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Received: 12 June 2022 | Revised: 28 February 2023 | Accepted: 23 March 2023

DOI: 10.1111/liv.15575

Liver INTERNATIONAL WILEY

ORIGINAL ARTICLE

Hepatic triglyceride content is intricately associated with numerous metabolites and biochemical pathways

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Funding information
King Abdullah Scholarship Program and King Faisal Specialist Hospital & Research Center; VELUX Stiftung, Grant/Award Number: 1156; ZonMW-VENI, Grant/Award Number: 916.14.023

Handling Editor: Luca Valenti

Abstract

Background and Aims: Non-alcoholic fatty liver disease (NAFLD) is characterized by the pathological accumulation of triglycerides in hepatocytes and is associated with insulin resistance, atherogenic dyslipidaemia 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 with 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 1363 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

Abbreviations: ^{1}H-MRS, proton magnetic resonance spectroscopy; AKG, alpha ketoglutarate; BCAA, branched chain amino acids; BCKA, branched chain keto acids; BMI, body mass index; CVD, cardiovascular disease; EBIC, extended Bayesian information criterium; GGM, Gaussian graphical model; GPC, glycerophosphorylcholine; GSMM, genome scale metabolic model; HCER, hexosyl/glucosylceramide; HILIC, hydrophilic interaction liquid chromatography; HOMA1-IR, homeostatic model assessment index for insulin resistance; HTGC, hepatic triglyceride content; Human1, human genome-scale metabolic model; IR, insulin resistance; LCER, lactosylceramide; MRI, magnetic resonance imaging; NAFLD, non-alcoholic fatty liver disease; NEO, the Netherlands Epidemiology of Obesity study; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositol; T2D, type 2 diabetes; UHPLC–MS/MS, ultra-high-performance liquid chromatography mass spectrometry.

created using correlation-based Gaussian graphical model (GGM) and genome scale metabolic model network analyses. Pathways associated with the clinical prognosis marker fibrosis 4 (FIB-4) index were tested using a closed global test.

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-ceramide and lactosyl-ceramide. We also identified a novel possible HTGC-related pathway connecting glutamate, metabolonic lactone sulphate and X-15245 using the GGM network. These pathways were confirmed to be associated with the FIB-4 index as well. 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 and the FIB-4 index. Moreover, we report a novel pathway glutamate-metabolonic lactone sulphate-X-15245 with a potential strong association with HTGC. These findings can aid elucidating HTGC metabolomic profiles and provide insight into novel drug targets for fibrosis-related outcomes.

KEYWORDS
dysregulation, genetic targets, liver triglyceride content, metabolomics, pathway analysis

1 | INTRODUCTION

Non-alcoholic 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, dyslipidaemia 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, among 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 IR 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]

Key points

In this study, we aimed to investigate the biological factors that contribute to the accumulation of triglycerides in the liver, which is associated with non-alcoholic fatty liver disease. To achieve this, we measured metabolites, which are the end products of chemical reactions in the body's metabolism. Our study found 118 metabolites associated with higher levels of liver fat. Some of these associations were previously unknown and may be useful for future studies and drug development. We also provided an interactive atlas of networks to aid researchers explore the complex relationships between metabolites and liver fat accumulation that we reported in this study.

Several studies have been performed to gain insight into the complex aetiology of NAFLD by applying metabolomics.[9] Changes in circulating metabolites are thought to reflect the composite of 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.[9,14]

Here we aimed to elucidate the HTGC metabolomic profile as assessed by ^{1}H-MRS, in a middle-aged population ($N = 496$) using an untargeted metabolomics platform (1363 metabolites). We further created a comprehensive atlas of HTGC-associated metabolites and pathways from our results using two pathway analysis approaches that allow flexible and interactive examination of our results. In addition, we examined the associations using global testing between the different pathways with the fibrosis 4 (FIB-4) index.

2 | METHODS

2.1 | 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 this 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, 1363 serum metabolites were measured of which 840 metabolites were from various endogenous pathways (56% lipids, 31% amino acids and 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[15,16] 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.[15]

2.2 | Multiple linear regression

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.

In addition, 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 whether the associations of metabolites with HTGC were dependent on IR, particularly for those known to be associated with IR, that is, amino acids and carbohydrates.

2.3 | Pathway analysis

Significant metabolites from the main analysis and the sex stratified analysis were subsequently analysed 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 dense metabolomic datasets from untargeted platforms.[10,17,18] In contrast, GSMM methods are based on a priori defined and curated pathways and have also been used in metabolomic and non-metabolomic studies.[19] In addition, we used an inhouse developed GSMM tool to construct the networks. Both methods are thus complementary in their basis, that is, 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/.

2.4 | Global test between pathways and FIB-4

To further explore the clinical relevance of the biochemical pathways associated with HTGC, we performed a global test to assess association of reported biochemical pathways with the FIB-4 index for liver fibrosis. The FIB-4 index is a non-invasive diagnosis tool used in the clinic to drive decisions regarding the risk of fibrosis and prioritizing appropriate treatment.[20] The FIB-4 index was first calculated for 394 individuals using the measurements for aspartate transaminase (AST), alanine aminotransferase (ALT) and platelet (PLT) count in the formula Age (years)×AST(IU/L)/PLT (109/L)×ALT1/2 (IU/L). We used multiple imputation using chained equations to impute the FIB-4 index values for an additional 100 individuals with AST and ALT values, but missing PLT count measurements. Sex, age, triglyceride concentration, alcohol consumption, fat percentage, AST and ALT were used as the imputation

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 (%).

		Men	Women	Women	Women
	Total	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)
FIB-4	1.2 (0.4)	1.3 (0.4)	1.2 (0.4)	1.3 (0.3)	1.0 (0.4)
FIB-4 imputed	1.2 (0.4)	1.3 (0.4)	1.2 (0.4)	1.3 (0.4)	1.0 (0.4)
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)

Abbreviations: CVD, cardiovascular disease; FIB-4, fibrosis 4 index; HOMA1-IR, homeostatic model assessment index for insulin resistance; HTGC, hepatic triglyceride content; IQR, interquartile range.

covariates. Two participants did not have measurements for AST, ALT or PLT and were not imputed. Therefore, the FIB-4 index was derived for 494/496 participants in total. The participants with FIB-4 values were dichotomized into high risk vs low risk of fibrosis progression using the standard cut off point 1.45 (Sterling, Lissen et al. 2006). In the unimputed dataset, 97 of the 394 participants with the calculated FIB-4 index had a FIB-4 > 1.45. After imputation, 116–124 participants out of 494 were had a FIB-4 index of 1.45 or more in the imputed dataset. These imputed datasets were combined for the subsequent global test.

For metabolomics data, the closed global test has been developed to identify biochemical pathways associated with an outcome.[21] We exploited this variant of the global test to assess the associations of the different metabolite pathway groups with the FIB-4 index. The global test is considered an unbiased method for assessing the overall effects of groups of exposures, particularly those with weak pathway effects in high-dimensional data.[21,22] We used the pathways defined in the Metabolon dataset as prior for selecting the metabolite pathway groups. Those pathways were lipids, amino acids, carbohydrates, xenobiotics, 'cofactors and vitamins', nucleotides, energy-related metabolites, peptides, uncharacterized and partially characterized molecules. As the partially characterized molecules group was small ($N = 7$), we combined them with

uncharacterized group due to their similarity. Similar to the linear regression analyses, we adjusted for confounding by including sex, age, fat percentage, statin use and alcohol consumption as covariates. Within the global test, familywise error rate is used to control for multiple testing within each pathway group tested.[21] The full methodology is described in detail in previous works.[21] The null hypothesis was defined as the absence of any associations within the pathway group with the FIB-4 index. If the null hypothesis is rejected, we further report the proportion of true discovery in the pathway, that is, the number of associations within the pathway groups with the FIB-4 index. For each group, we set the maximum number of test iterations to 5000.

3 | RESULTS

3.1 | Association analyses of metabolites and HTGC

Univariate linear regression analyses were performed to examine the associations between the 1365 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 $<6.59\times10^{-5}$), 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 (Table S1). Excluding individuals with lipid-lowering medications did not alter the results (data not shown).

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.

3.2 | 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 IR-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. These results are detailed and discussed in the Supplementary Materials and Figure S1.

3.3 | 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 as follows: palmitoyl-oleoyl-glycerol (16:0/18:1) [β: 2.23 (95% CI 1.72; 2.73)], 1-palmitoyl-2-palmitoleoyl-glycerophosphorylcholine (GPC; 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, among 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, 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)].

3.4 | Other metabolites

Metabolonic lactone sulphate, 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;

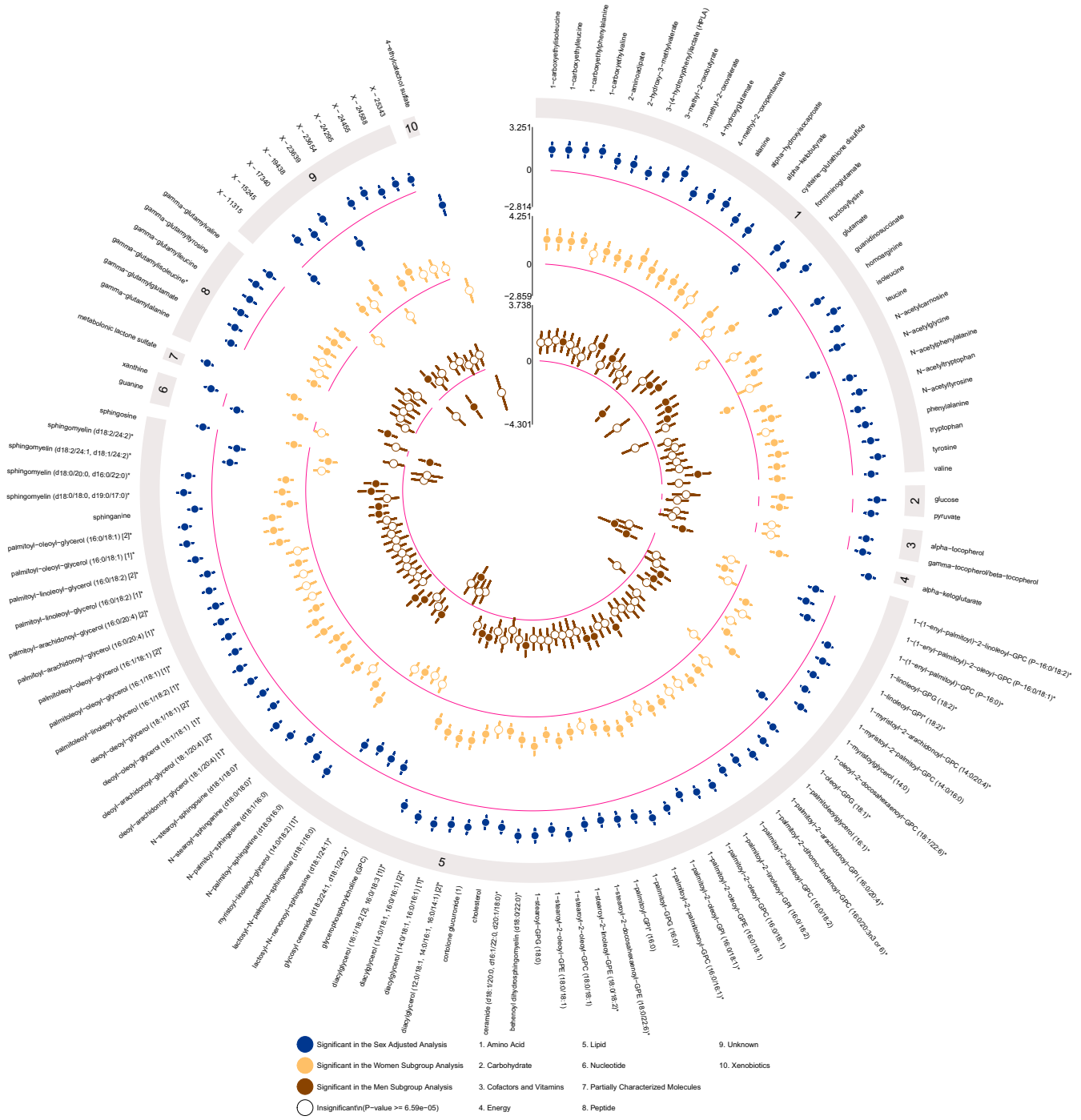

FIGURE 1 Comparison of the point estimates and confidence intervals for the 118 metabolites associated with hepatic triglyceride content (HTGC) in the primary analysis and the sex stratified analysis. The first ring shows the effect estimates in the sex combined analysis. The second ring shows analysis 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.

−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.

3.5 | Correlation-based network analysis using GGM

GGM networks were generated including all significantly (p-value $<6.59\times10^{-5}$) 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 three groups: (1) primary amino acids, for example, glutamate; (2)

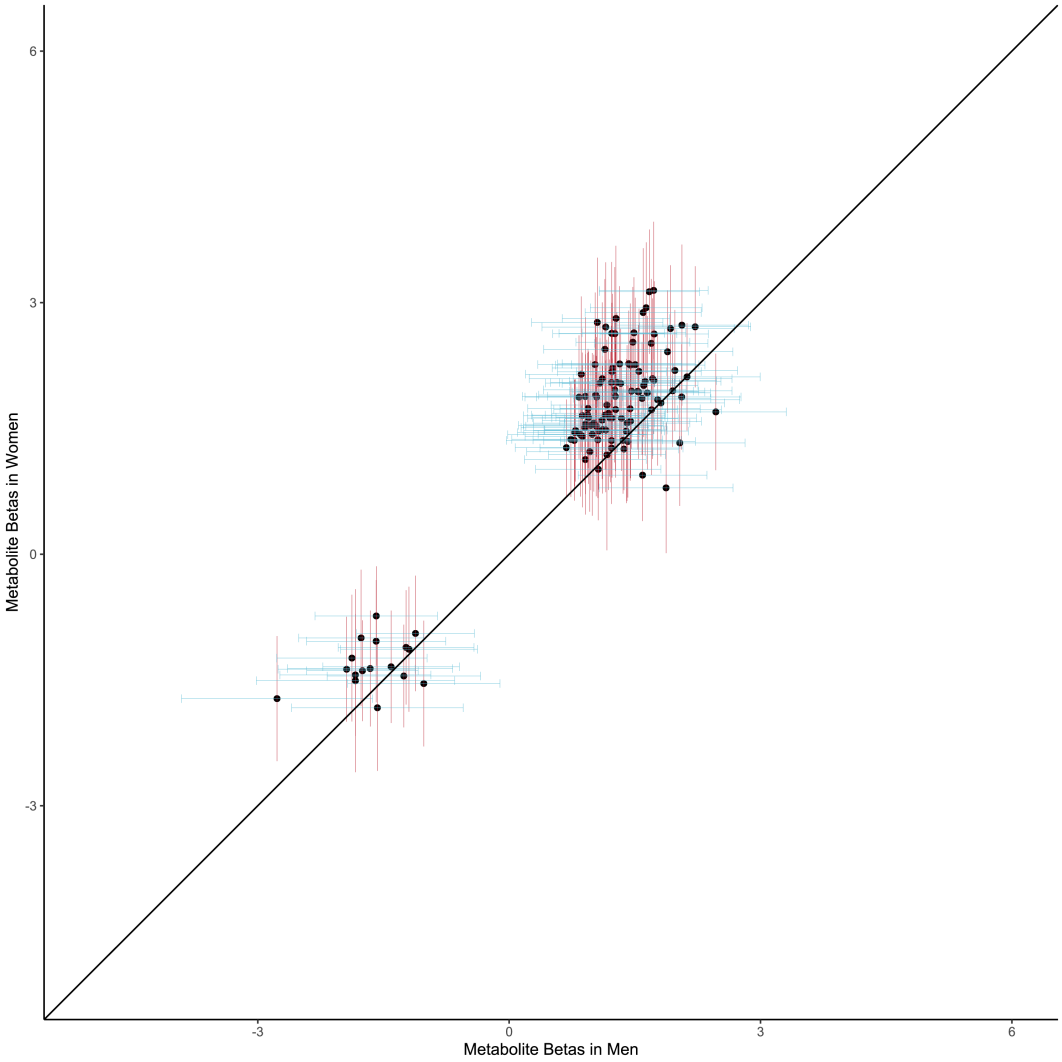

FIGURE 2 Beta–Beta plot comparing the effect estimates of 118 metabolites associated with hepatic triglyceride content (HTGC). Overall, effect estimates of the metabolites were stronger in women than men.

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-palmitoyl-sphinganine (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 sulphate 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 GGM network and, interestingly, alpha-tocopherol was also connected to cholesterol.

3.6 | 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 Human1 was performed (Figure 4). The network showed several amino acids subclusters that were associated with higher HTGC, among which the BCAAs and their keto and 2-hydroxy form, the

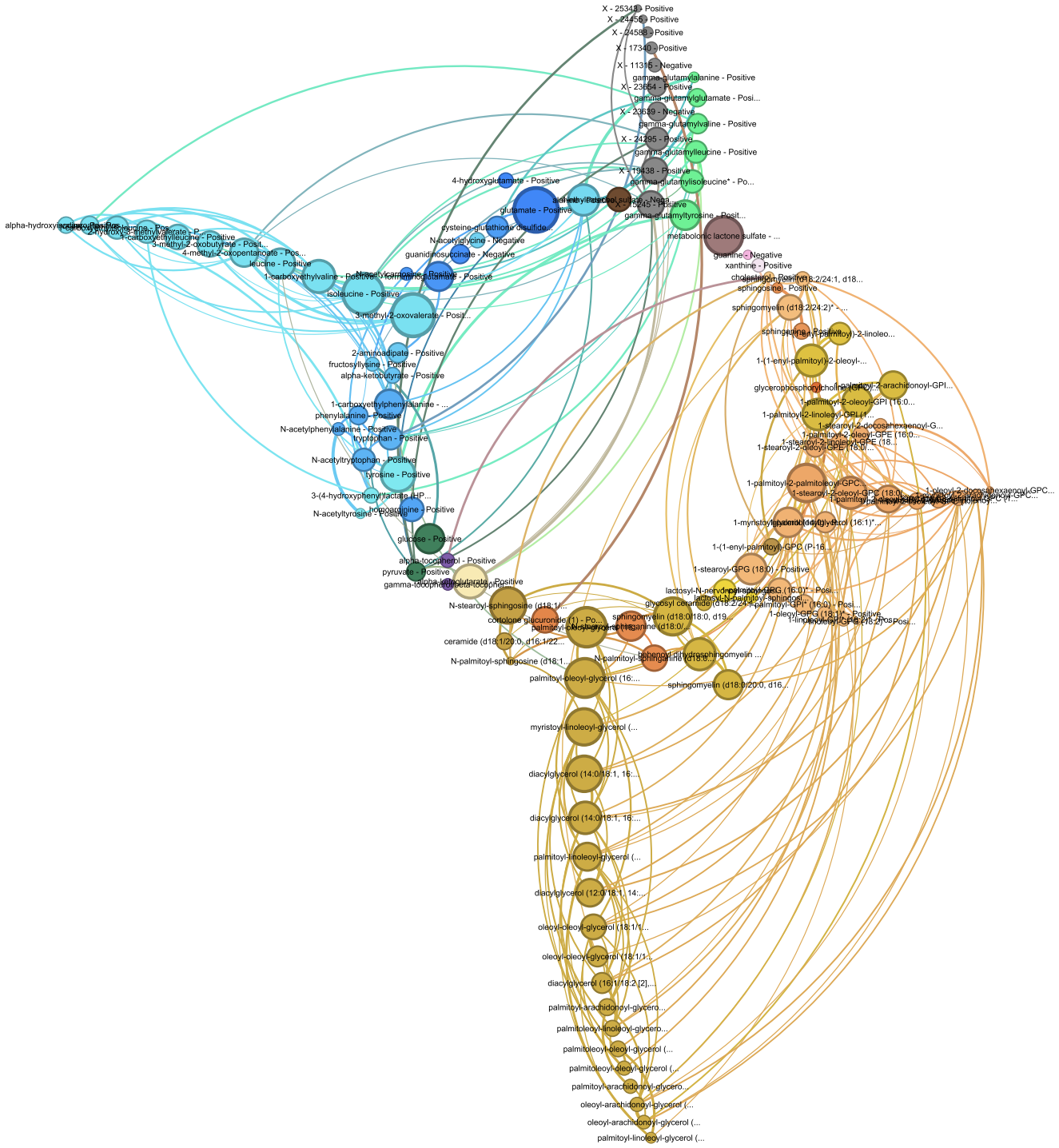

FIGURE 3 Gaussian Graphical Model for the sex adjusted network showing the amino acids cluster in blue and the lipids clusters in orange/yellow. Size of the metabolite circles (nodes) are proportional to the effect size on hepatic triglyceride content (HTGC). Lines between the nodes (edges) are proportional to the correlation between the metabolites.

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 (labelled 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/.

3.7 | Global test analysis

The null hypothesis for the closed global test was rejected for four of the nine pathway groups (Table 2). These were lipids (number of true discoveries (TD) = 168), amino acids (TD = 48), xenobiotics (TD = 1) and the uncharacterized/partially characterized groups (TD = 68). These tests did not require any further test iterations to reject the null hypothesis (with the exception on xenobiotics which required 8 iterations), indicating that the associations were strong between the metabolites in these pathways and the FIB-4 index. Results for the nucleotides were inconclusive and the null hypothesis was not rejected.

4 | 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

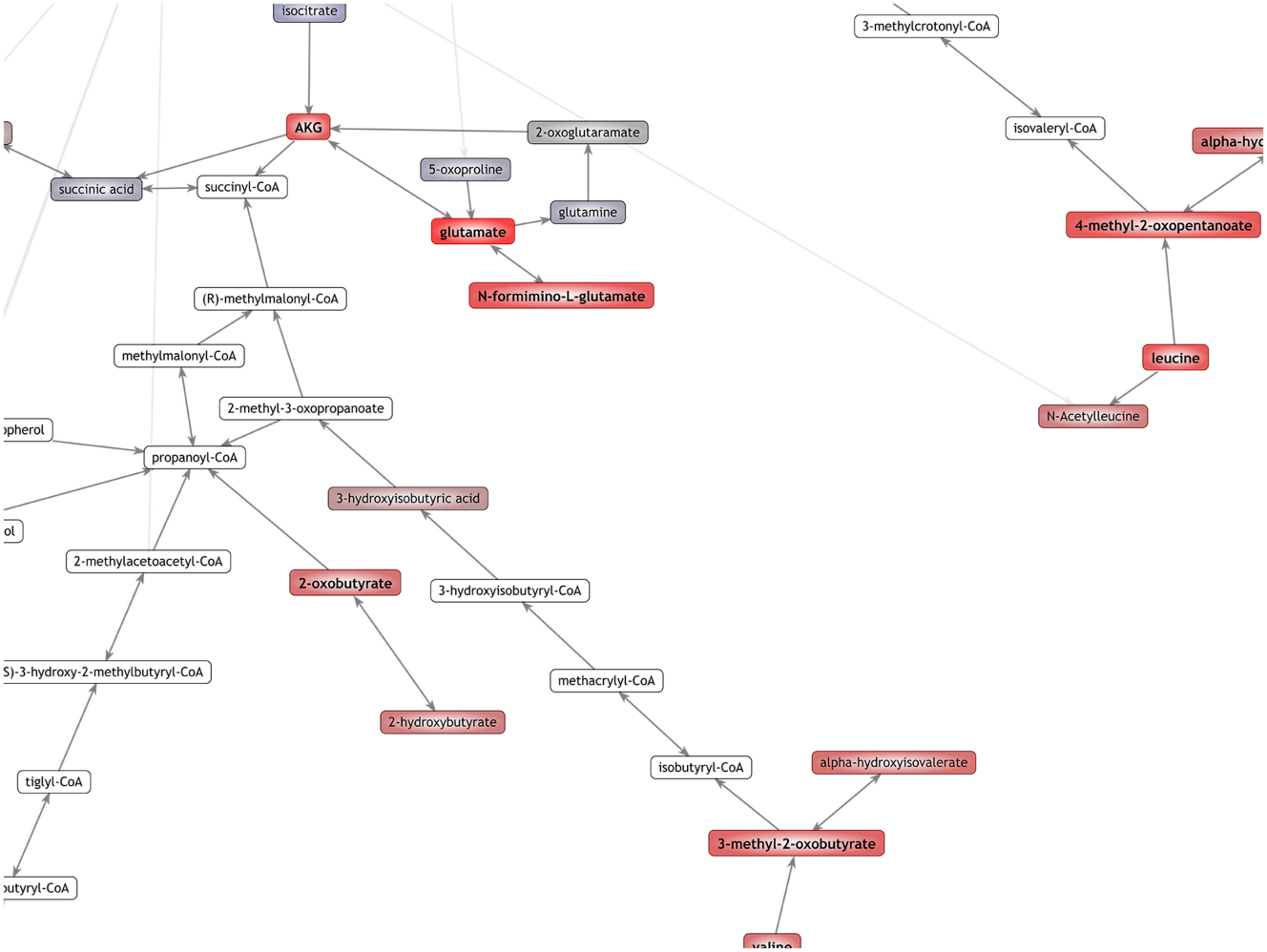

FIGURE 4 A section of the full genome scale metabolic model-based network showing how metabolites (nodes) that are significantly associated with hepatic triglyceride content (HTGC) are related to each other via biochemical reaction paths (edges). Metabolites are colour coded according to their β value, where red indicates a positive β and blue a negative β. The network also contains metabolites that are not associated with HTGC but that lie in the conversion path between significant metabolites. Intermediate metabolites that have not been measured are coloured white. To facilitate the interpretation of the network visualization, all intermediates of the traditional pathways of glycolysis, branched amino acid (BCAA) degradation and the Krebs cycle have been added, irrespective of whether they have been measured or not.

TABLE 2 Closed global test results for the pathways associated with the FIB-4 index as well as the number of true discoveries in each pathway.

Pathway	Number of metabolites	Global test hypothesis	Number of iterations	Number of true discoveries
Lipids	475	Rejected	0	168
Amino acids	215	Rejected	0	41
Carbohydrates	22	Not rejected	0	0
Xenobiotics	220	Rejected	8	1
Cofactors and vitamins	30	Not rejected	0	0
Nucleotides	38	Unsure	5000	0
Energy	11	Not rejected	0	0
Peptides	49	Not rejected	0	0
Uncharacterized/partially characterized	303	Rejected	0	68

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 but similar mean FIB-4 index in men and women. Apparently, metabolites have strong correlations even with smaller HTGC ranges. Pathway analyses revealed two clusters of interrelated metabolites, one primarily involving amino acid-related metabolism and the other lipid metabolism.

4.1 | 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.[9,23,24] 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.[25,26] It is interesting that the BCAAs and derivates, known to be associated with body fat and IR,[25] remained associated with HTGC despite the adjustment for total body fat and after further adjustment for HOMA-IR in the sensitivity analysis. These results indicate that the associations of amino acids and carbohydrates metabolites with the levels of HTGC were overall largely 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.[9,27] Moreover, the proposed glutamate–serine–glycine index in those studies was not replicated in our analysis despite our larger sample size ($N=496$ vs. $N=64$ [$N=20$ controls]). The previous studies focused on NAFLD and NASH patients in a case–control study design in contrast to our study that included participants from the general population with an average HTGC of 5.6%. Therefore, it may be possible that serine and glycine are better markers for advanced stages of HTGC, as observed in NAFLD/NASH patients, due to the stronger metabolic dysregulation but are not markers for lower levels of HTGC.

4.2 | 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.[28] 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.[29] Overall, most of the lipids (52/62) 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 GGM 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.[30]

The associations of the ceramides and dihydroceramides with higher HTGC are analogous to previous studies on NAFLD.[31–33] However, the associations of sphingomyelins and dihydrosphingomyelins with HTGC and NAFLD in humans are less studied and understood compared to the associations of ceramides.[34] Since dihydroceramides are precursors for the synthesis of dihydrosphingomyelins,[33,35] it could explain their association with higher HTGC. However, a small study by Lovric et al. ($N = 75$)[36] 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 in HTGC in the liver.[37] Regarding HCER and LCER, as stated earlier, HCER is involved in the synthesis of LCER, ceramide and glucose.[30] In addition, both LCER and HCER are involved in glycosphingolipid metabolism.[38] One small study ($n = 28$) reported a positive association HCER and LCER with NASH.[34] Finally, a recent a study reported similar results of higher dihydrosphingolipid classes being associated with increased fibrosis in animal models and NAFLD patients.[39]

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.

4.3 | 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 sulphate (formerly assigned the ID X-12063). Metabolonic lactone sulphate 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[40–42] and acute-on-chronic liver failure.[43] Moreover, metabolonic lactone sulphate was associated with cardiometabolic disease[44] and was positively correlated with BMI, waist hip ratio and HOMA-IR.[45] Metabolonic lactone sulphate was also reported to be associated with the *CYP3A5/ZSCAN25* locus via the rs10242455[46] and rs7808022[47] single nucleotide polymorphisms (SNPs), respectively. This locus is highly expressed in liver and shares the same regulatory promoters.[48] *CYP3A* is particularly expressed in the liver and produces the CYP3A protein, a highly abundant drug-metabolizing liver enzyme.[44,48] Although the specific functionality and underlying biological pathways of metabolonic lactone sulphate remains unelucidated, the evidence indicates a link with the liver, cardiometabolic disease, and our findings with higher HTGC levels.

4-Ethylcatechol is a xenobiotic metabolite that is primarily acquired from the ingestion of coffee beverages and products.[49,50] In our analysis, 4-Ethylcatechol was negatively associated with HTGC levels and was not connected to any metabolites in the GGM or GSMM networks. Protective effects of coffee and caffeine intake against liver fibrosis have been suggested before due to its antifibrotic and antioxidant effects.[51,52] Several studies examining the association of coffee consumption with liver fat found similarly reduced HTGC.[51,53,54] 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.[51] Results for patients with NAFLD were less conclusive with some showing that increased coffee consumption was associated with reduction of NAFLD.[54]

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.[55] 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.[56] 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.[57,58]

4.4 | Pathway analysis

Pathway analysis was performed using two approaches. The first approach—GGM—is data driven and uses 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 unannotated 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.[17] 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,[17] which showed that strongly associated metabolites generally corresponded to the same pathways.

4.5 | Pathways associated with the FIB-4 index

The results of the closed global test were in line with the findings we did for HTGC in the linear regression analyses and in the GGM and GSMM networks. Namely, all analyses identified the lipids and amino acids metabolite groups. In addition, the metabolite groups xenobiotics, uncharacterized and partially characterized groups were also found to be associated with the FIB-4 index. The failure to reject the null hypothesis for the carbohydrates was in line with the results of sensitivity analysis adjusting for HOMA-IR. This indicates that the carbohydrates were indeed weak and not directly associated with HTGC or the FIB-4 index. The FIB-4 index is clinical diagnosis tool to rule out high fibrosis risk[20,59] and has been shown to have a comparable performance as the assessment of liver biopsy samples.[59] Accordingly, the global test results provide a strong indication that our beforementioned metabolites and pathways, found to be associated with HTGC and the FIB-4 index, are also potentially clinically relevant.

4.6 | 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 GGM and GSMM networks, were particularly interesting due to the contrasting associations with HTGC. Thus, it appears that the metabolomic flux between these metabolite species is associated with an overall higher HTGC level. 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.[9,27] However, our pathway analyses revealed two metabolites to be strongly correlated with glutamate and were also shown to be associated with HTGC. These were metabolonic lactone sulphate 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 sulphate 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.[47] This particular SNP was reported to be strongly associated with NAFLD.[8] The aforementioned findings from the literature regarding the associations of metabolonic lactone sulphate with the liver and cardiometabolic disease support our reported association with HTGC and pathway associations with FIB-4 index from global test. Therefore, glutamate, metabolonic lactone sulphate 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.[60], 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 sulphate.

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.

4.7 | 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, HTGC measurements in this cohort were assessed by ^{1}H-MRS, which provides high accuracy and

sensitivity in measuring HTGC even at low levels.[61] 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. Moreover, we addressed this limitation by conducting a global test using the FIB-4 index, reported to be comparable in performance with liver biopsy, as the outcome. 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. However, we did not include a genetic analysis as our current sample size is underpowered for such analysis. 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.

5 | CONCLUSION

In this study, we performed 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 metabolites associated with HTGC using an untargeted platform that measured 1363 metabolites. Using this platform, associations were found between 118 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 BCAA, diglycerol, ceramide and sphingomyelin 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 potentially novel pathway linking glutamate with the novel metabolites metabolonic lactone sulphate and X-15245. Thus, our atlas provides novel insight in metabolomic profiles associated with liver fat accumulation and may facilitate further studies to find causal links between the metabolites reported here and liver fibrosis.

ACKNOWLEDGEMENTS

The authors of the NEO study thank all participants, all participating general practitioners for inviting eligible participants, all research nurses for data collection and the NEO study group: Pat van Beelen, Petra Noordijk and Ingeborg de Jonge for coordination, laboratory and data management.

FUNDING INFORMATION

The NEO study is supported by the participating Departments, the Division, and the Board of Directors of the Leiden University Medical Centre, and by the Leiden University, Research Profile Area 'Vascular and Regenerative Medicine'. The analyses of metabolites are funded by the VENI grant (ZonMW-VENI Grant 916.14.023) of D.O.M.-K., D.v.H. and R.N. were supported by a grant of the VELUX Stiftung [grant number 1156]. T.O.F. was supported by the King Abdullah Scholarship Program and King Faisal Specialist Hospital & Research Center [No. 1012879283].

CONFLICT OF INTEREST STATEMENT

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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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Faquih TO, van Klinken JB, Li-Gao R, et al. Hepatic triglyceride content is intricately associated with numerous metabolites and biochemical pathways. *Liver Int*. 2023;43:1458-1472. doi:10.1111/liv.15575