasLiver/Networks/LiverFatNetworks/Model2/SexAdj/network/). Moreover, all 10 of the negatively associated lipids—which included sphingomyelins, ether PC, and glucosyl- and lactosylceramide species—were connected to each other and formed a subcluster within the lipids. The partially characterized metabolite metabolonic lactone sulfate showed a positive correlation with glutamate only in the stratified analyses in men and post-menopausal women (data not shown). Alpha-tocopherol and gamma-tocopherol/beta-tocopherol were correlated and connected in the GMM network and, interestingly, alpha-tocopherol was also connected to cholesterol.

4.6 GSMM Pathway-based Analyses

To assess how and whether HTGC associated metabolites were linked biochemically to one another through metabolic pathways, a network analysis based on the genome scale metabolic model HRM2 was performed (Figure 4). The network showed several amino acids subclusters that were associated with higher HTGC, amongst which the BCAAs and their keto and 2-hydroxy form, the clusters of aromatic amino acids and intermediates, and glutamate and alpha-ketoglutarate. The lipid cluster showed the relation between the positively and negatively correlated lipids, providing insight into the enzymatic conversions which are potentially affected by HTGC. The pathway mapping of the 10 lipid metabolites that were associated with lower level HTGC reported in GGM network were also confirmed in the GSMM. Biochemical reactions were found to connect sphingomyelin species to the glucosylceramide and lactosylceramide species (labeled as LacCer in the GSMM network) via the ceramides. An additional connection not shown in the GSMM network included the reactions connecting glucosylceramide to glucose via its breakdown to ceramide and glucose.

To improve the interpretability of the network, only the main lipid classes were shown as nodes. The full network of associated metabolites is available as an interactive, single page website: https://tofaquih.github.io/AtlasLiver/Networks/LiverFatNetworks/.

5 DISCUSSION

In the present work, we establish a comprehensive atlas of metabolites associated with HTGC in a Dutch population composed of middle-aged men and women. We used an untargeted metabolomics approach to measure 1361 metabolites and ^{1}H-MRS measurement of HTGC. In total, 118 metabolites were associated with HTGC after correction for sex and other confounders, of which 101 associated with higher levels of HTGC. Stratification by sex and menopause in women revealed that a larger number of associations was significant in the women strata and the estimates of the effect sizes were higher in women, particularly post-menopause. However, the directions of the effects for the overlapping metabolite-HTGC associations were uniform in all strata. This difference was observed despite a higher average level of HTGC in men. Apparently, metabolites have strong correlations even with smaller HTGC ranges. Pathway analyses revealed 2 clusters of interrelated metabolites, one primarily involving amino acid related metabolism and the other lipid metabolism.

5.1 91. Branched Chain Amino Acids and Branched Chain Keto Acids

Our analysis showed that amino acids levels were overall associated with higher levels of HTGC. Moreover, these amino acids had clear clustering in both the GGM- and the GSMM-based pathway network analyses. These results coincided with previous literature findings that elevated levels of BCAAs associated with HTGC and NAFLD (9, 18, 19). The primary metabolites from the amino acids associated with higher HTGC were glutamate, leucine, isoleucine, and valine. In addition, untargeted metabolomics enabled us to screen metabolite derivatives and subclasses, several of which we also found to be strongly associated with the higher levels of HTGC. These derivatives form during normal or abnormal breakdown of BCAAs and include BCAAs with acetyl, lactoyl or methyl groups, as well as metabolites categorized as branched chain keto acids (BCKA), their 2-hydroxy form, and gamma-glutamyl alpha-amino acids. Furthermore, these metabolites were shown in the GGM and GSMM at the intersection of the BCAAs, glutamate, and pyruvate. The derivatives, particularly BCKAs, are an indication of an association between BCAA catabolism with HTGC levels (20, 21). It is interesting that the BCAAs and derivates, known to be associated with body fat and insulin resistance (20), remained associated with HTCG despite the adjustment for total body fat and after further adjustment for HOMA-IR in the sensitivity analysis. These results indicate that these associations were overall independent from body fat and IR. A few amino acids that were previously found to be associated with HTGC and NAFLD using ultrasonography assessment did not replicate in our study. For example, serine and glycine were not associated with HTGC in our analysis contrary to the results of previous studies (9, 22). Moreover, the proposed glutamate-serine-glycine index in those studies was also not observed in our analysis despite our larger sample size (N= 496 versus N=64 [N=20 controls]). This discrepancy could possibly be due to our study included participants from the general population with an HTGC range around 5.6% and did not focus on NAFLD or NASH patients specifically in a case-control study design. Therefore, it is possible that serine and glycine are better markers for advanced stages of HTGC, as observed in NAFLD/NASH patients, due to the stronger effect of metabolic dysregulation, but are not strong markers for general levels of HTGC.

5.2 92. Lipid Metabolites

Lipids have the largest effect estimates in the GGM and GSMM networks and reflect a strong association of lipid metabolism with HTGC. This cluster contained lipids from various subclasses of which di- and mono-glycerides were predominant. These associations with HTGC, particularly from di- and mono-glycerides, are supported by previous studies (23). The GGM network showed strong correlations between diglycerols, PCs, PEs, PIs, and other lipid subclasses, consistent with our GSMM based network and the established biological pathways (24). Overall, most of the lipids (N=52 of 62) were associated with higher HTGC levels. Notable exceptions were the group of 10 lipids comprising of ether-PC species, sphingomyelin, HCER, and LCER species, which the highest reduction effect on HTGC levels in our results. In addition, these lipids were highly connected in the GMM network and shared the same biological pathways in the GSMM network. Interestingly, the aforementioned sphingomyelins with long fatty acyl chains reduced HTGC levels, in contrast to dihydrosphingomyelins which associated with higher HTGC. Other interesting metabolite groups were the beforementioned HCER and LCER. Unlike the ceramide and dihydroceramide metabolites, which associated with higher HTGC level, both HCER and LCER had the opposite effect. In this case, the breakdown of these metabolites seems to contribute to the upregulation of ceramides and dihydroceramides, as well as glucose, specifically via HCER (25).

The associations of the ceramides and dihydroceramides with higher HTCG are analogous to previous studies on NAFLD (26-28). However, the associations of sphingomyelins and dihydrosphingomyelins with HTGC and NAFLD in humans are less studied and understood compared to the associations of ceramides (29). Since dihydroceramides are precursors for the synthesis of dihydrosphingomyelins (28, 30), it could explain their association with higher HTGC. However, a small study by Lovric et al. (N=75) (31) reported contrary pattern using the same metabolomic platform used in our study, in which higher sphingomyelins but lower dihydrosphingomyelins were associated with NAFLD. In mice, dietary sphingomyelins were associated with a decrease of HTGC in the liver (32). Regarding HCER and LCER, as stated earlier, HCER is involved in the synthesis of LCER, ceramide, and glucose (25). In addition, both LCER and HCER are involved in glycosphingolipid metabolism (33). One small study (n=28) reported a positive association HCER and LCER with NASH (29). Finally, a recent a study reported similar results of higher dihydrosphingolipid classes being associated with increased fibrosis in animal models and NAFLD patients (34).

Overall, our study presents a deeper look into these metabolite subclasses in a larger sample size in a general population. Further studies focusing on the contrasting associations of sphingomyelins versus dihydrosphingomyelins and ceramide versus HCER could elucidate their specific function and relationship to HTGC and NAFLD in humans.

5.3 Other Metabolites Strongly Associated with HTGC

In addition to the amino acids and lipids results, our analysis and the GGM network included uncharacterized and xenobiotic metabolites, not previously reported in HTGC or NAFLD studies. A particularly interesting finding was the relatively novel metabolite metabolonic lactone sulfate (formerly assigned the ID X-12063). Metabolonic lactone sulfate had a large effect estimate in the adjusted and the stratified models and was associated with higher HTGC levels. Previous studies have found this metabolite to be a biomarker and a predictor for T2D (35-37) and acute-on-chronic liver failure (38). Moreover, metabolonic lactone sulfate was associated with cardiometabolic disease (39) and was positively correlated with BMI, waist hip ratio, and HOMA-IR (40). Metabolonic lactone sulfate was also reported to be associated with the *CYP3A5*/*ZSCAN25* locus via the rs10242455 (41) and rs7808022 (42) single nucleotide polymorphisms (SNPs), respectively. This genes in this region are highly expressed in the liver and share the same regulatory promoters (43). The gene *CYP3A* is particularly expressed in the liver and encodes for the CYP3A protein, a key drug-metabolizing liver enzyme (39, 43). Although the specific functionality and underlying biological pathways of metabolonic lactone sulfate remains unelucidated, the available evidence suggests a link with the liver function, cardiometabolic diseases. This supports our findings regarding the association of metabolonic lactone sulfate with higher HTGC levels and possibly NAFLD.

4-Ethylcatechol is a xenobiotic metabolite that is primarily acquired from the ingestion of coffee beverages and products (44, 45). In our analysis, 4-Ethylcatechol was associated with lower HTGC levels and was not connected to any metabolites in the GGM or GSMM networks. Protective properties of coffee and caffeine intake against liver fibrosis have been suggested before due to its anti-fibrotic and antioxidant effects (46, 47). Several studies examining the association of coffee consumption with liver fat found similarly reduced HTGC (46, 48, 49). For instance, a recent large systematic review and meta-analysis study found that coffee consumption was negatively associated with liver fibrosis and suggested protection from severe liver fibrosis and cirrhosis (46). Results for patients with NAFLD were less conclusive with some showing that increased coffee consumption was associated with reduction of NAFLD (49).

Uncharacterized metabolites, such as X-15245, X-24295, X-19438, and X-25343, are associated with HTGC with strong connections to pyruvate, glucose, and AKG. Further analysis into the structure of these metabolites is needed to determine their identities and their biological relationships to HTGC and the other metabolites in the network.

Our analysis also shows associations with vitamin E metabolites. Briefly, vitamin E metabolites (alpha-tocopherol and gamma-tocopherol/beta-tocopherol) are positively associated with HTGC, and alpha-tocopherol was directly correlated with cholesterol in the GGM network. This finding is supported by one study that found a positive correlation between vitamin E and NAFLD (50). Moreover, vitamin E is known to bind to lipoproteins in the blood which promoted the usage of cholesterol-adjusted vitamin E in several studies as a superior measurement of vitamin E (51). However, the mechanism linking vitamin E with HTGC remains unclear and clinical trials present mixed results regarding the relationships. Some studies have shown a negative or no relation while others suggested possibly therapeutic benefits of vitamin E supplementation for NAFLD and NASH patients via the suppression of HTGC (52, 53).

5.4 Pathway Analysis

Pathway analysis was performed using two approaches. The first approach—GMM—is data-driven and calculates the partial correlations between the HTGC associated metabolites to create a network. The second approach maps metabolites to a GSMM, which consists of known functionally annotated biochemical conversions that can occur in humans. An advantage of the GGM is that all measured and associated metabolites in the study are included in the network, even the uncharacterized and xenobiotic metabolites ones. However, a GGM is data driven and does not necessarily reflect actual biological pathways, and only shows metabolites measured by the platform. That is, metabolites can be directly linked in the GGM even though they are distant in terms of intermediate biochemical reactions (16). The GSMM based network analysis, on the other hand, includes measured associated metabolites as well as relevant intermediate metabolites regardless of whether they were measured or associated with HTGC. In addition, the created network shows the directionality of the biological reactions for the biosynthesis and degradation of the metabolites and provides relevant details regarding the enzymes involved in each reaction.

Although both types of pathway analysis are complementary in their approach, we found that the network resulting from GGM had a good alignment with the metabolic reaction paths that resulted from the GSMM approach. This is in concordance with previous work (16), which showed that strongly associated metabolites generally corresponded to the same pathways.

5.5 Key Findings and Potential Targets for Genetic and Drug Research

In summary, our analyses and atlas showed interesting pathway associations for HTGC which may be relevant for fatty liver disease research. The pathways connecting glutamate, BCAAs, and derivatives of BCAAs during normal/abnormal metabolism were particularly associated with the higher HTGC levels. Among the wide variety of lipid metabolites, higher metabolite concentrations in the pathways connecting diglycerols, PCs, PEs, and PIs had a strong association with higher HTGC levels. Notably, the pathways connecting the ceramide species and their relationship with sphingomyelin species, as shown in the GMM and GSMM networks, were particularly interesting due to the contrasting associations of HTGC. Thus, it appears that the metabolomic flux between these metabolite species is associated with an overall higher HTGC levels. These metabolites and pathways are of interest to explore in future HTGC and liver fibrosis studies.

The most interesting finding was the in our study was the strong association of glutamate and the novel metabolites correlated with it. Glutamate itself is a well-known biomarker for liver fat and NAFLD (9, 22). However, our pathway analyses revealed 2 metabolites to be strongly correlated with glutamate and were also shown to be associated with HTGC. These were metabolonic lactone sulfate and the uncharacterized metabolite X-15245. These metabolites were associated with HTGC in all models and specifically interconnected with glutamate in the GGM networks for men and postmenopausal women, hence indicating that these metabolites share common biochemical pathways. As discussed earlier, metabolonic lactone sulfate has been found to be associated with several cardiometabolic related outcomes in other studies. For example, the uncharacterized metabolite X-15245 is associated with rs1260326 SNP in the *GCKR* gene (42). This particular SNP was reported to be strongly associated with NAFLD (8). Based on these findings, glutamate, metabolonic lactone sulfate and X-15245 and their potentially shared pathway are important candidates for further etiological studies on HTGC and NAFLD. Furthermore, the genetic SNPs associated with these metabolites can be used as possible new genetic marker for HTGC/NAFLD. This can be achieved similar to the approach used by Mancina et al. (54), in which SNPs associated with triglycerides were tested for their association with liver fat and subsequently used to identify a protective link between *PSD3* and HTGC. Moreover, future studies should aim to identify the uncharacterized metabolite X-15245 and elucidate the biological properties for it and metabolonic lactone sulfate.

Exploration of the aforementioned metabolites and the various other metabolites and pathways we have discussed here, in combination with metabolomic and genetic studies, can be key to identifying causal associations and possible drug targets for liver fibrosis in the future.

5.6 Strengths and Limitations

Previous literature on metabolite-HTGC associations focused mainly on a small number of well-established metabolites or metabolites involved in specific pathways. A strength of our study is the use of an untargeted metabolomics platform with over a thousand measured metabolites from 10 metabolite pathway classes in a relatively large population of middle-aged individuals in the Netherlands. Our study population was a random selection of volunteers from the Leiderdorp area and was not selected on NAFLD or NASH diagnosis. Moreover, HTCG measurements in this cohort were assessed by ^{1}H-MRS, which provides high accuracy and sensitivity in measuring HTGC even at low levels (55). Furthermore, we expanded our analysis by combining linear regression with correlation and biochemical pathway analysis methods to construct a comprehensive atlas of metabolomic profiles of HTGC and an atlas for men and women separately. A limitation of our study was the selection of only individuals of white ethnicity from a high social economic status area, which limits generalizability to other ethnicities and social status. Another limitation of our study is the lack of biological validation. Future studies with liver biopsy samples will aid in validating our findings. However, studies on NAFLD are extensive but usually focused on specific metabolites such as amino acids or lipids. Our study itself can be considered as a validation of the various results from the previous literature by taking advantage of the wide range of the metabolites from various biochemical pathway classes. The GSMM network provided useful insight into the biochemical relation between the metabolites associated with HTGC and their intermediates. The directionality of the edges in the network, however, is based on knowledge regarding the thermodynamic reversibility of the corresponding reactions and makes no assertion about causality or potential association with HTGC. Although the GSMM network provides some information regarding known gene-pathway associations, genetic analysis for some HTGC or metabolite related SNPs would have benefited our study. Finally, further investigation for sex differences in the number of

metabolite associations with HTGC levels, particularly for postmenopausal women, requires a larger longitudinal study for validation.

6 CONCLUSION

In this study, we conducted a cross sectional analysis in 496 middle aged men and women to gain insight the metabolomic profile associated with hepatic triglyceride accumulation as assessed by ^{1}H-MRS. We used a hypothesis-free approach to study a myriad of metabolites associated with HTGC using an untargeted platform that measured 1,363 metabolites. Using this platform, associations were found between 118 well-known, lesser-known, and novel metabolites with HTGC levels. These findings were combined by pathway analyses using a correlation driven network (GGM) and a biologically driven network (GSMM) to create an atlas of metabolites associated with HTGC. Analysis of these networks indicated strong associations between the BCAAs, diglycerols, ceramides, and sphingomyelins pathways with HTGC levels. These pathways were additionally found to reject the null hypothesis of the closed global test when using the FIB-4 index as the outcome. In addition, our atlas of networks, enriched with pathway knowledge, provided interesting insights regarding pathways associated with HTGC. These included the pathways connecting BCAA and BCAA derivates, the flux between the ceramide species (i.e., HCER and LCER), sphingomyelins and their dihydro forms, and the potential novel pathway linking glutamate with the novel metabolites metabolonic lactone sulfate and X-15245. Thus, our atlas is potentially essential for understanding metabolomic profiles associated with liver fat accumulation. In turn facilitating further studies to find causal links between the metabolites reported here with liver fibrosis.

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Table 1: Characteristics of the participants from Leiderdorp with metabolomics and HTGC measurement. Continuous variables are represented by mean (SD) unless stated otherwise; dichotomous variables are represented by percentage (%). Abbreviations: HTGC, hepatic triglyceride content; IQR, interquartile range; CVD, cardiovascular disease; HOMA1-IR, homeostatic model assessment index for insulin resistance.

	Total	Men	Women	Women	Women
		All	All	Postmenopausal	Premenopausal
n	496	233	263	159	104
Age (years)	55.8 (6)	55.9 (6.2)	55.6 (5.8)	59.4 (3.8)	49.9 (2.7)
HTGC (mean; median [IQR])	6.1; 2.74 [1.36, 6.75]	7.5; 4.18 [2.18, 9.74]	4.8; 1.79 [1.08, 4.27]	5.5; 2.09 [1.23, 5.90]	3.8; 1.36 [0.89, 3.17]
HTGC >= 5.56 (%)	153 (30.8)	96 (41.2)	57 (21.7)	43 (27.0)	14 (13.5)
BMI (kg/m^2)	25.9 (4.1)	26.6 (3.4)	25.3 (4.5)	25.5 (4.4)	25.0 (4.7)
Total Body Fat	30.6 (8.3)	24.5 (5.1)	36.1 (6.5)	35.2 (6.7)	36.6 (6.4)
Aspartate Transaminase (IU/L)	24.4 (6.6)	25.6 (6.2)	23.2 (6.6)	24.0 (5.9)	22.0 (7.5)
Alanine Aminotransferase (IU/L)	25.3 (51.4)	28.9 (11.8)	22.2 (9.2)	22.8 (7.7)	21.2 (11.0)
Platelet Count (10^9/L)	236.2 (11.0)	219.1 (47.2)	252.3 (50.2)	251.1 (492)	254.1 (51.8)
Alcohol Consumption (g/day)	14.2 (15.9)	19.5 (19.3)	9.4 (10)	9.7 (9.8)	8.9 (10.3)
Smoking (%)					
Never	202 (40.7)	89 (38.2)	113 (43.0)	59 (37.1)	54 (51.9)
Former	237 (47.8)	117 (50.2)	120 (45.6)	81 (50.9)	39 (37.5)
Current	57 (11.5)	27 (11.6)	30 (11.4)	19 (11.9)	11 (10.6)
HOMA1-IR	2.6 (2.4)	2.92 (2.9)	2.2 (1.7)	2.4 (1.7)	2.02 (1.8)
Hypertension (%)	183 (36.9)	96 (41.2)	87 (33.1)	59 (37.1)	28 (26.9)
CVD (%)	21 (4.3)	11 (4.7)	10 (3.8)	8 (5.1)	2 (1.9)
Fasting plasma glucose (mmol/L)	5.5 (1.0)	5.63 (1.2)	5.3 (0.9)	5.5 (0.9)	5.09 (0.5)
Serum triglycerides (mmol/L)	1.2 (0.8)	1.5 (0.9)	1.0 (0.6)	1.1 (0.7)	0.9 (0.5)
LDL (mmol/L)	3.6 (1.0)	3.6 (0.9)	3.6 (1.0)	3.4 (0.8)	3.4 (0.7)
HDL (mmol/L)	1.6 (0.5)	1.3 (0.3)	1.8 (0.4)	1.8 (0.4)	1.8 (0.4)
Cholesterol (mmol/L)	5.7 (1.1)	5.6 (1.0)	5.8 (1.1)	5.3 (0.8)	5.6 (0.8)
Hypertension Medication (%)	95 (19.2)	43 (18.5)	52 (19.8)	39 (24.5)	13 (12.5)
Lipid Lowering Medication (%)	41 (8.3)	26 (11.2)	15 (5.7)	15 (9.4)	0 (0.0)

Figure 1: Comparison of the point estimates and confidence intervals for the 118 metabolites associated with HTGC in the primary analysis and the sex stratified analysis. The first ring shows the effect estimates in the sex combined analysis. The second ring shows analysis in women; and the final ring is for the men stratified analysis. Hollow circles are estimates that are not significant (p-value ≤ 6.5E^{-5}). Metabolites are grouped by super pathway in each section.

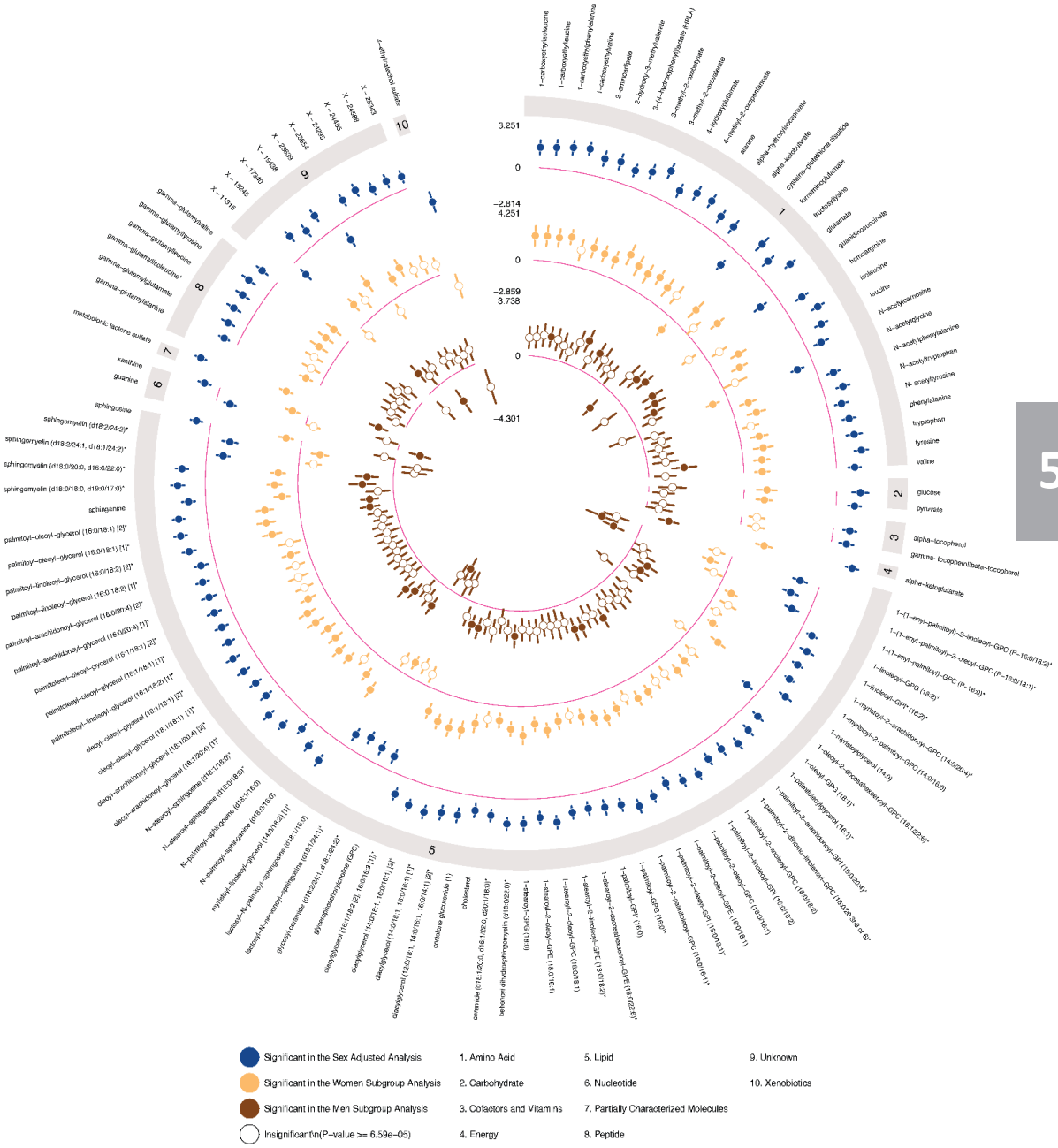
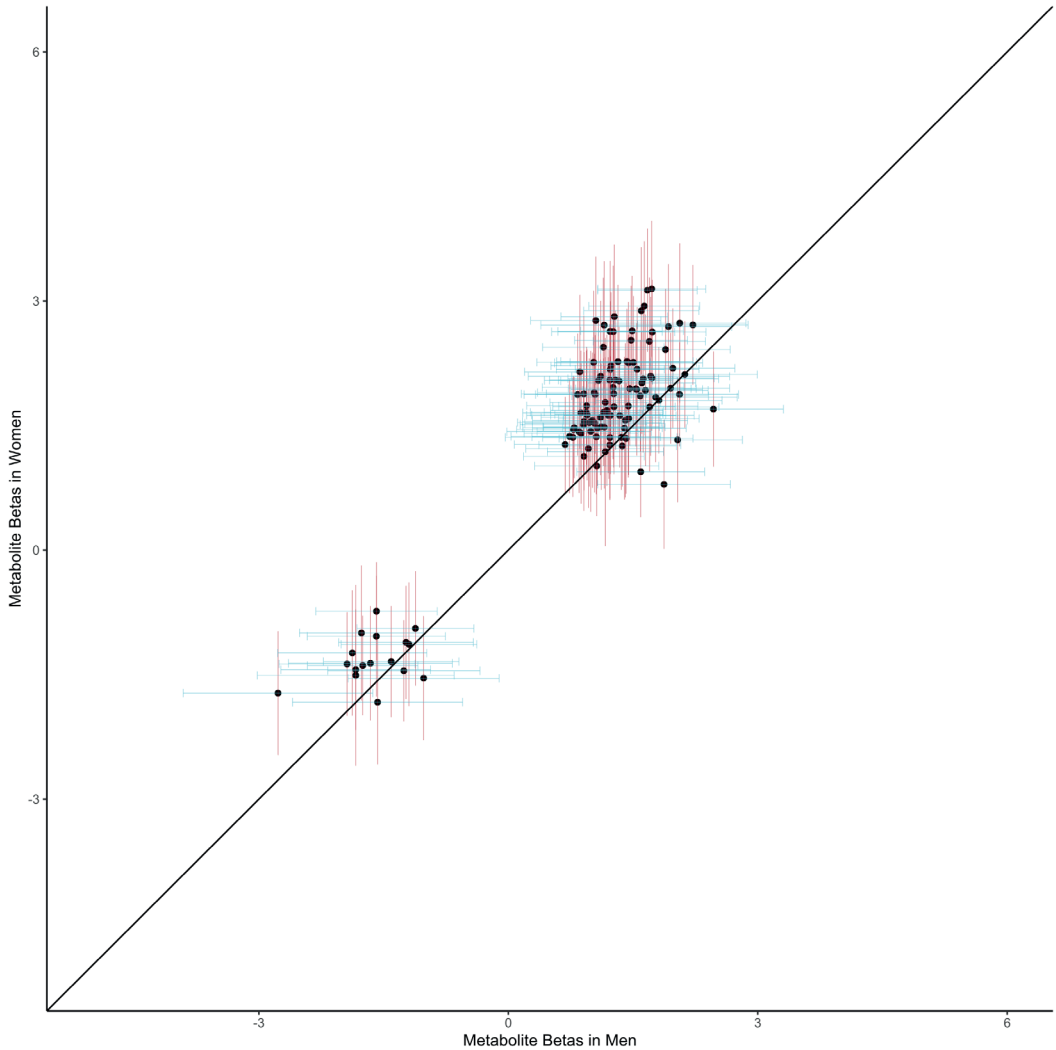


Figure 2 Beta-Beta plot comparing the effect estimates of 118 metabolites associated with HTGC. Overall, effect estimates of the metabolites were stronger in women than men.



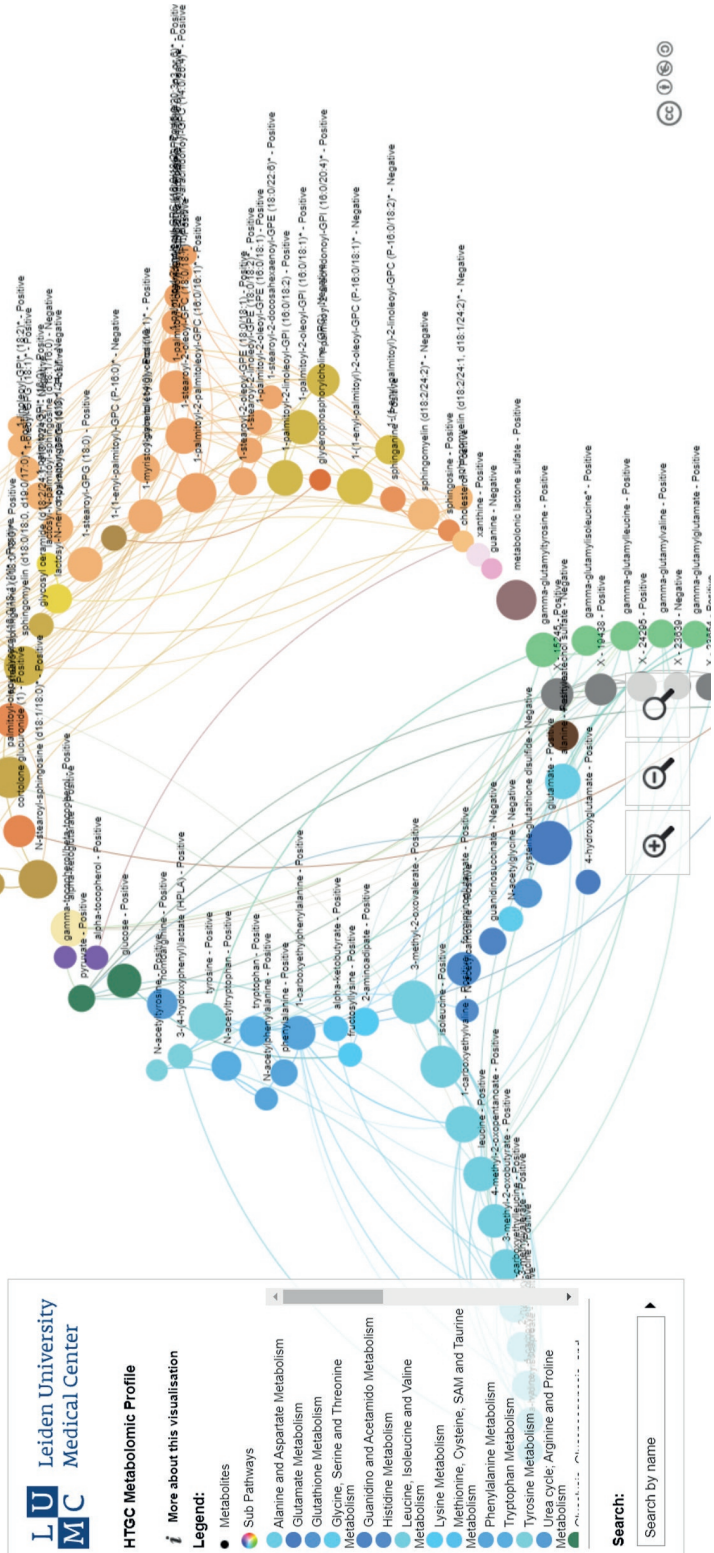
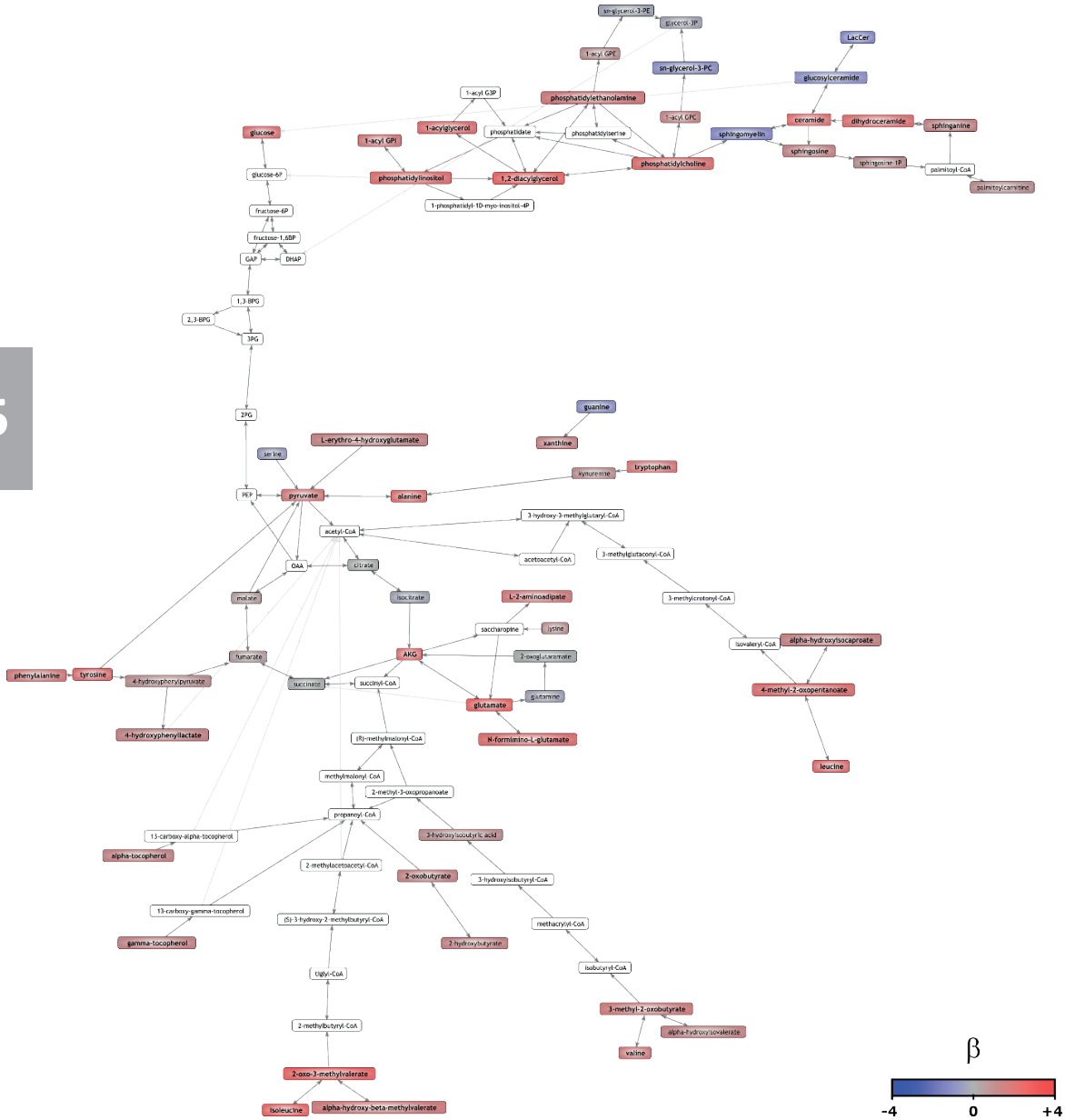


Figure 3: Gaussian Graphical Model for the sex adjusted network showing the amino acids cluster in blue and the lipids clusters in orange/yellow.

Figure 4: Genome scale metabolic model-based network showing how metabolites (nodes) that are significantly associated to HTGC are related to each other via biochemical reaction paths (edges). Metabolites are color coded according to their *b* value, where red indicates a positive *b* and blue a negative *b*. The network also contains metabolites that are not associated with HGTC but that lie in the conversion path between significant metabolites. Intermediate metabolites that have not been measured are colored white. To facilitate the interpretation of the network visualization, all intermediates of the traditional pathways of glycolysis, BCAA degradation and the Krebs cycle have been added, irrespective of whether they have been measured or not.



8 SUPPLEMENTARY MATERIALS:

8.1 Study Design

The Netherlands Epidemiology of Obesity (NEO) study is a prospective, population-based cohort study aimed at investigating the pathways leading to obesity-related conditions (1)ÿ(ÿÿ. During the period between 2008 and 2012 a total of 6671 participants aged 45–65 years, with an oversampling of overweight persons, from the Leiden greater area were included. Persons aged between 45 and 65 years with a self-reported body mass index (BMI) of ≥ 27 kg/m^2 were invited to participate through letters from their general practitioner and municipalities, as well as through local advertisements. Additionally, all inhabitants aged 45–65 years from one municipality, Leiderdorp, were invited irrespective of their BMI.

Approximately 35% (n = 2580) of the NEO participants without potential magnetic resonance imaging contra-indications were randomly selected to undergo direct assessment of the hepatic triglyceride content (HTGC) by proton magnetic resonance spectroscopy (^{1}H-MRS), which was described in detail in our previous work(1, 2).

Participants were invited for a baseline visit at the NEO study center of the Leiden University Medical Centre (LUMC) after an overnight fast. Prior to this study visit, participants completed a general questionnaire at home to report demographic, lifestyle, and clinical information. Data regarding alcohol intake (g/day) and menopausal status for women was recorded from the participants response in the questionnaire. Participants were asked to bring all medication they were using in the month preceding the baseline study visit and research nurses recorded names (e.g., lipid-lowering medication) and dosages of all medication. Participants came to the research site in the morning and completed a screening form, asking about anything that might create a health risk or interfere with MRI (most notably metallic devices, claustrophobia, or a body circumference of more than 1.70 m). All participants underwent an extensive physical examination, including anthropometry and blood sampling. The percent body fat of the participants was estimated by the Tanita bio impedance balance (TBF-310, Tanita International Division, UK) without shoes and one kilogram was subtracted to correct for the weight of clothing.

8.2 Untargeted Metabolomics Measurements

Fasting state serum samples of the Leiderdorp general subpopulation which had a normal BMI distribution (N=599 in total) from the NEO study were sent for untargeted metabolomics measurements at Metabolon Inc. (Durham, North Carolina, USA) using Metabolon™ Discovery HD4 platform. In brief, this process involves four independent ultra-high-performance liquid chromatography mass spectrometry (UHPLC-MS/MS) platforms (3, 4). Two platforms use positive ionization reverse phase chromatography, one uses negative ionization reverse phase chromatography, and one uses hydrophilic interaction liquid chromatography (HILIC) negative ionization (4). Known metabolites were annotated at Metabolon Inc. with their chemical names, super pathways, sub pathways, compound identifiers from various metabolite databases, and information regarding their biochemical properties.

8.3 Methods

8.3.1 Imputation and Scaling of Metabolite Relative Concentrations

In brief, for the endogenous and unannotated metabolites we applied the multiple imputation method and used the outcome variable, HTGC, along with a select number of correlated metabolites as auxiliary variables to generate 5 imputed datasets. Xenobiotic metabolites were imputed to zero to account for true missingness. All metabolites were natural log-transformed and scaled on their means during the imputation process (5). Following the imputation, metabolite levels were range scaled (also known as min-max scaling) by using the differences of the biological range between the maximum and minimum values for each metabolite and setting the range of all measured metabolites between -1 and 1. This method of scaling was shown to perform well for metabolomic data, particularly untargeted metabolomics data with large units of relative measurement and wide variance between the metabolites. In turn, range scaling provides equal importance to the metabolites in a more biologically relevant manner (6).

For each imputed dataset we performed the regression to assess the association between HTGC and each measured metabolite independently. Each metabolite was used as the exposure variable with the HTGC as the outcome in univariate linear regression analyses. Furthermore, we adjusted for sex, age, total body fat, alcohol intake, and lipid lowering medication. The results of the regression analyses in the imputed datasets were subsequently combined using Rubin's rules (7). All estimates represent the effect on the natural log of HTGC levels per one scaled unit of the metabolite concentration.

To adjust for multiple testing, we applied Bonferroni correction and adopted the method VeffLi estimate described by Ji and Li (8)to account for the number of independent components underlying all 1,363 metabolite measures. This method considers the strong correlations between metabolites from mutual pathways and the inclusion of multiple isomers of the same metabolites to calculate the effective number of independent variables. Accordingly, 758 independent variables were identified, and the calculated Bonferroni correction threshold was set to $0.05/758 = 6.59\times10^{-5}$. The analysis and forest plots were all conducted using R version 3.6.1 (9) and Python 3.7.6 (10). The circular plot was made using the EpiViz R package (11, 12).

8.3.2 Gaussian Graphical Model (GGM)

Significant metabolites from the main analysis and the sex stratified analysis were used to develop the GGM networks separately. To create the network, we used the full measurements of the metabolites from our imputed dataset. Since we used multiple imputation, we could only select one imputed dataset. The correlations between the metabolites were calculated using sparse Gaussian Graphical Models with graphical LASSO using the glasso function in R (qgraph R package version 1.6.9) (13) coupled with the Extended Bayesian Information Criterium (EBIC) to tune the LASSO shrinkage factor (14). EBIC has been reported in simulation studies to perform better than the standard BIC, particularly for controlling false positivity rates. Here, we set the EBIC tuning parameter to the default value of 0.5 (wherein a value of 0 uses standard BIC) for a balanced tuning (15). Based on the correlation coefficients, edges were drawn between the correlated metabolite nodes with the width corresponding to the coefficient strength. The size of the nodes was determined by the absolute value of the effect estimate from the multivariable linear regression analysis. The metabolite nodes were colored based on their biochemical super and sub pathway classes and organized in ascending order based on their p-values. Visualization and layout of the networks were created in Gephi and exported as an interactive HTML5 using the sigmaExporter plugin (16). All GMM networks can be accessed online on https://tofaquih.github.io/AtlasLiver/.

8.3.3 Genome Scale Metabolic Model-based (GSMM) pathway analyses

We mapped the metabolite names onto a curated version of the Human Metabolic Reactions database (HMR 2) genome-scale metabolic model (GSMM) (17) and calculated which metabolite pairs were ≤ 2 reaction steps apart in the model. The GSMM-HMR2 was used to determine the biochemical interconversions between the metabolites that were measured on the Metabolon platform. All computations described in this section were performed in MATLAB R2019b. To facilitate the mapping of metabolomics data to the GSMM, we enriched the model with compound synonyms and external identifiers from the Chemical Entities of Biological Interest (ChEBI) database (18), where ChEBI identifiers and synonyms of conjugate acids and bases were also included. Of the 1067 metabolites with known identity that were quantified, 400 mapped properly to the GSMM. All reactions and compounds in HMR2 were checked for mass and redox balance and were adjusted when necessary.

Biochemical interactions between metabolites were determined by converting the GSMM into a weighted directed graph where nodes represent metabolites and edges represent reactions. Subsequently all reaction paths between the measured metabolites that involved one or two reaction steps were determined using a generic path finding algorithm that was developed *in house*. To ensure that the reaction paths represented relevant biochemical conversions, each path was checked for stoichiometric and thermodynamic consistency. In addition, only substrate-product mappings were considered that involved the transfer of carbon-based moieties, except for CO2. Therefore, half-reactions involving the transfer of electrons, amino or phosphate groups were decoupled from the main reaction in the path finding procedure. For example, in the reaction NADH + pyruvate <=> NAD^+ + lactate, only NADH and NAD^+ are linked in the graph and pyruvate and lactate are linked. Likewise, in the reaction glutamate + pyruvate <=> AKG + alanine, only glutamate and AKG are linked, and pyruvate and alanine are linked. In this way we prevented the creation of crowded, highly connected networks in which most metabolites are connected to a few hub metabolites such as H+, H2O, ATP and NADH.

The weight of the edges in the GSMM-based network was set to 1 for all reactions, except for transporter reactions that transferred compounds over the cellular membranes and (half) reactions that involved uniquely produced metabolites; both these types of reactions were assigned a weight of zero. A consequence of the second exception is that linear reaction chains in which the intermediates were not produced by other reactions were counted as a single reaction step during the path search. Finally, we added a list of intermediates from the glycolysis, Krebs cycle and BCAA degradation pathways that could not be quantified on the metabolomics platform, to prevent gaps in the traditional pathways.

This resulted in a network of connected metabolites that we integrated with biochemical reaction and pathway knowledge into an interactive HTML/JavaScript document, which can be accessed on https://tofaquih.github.io/AtlasLiver/. The results from the network analysis include measured associated metabolites as well as relevant intermediate metabolites regardless of whether they were measured in the dataset. In addition, the network shows the directionality of the biological reaction paths for the biosynthesis and degradation of the metabolites and includes details regarding the involved genes and intermediate reaction steps. Furthermore, for all metabolites and genes, also those not included in the network, hyperlinks to external databases and gene expression profiles are provided. Reaction information was enriched by importing tissue-specific gene expression from the Human Protein Atlas (HPA)(19) and Genotype-Tissue Expression (GTEx) project(20).

8.4 Supplementary Results

Amino Acids Associated with HTGC Independently after Adjustment for Insulin Resistance

Insulin resistance (IR) and HTGC have a complex bi-directional relationship and share overlapping metabolomic pathways, particularly the amino acid metabolism (21). Both of these disease are associated with an elevation and dysregulation of amino acids (22)ÿ. However, it remains uncertain if IR causes HTGC or vice versa (23). To inspect the strong and complex link between IR, HTGC, and amino acids, we performed a post hoc multivariable linear regression analysis and adjusted for IR in addition to sex, age, total body fat, alcohol intake, and lipid lowering. In this analysis we examined 43 metabolites, including amino acids, carbohydrates, metabolonic lactone sulfate, and vitamin E metabolites. Only 10 metabolites lost their association with HTGC, notably pyruvate, while a small reduction of the effect estimates occurred for the remaining metabolites. Overall, the association of the amino acid metabolites with HTGC in our main analysis remained after adjustment for IR (Supplementary Figure 1).

8.4.1 HTGC Associations with Men, Women, and Post-Menopausal Women

NAFLD has been previously shown to be a sexually dimorphic disease in mice and humans (7). Moreover, men have a higher risk of NAFLD than premenopausal women and a similar risk as postmenopausal women, indicating an increased risk of NAFLD after menopause(24-26). However, it has been shown that postmenopausal women have a higher prevalence of severe forms of fibrosis than men and premenopausal women(26). This increased risk was found to be associated with early menarche and estrogen deficiency(25-27). Sex-stratified analysis was performed to observe the general differences of metabolomic associations with HTGC in men vs women and premenopausal vs postmenopausal women (Supplementary Figure 1 and Supplementary Figure 2). We performed secondary analyses using multivariable linear regression in women stratified by menopause and adjusted for age, total body fat, alcohol intake, and lipid lowering medication (Supplementary Figure 2 and Supplementary Figure 3). We found that the number of associations and effect sizes effects were even higher in post-menopausal women while only few metabolites were even associated with outcomes HTGC in the premenopausal women. These findings are in line with previous findings of increased amino acids, such as glutamate and tyrosine, and increased lipids, such as triglycerides and phosphatidylcholines, in postmenopausal women (25, 28).

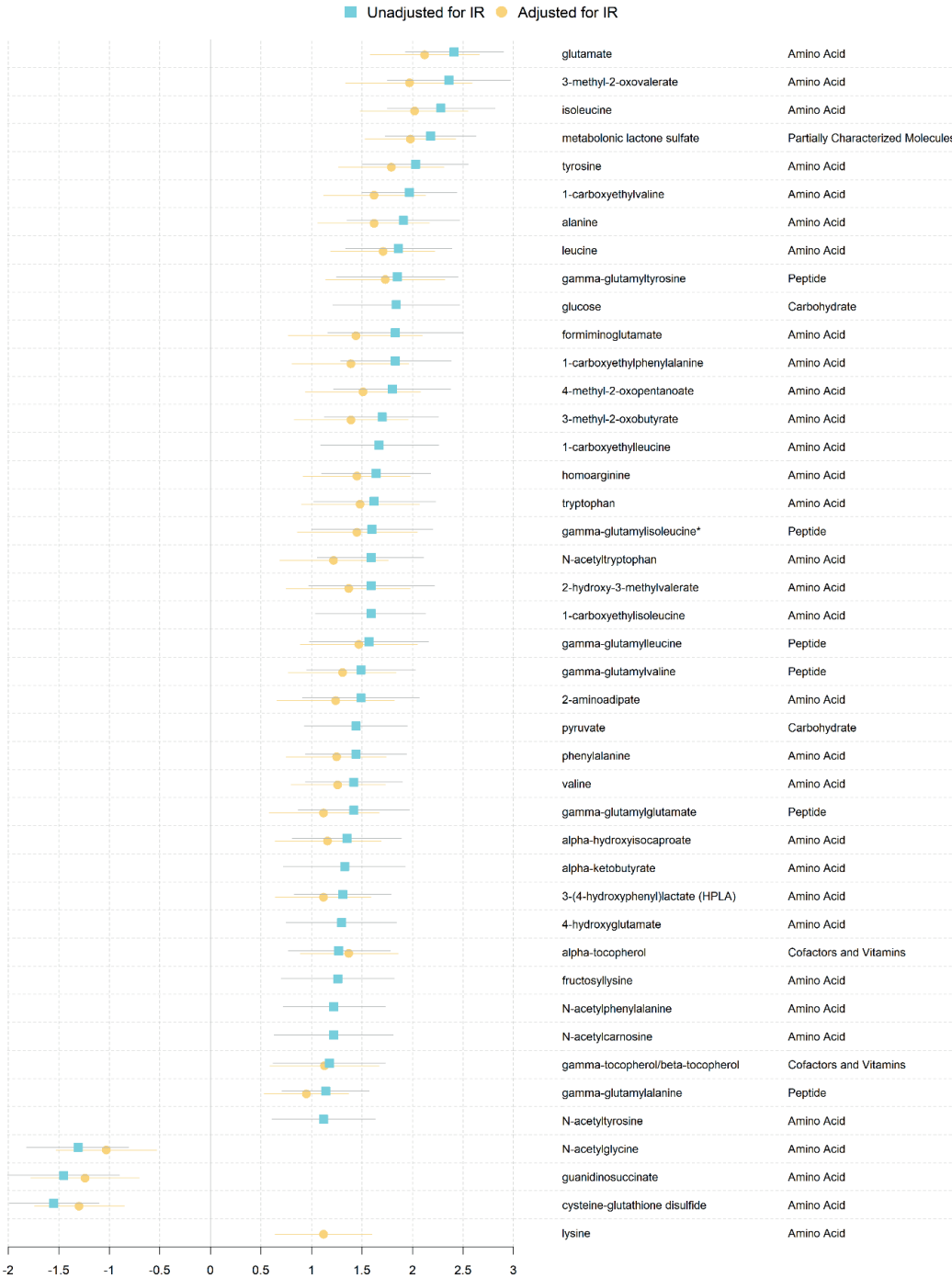
In addition to the general differences between the sex-specific metabolic profiles, several associated metabolites were not shared between men and women (Supplementary Table 1). Two metabolites of interest in men were etiocholanolone, and pregnanediol 3-O-glucuronide. Etiocholanolone glucuronide, a testosterone metabolite(29), and pregnanediol 3-O-glucuronide were negatively associated with HTGC. However, previous studies on this association show inconsistent results(26, 30-32).

Overall, directions of all associations are identical in men, pre- and postmenopausal women. The larger effect sizes in women resulted in a larger number of metabolites associated with HTGC than in men. However, in our analysis the small sample size in the sex-stratified analyses resulted in a loss of power. Thus, we did not perform any specific analysis between men and women and only describe the general observations between the two profiles and provide them as part of the GMM and GSMM networks. Our data indicate that similar pathways are associated with HTGC in men and women before and after menopause. The fact that we do not find obvious sex differences beyond effect size may be due to the homogenous and relatively healthy cohort. Further insight into the metabolite-HTGC differences between men and women, and pre and postmenopausal woman would require analysis in a larger study.

Supplementary Table 1: Metabolites associated only in men or women univariate analysis

Sex	Metabolites	P Value	Estimate (95% CI)	Pathway	Sub Pathway
Men	pregnanediol-3-glucuronide	6.48e-05	-2.98 (-4.42 – -4.42)	Lipid	Progestin Steroids
Men	etiocholanolone glucuronide	3.99e-05	-1.44 (-2.11 – -2.11)	Lipid	Androgenic Steroids
Women	3-aminoisobutyrate	5.98e-05	-1.23 (-1.83 – -1.83)	Nucleotide	Pyrimidine Metabolism, Thymine containing
Women	N-acetylleucine	2.20e-05	1.63 (0.89 – 0.89)	Amino Acid	Leucine, Isoleucine and Valine Metabolism
Women	2-hydroxyarachidate*	6.47e-06	1.78 (1.02 – 1.02)	Lipid	Fatty Acid, Monohydroxy

Supplementary Figure 1: Forest plot comparing the effect estimates of amino acids and carbohydrates on HTGC with and without the adjustment for insulin resistance (IR).



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