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ARTICLE

Prediction of non-alcoholic fatty-liver disease and liver fat content by serum molecular lipids

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Abstract

Aims/hypothesis We examined whether analysis of lipids by ultra-performance liquid chromatography (UPLC) coupled to MS allows the development of a laboratory test for non-alcoholic fatty-liver disease (NAFLD), and how a lipid-profile biomarker compares with the prediction of NAFLD and liver-fat content based on routinely available clinical and laboratory data.

Methods We analysed the concentrations of molecular lipids by UPLC-MS in blood samples of 679 well-characterised individuals in whom liver-fat content was measured using proton magnetic resonance spectroscopy (^{1}H-MRS) or liver biopsy. The participants were divided into biomarker-discovery (n=287) and validation (n=392) groups to build and validate the diagnostic models, respectively.

Results Individuals with NAFLD had increased triacylglycerols with low carbon number and double-bond content while lysophosphatidylcholines and ether phospholipids were diminished in those with NAFLD. A serum-lipid signature comprising three molecular lipids ('lipid triplet') was developed to estimate the percentage of liver fat. It had a sensitivity of 69.1% and specificity of 73.8% when applied for diagnosis of NAFLD in the validation series. The usefulness of the lipid triplet was demonstrated in a weight-loss intervention study.

Conclusions/interpretation The liver-fat-biomarker signature based on molecular lipids may provide a non-invasive tool to diagnose NAFLD, in addition to highlighting lipid molecular pathways involved in the disease.

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Keywords Lipidomics · Mass spectrometry · Non-alcoholic fatty-liver disease

Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
^{1}H-MRS	Proton magnetic resonance spectroscopy
LC	Lipid cluster
LysoPC	Lysophosphatidylcholine
NAFLD	Non-alcoholic fatty-liver disease
NASH	Non-alcoholic steatohepatitis
PC	Phosphatidylcholine
ROC	Receiver operating characteristic
TG	Triacylglycerol
UPLC	Ultra-performance liquid chromatography

Introduction

Non-alcoholic fatty-liver disease (NAFLD) is defined as hepatic fat accumulation exceeding 5–10% of liver weight that is not due to alcohol, autoimmune, viral or other known causes [1]. It has been estimated that 30% of adults have a fatty liver due to non-alcoholic causes [1, 2]. NAFLD predicts non-alcoholic steatohepatitis (NASH), type 2 diabetes and cardiovascular disease, independent of obesity [3–5]. NAFLD has also become a prevalent indication for liver transplantation [6]. As the diagnosis of NAFLD is not as straightforward as, for example, that of type 2 diabetes, there is a need to develop diagnostic tools to identify individuals with NAFLD.

Liver-fat content can be quantified invasively by a liver biopsy and non-invasively by proton magnetic resonance spectroscopy (^{1}H-MRS), but these methods are unavailable in clinical practice. Other radiological techniques provide qualitative rather than quantitative estimates, and their sensitivity is limited [7]. Elevated liver-function tests alone are unspecific and insensitive markers of a fatty liver [8]. Several biomarkers based on simple biochemical and clinical data have been developed to predict NAFLD, such as the fatty-liver index (BMI, waist, triacylglycerols [TGs], γ-glutamyltransferase) and the commercially available Steatotest (12 variables in an undisclosed formula) (reviewed in Machado and Cortez-Pinto [9]). We have recently developed an NAFLD liver-fat score, which allows identification of individuals with NAFLD with sensitivity of 86% and specificity of 71% using clinical and laboratory data (the metabolic syndrome, type 2 diabetes, fasting insulin and serum alanine aminotransferase [ALT] and aspartate aminotransferase [AST] concentrations) [10]. However, it is cumbersome and more expensive to assess compared with a simple laboratory test.

Ideally, diagnostic tests for NAFLD would directly reflect the changes in lipid composition of the liver and respond to molecular changes accompanying changes in liver fat such as those induced by weight loss. We have previously shown in liver-biopsy studies [11] and by measuring the rates of lipid production across the splanchnic bed that the more fat there is in the liver, the greater is the relative abundance of TGs containing saturated or monounsaturated fatty acids [12]. These changes could reflect an increase in de novo lipogenesis, which is increased in NAFLD [13] and produces only saturated fatty acids, especially palmitic and stearic acids, which are then converted to respective monounsaturated fatty acids via stearoyl-CoA desaturase-1 (SCD-1) [14]. As there is considerable similarity between the sources of fatty acids for intrahepatocellular TGs and for TG secretion by the liver in lipoproteins [13], detailed analysis of circulating molecular lipids might enable identification of biomarkers for NAFLD that reflect its pathogenesis. The aim of the present study was thus to identify novel serum-lipid markers to estimate liver-fat content and diagnose NAFLD. We analysed the concentrations of lipids in serum samples of 679 well-characterised individuals in whom liver-fat content was measured using ^{1}H-MRS or a liver biopsy. The analyses were performed using an established metabolomics platform for profiling molecular lipids [15].

Methods

Participants The individuals who participated in the metabolic studies performed at the Universities of Helsinki and Turku (Finland) were recruited by newspaper advertisements, contact via the occupational health services and from patients referred to the department of gastroenterology because of chronically elevated serum transaminase concentrations. The inclusion criteria were: age 18 to 75 years; no known acute or chronic disease except obesity or type 2 diabetes, based on medical history, physical examination and standard laboratory tests (blood counts, serum creatinine, thyroid-stimulating hormone, electrolyte concentrations) and electrocardiogram; and alcohol consumption less than 20 g per day. Hepatitis B and C serology, transferrin saturation, anti-smooth muscle antibodies, anti-nuclear antibodies and anti-mitochondrial antibodies were measured in all patients referred to the gastroenterologist because of chronically elevated liver-function tests. Exclusion criteria included use of glitazones and pregnancy. Elevated liver enzymes (serum ALT or AST) were not exclusion criteria. The cohort who participated in the weight-loss intervention study were recruited at the Antwerp University Hospital (Belgium) from patients referred to the obesity clinic. The design and enrolment criteria for this study have been previously reported [16].

The study protocol was approved by the ethics committees of the University Hospitals of Helsinki, Turku and Antwerp. Each participant provided written informed consent.

Study design The participants were randomly divided into biomarker-discovery (n=287) and validation (n=392) groups to build and validate the model, respectively. All study participants (n=679) were used as the second validation group. The validation and biomarker-discovery groups were comparable with respect to age, sex, BMI, liver-fat content, prevalence of NAFLD, waist circumference, type 2 diabetes, BP and fasting serum glucose, TG, HDL-cholesterol and insulin concentrations (Table 1). The prevalence of metabolic syndrome and liver-enzyme concentrations were slightly higher and those of LDL-cholesterol and total serum cholesterol slightly lower in the validation compared with the discovery group (Table 1). An unthawed blood sample was available from all participants. Of the blood samples used, 78% were citrate plasma, 16% were heparin plasma and 7% were serum samples. Studies comparing citrate and heparin plasma and serum samples from the same participants showed comparable lipidomics data using each sample type (data not shown).

Liver-fat content In 369 participants (54%), liver-fat content was measured using ^{1}H-MRS as previously described [10]. This measurement has been validated against histologically determined lipid content [17] and against estimates of fatty infiltration by computed tomography [18] and MRI [19]. The reproducibility of repeated measurements of liver fat in non-diabetic participants as determined on two separate occasions in our laboratory is 11% [20].

In 46% of the participants, liver fat was measured using a liver biopsy. The fat content of the liver biopsy specimens (percentage of hepatocytes with macrovesicular steatosis) was determined for all participants, based on haematoxylin-eosin staining, by an experienced liver pathologist (Johanna Arola) in a blinded fashion. The percentage of macrovesicular steatosis was converted to the liver-fat percentage measured by ^{1}H-MRS and liver histology as previously described [17]. NAFLD was defined as liver fat ≥55.6 mg TG/g liver tissue or ≥5.56% liver tissue weight [21]. Descriptions of other clinical and biochemical measurements are provided in the electronic supplementary material (ESM) Methods section.

Lipidomic analysis with ultra-performance liquid chromatography coupled to MS An established platform based on ultra-performance liquid chromatography (UPLC; Acquity Ultra Performance LC; Waters, Milford, MA, USA) coupled to time-of-flight MS was applied to analyse the aliquots (10 μl) of serum or plasma sample [15]. The data were processed by using MZmine 2 open source software [22] (http://mzmine.sourceforge.net/) and the lipid identification was based on an internal spectral library or de novo identification using tandem MS [15]. A detailed description of the lipidomic-analysis method is provided in ESM Methods.

Table 1 Characteristics of the biomarker-discovery and validation groups

Characteristic	Biomarker-discovery group	Validation group	p value
n (% men)	287 (33)	392 (39)	0.11[a]
Age (years)	47±11	47±12	0.58
BMI (kg/m^2)	34.7 (30.6, 40.2)	34.8 (30.2, 41.0)	0.37
Waist (cm)	111±17	113±18	0.48
Type 2 diabetes (%)	21	22	0.50[a]
Metabolic syndrome (%)	61	69	0.034[a]
Liver fat (%)	4.9 (1.7, 12.4)	4.8 (1.0, 12.1)	0.48
NAFLD (%)	45	45	0.94[a]
fP-glucose (mmol/l)	5.9±2.0	6.0±1.8	0.28
fS-TGs (mmol/l)	1.40 (1.00, 2.00)	1.40 (1.00, 1.99)	0.98
fS-HDL-cholesterol (mmol/l)	1.25 (1.06, 1.57)	1.21 (1.04, 1.51)	0.33
fS-LDL-cholesterol (mmol/l)	3.01±0.93	2.83±0.90	0.012
Total serum cholesterol (mmol/l)	5.1±1.1	4.9±1.0	0.012
Systolic BP (mmHg)	133±17	134±17	0.28
Diastolic BP (mmHg)	83±11	85±11	0.062
fS-insulin (mU/l)	12 (7, 17)	12 (7, 17)	0.55
S-ALT (U/l)	32 (23, 48)	36 (25, 54)	0.010
S-AST (U/l)	28 (22, 37)	30 (24, 41)	0.005
S-GGT (U/l)	27 (19, 42)	32 (21, 57)	0.006
Use of statins (%)	14	17	0.29[a]
Use of BP medications (%)	29	36	0.047[a]
Use of glucose-lowering medication (%)	9	17	0.003[a]

[a] χ^2 test

Abbreviations: fP, fasting plasma; fS, fasting serum; GGT, γ-glutamyltransferase, S-, serum

Multivariate statistical analysis of lipidomics data The data were rescaled to zero mean and unit variance to obtain metabolite profiles comparable with each other for clustering. Bayesian-model-based clustering was applied on the scaled data to group lipids with similar profiles across all samples. The analyses were performed using the MCLUST [23] method, implemented in R software (www.r-project.org/).

Diagnostic model In order to perform variable selection we developed a new algorithm based on the method of artificial life [24, 25]. In brief, the algorithm performs a heuristic search for combinations of variables that could predict a specific output variable (either dichotomous or real valued), by evaluating a population of thousands of evolving models, in which better models evolve by combining with each other to produce the next generations of models. The simulation is continued until the variables selected in the model population do not change further. We used logistic regression [26] for dichotomous output (i.e. classification) and ridge regression [27] for real valued output (i.e. regression) as the modelling method. The AUCs of the receiver operating characteristic (ROC) curves were compared using generalised *U* statistics [28], implemented with the R package pROC [29].

Results

Diagnosis of NAFLD Using the UPLC-MS-based analytical platform, a total of 413 molecular lipids were measured in the biomarker-discovery group. The global lipidome was first surveyed by clustering the data into a subset of clusters using Bayesian-model-based clustering [23]. The lipidomic platform data were decomposed into nine lipid clusters (LCs). Descriptions of each cluster and the representative lipids are shown in Table 2. As expected, to a large degree, the clusters followed different lipid functional or structural groups.

As shown in Fig. 1, several of the clusters had different average metabolite profiles in participants with NAFLD (NAFLD+ group) compared with those with normal liver fat (NAFLD− group). Specifically, there was an overall and highly significant trend in NAFLD towards increased concentrations of TGs containing saturated and monounsaturated fatty acids (LC9), as well as a lower concentration of lysophosphatidylcholine (lysoPC) from LC2 and ether lipids (LC4). In a plot of representative lipids from significantly altered clusters against liver-fat content (Fig. 2), each of the lipids in the lipid triplet was significantly correlated with liver-fat content.

Next, we aimed to establish if specific molecular lipids can be used for the diagnosis of NAFLD. Based on lipidomics in the biomarker-discovery series, a predictive model comprising a lipid triplet (TG[16:0/18:0/18:1], phosphatidylcholine [PC][18:1/22:6], PC[O-24:1/20:4]) was derived using an evolutionary algorithm. The results were compared with the reference model (NAFLD score) derived from selected clinical variables [10] (ESM Fig. 1). The mass spectra confirming the identity of the minor lipid (PC[O-24:1/20:4]) is shown in ESM Fig. 2. In the biomarker-discovery series, the lipid-derived model was comparable with the reference model (no significant difference between the ROC curves).

The model was independently tested in the validation series. The lipid triplet showed good generalisation in the prediction of NAFLD in the validation series (ESM Fig. 1), with no significant difference in AUC compared with the reference model. We defined the optimal cut-off point for classification using the Youden's index on the biomarker-discovery series ROC curve. For the diagnostic model based on the lipid triplet, the optimal cut-off (0.463) resulted in a diagnostic test with a sensitivity of 69.5% and specificity of 78.6%, 72.4% positive predictive value (PPV), and 76.2% negative predictive value (NPV). When applied to the validation series, the sensitivity and specificity were 65.2% and 72.9%. The reference model in combination with its optimal cut-off point attained a test with a sensitivity of 72.4% and specificity of 74.1% in the discovery series. The sensitivity and specificity of the reference model when applied to the validation series was 75.9% and 59.6%, respectively. Additionally, for the lipid model, we defined cut-off points for 'ruling in' and 'ruling out' NAFLD for potential use in pre-screening [30]. The 'ruling in' cut-off point 0.661 corresponded to a test with 39.1% sensitivity and 95% specificity in the discovery series, and showed 44.9% sensitivity and 84.6% specificity when applied to the validation series. The 'ruling out' cut-off point 0.215 corresponded to a test with 95.3% sensitivity and 31.4% specificity in the discovery series, and showed 85.4% sensitivity and 54.2% specificity when applied to the validation series (ESM Fig. 1 and ESM Table 1).

As the lipid triplet included in the NAFLD model included an abundant TG together with two polyunsaturated fatty acid (PUFA)-containing lipids that were both negatively associated with liver fat (Table 2) and are both common constituents of HDL [31–33], we also tested if TG(16:0/18:0/18:1) together with HDL-cholesterol would predict NAFLD. The model was developed from the biomarker-discovery series using the same method as described above. It performed reasonably well, with an AUC 0.74 (95% CI 0.69, 0.80) in the discovery series and AUC 0.71 (95% CI 0.67, 0.77) in the validation series (ESM Fig. 3).

Biomarker discovery of liver fat from serum lipidomic profile The significant associations of specific lipid and polar metabolite concentrations with liver-fat content (Fig. 2) suggested that circulating molecular lipids may be predictive of liver fat. Using the lipidomics data from the discovery series, we derived a model to determine the liver-fat content by applying the artificial-life algorithm and ridge regression as for the NAFLD model. Based on the data in the

Table 2 Metabolome and lipidome cluster descriptions

Cluster name	Cluster size	Cluster description	*p* value	Examples of metabolites
LC1	22	Diverse, odd- and short-chain TGs, ether lipids	0.50	PE(36:6e), TG(46:2) (↑), TG(42:0), TG(49:0) (↑)
LC2	130	Diverse, lysoPCs, SMs, PCs	0.033	lysoPC(16:0) (↓), lysoPC(18:0) (↓), SM(d18:0/18:0), SM(d18:1/24:1), SM(d18:1/16:0) (↓), PC(34:2), PC(18:1/22:6) (↓)
LC3	30	Ether lipids (PEs)	0.70	PE(34:6e), PE(34:4e), PE(34:3e)
LC4	82	Ether PCs, PEs	0.036	PC(O-24:1/20:4) (↓), PC(34:1e) (↓), PC(34:2p) (↓), PE(38:2) (↓), PE(36:2) (↓)
LC5	24	PUFA-containing PCs, PEs and TGs	0.070	PC(38:6), PC(40:6), PE(38:5), TG(58:8) (↑), TG(16:0/16:1/22:6) (↑)
LC6	32	Unknown	0.99	
LC7	57	C18:2-containing TGs	0.097	TG(16:0/18:2/18:1), TG(18:1/16:1/18:2), TG(18:1/18:2/18:1)
LC8	4	Unknown	0.11	
LC9	32	SFA- and MUFA-containing TGs	7.34×10^{-6}	TG(16:0/18:1/18:1) (↑), TG(16:0/16:0/18:1) (↑), TG(16:0/18:1/20:1) (↑)

(↑) and (↓) mark significant up- and downregulation, respectively, for individually listed metabolites

p values were determined using the two-sided *t* test (NAFLD+ vs NAFLD− groups in the biomarker-discovery cohort)

MUFA, monounsaturated fatty acid; PE, phosphatidylethanolamine; SFA, saturated fatty acid; SM, sphingomyelin

biomarker-discovery series, the following model for liver-fat content was derived using the concentration of the three model-selected molecular lipids (PC[O-24:1/20:4], PC[18:1/22:6] and TG[48:0]):

$$\log_{10}(\text{liver fat}[\%]) = 0.0314–0.575 \times \log_{10}(\text{PC}[\text{O}-24:1/20:4])–0.537 \times \log_{10}(\text{PC}[18:1/22:6]) + 0.810 \times \log_{10}(\text{TG}[48:0])$$

Figure 3 shows the performance of the model when applied to the combined biomarker-discovery and validation data (ESM Table 2 shows the performance of prediction on different amounts of liver fat). Performance in the discovery and validation series, when analysed separately, is shown in ESM Fig. 4. We also applied the liver-fat model above for the diagnosis of NAFLD. Figure 3 shows the ROC curves of the diagnostic performance of the model. Interestingly, this model not only shows better performance than the original NAFLD diagnostic model (ESM Fig. 1), but also performs as well as the reference model. However, it is not significantly better than the reference model. The optimal cut-off point, as determined by the Youden's index, resulted in a test with 69.5% sensitivity and 75.5% specificity in the biomarker-discovery series. When applied to the validation series, the sensitivity was 69.1% and specificity 73.8%. When applied to the combined biomarker-discovery and validation series, the sensitivity was 69.3% and specificity 74.5%. The sensitivity of the reference model was 74.6% and specificity 64.8% in the combined discovery and validation series. The sensitivities and specificities corresponding to the 'ruling in' and 'ruling out' cut-off points are marked in Fig. 3 and ESM Fig. 4.

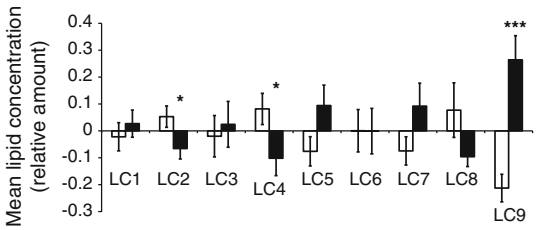

Fig. 1 Mean lipid levels within each cluster, shown separately for patients with NAFLD (NAFLD+, black bars) and without NAFLD (NAFLD−, white bars) in the biomarker-discovery cohort. The data for each lipid are scaled to zero mean and unit variance. Statistical comparison was performed using the two-sided *t* test. The cluster summaries are shown in Table 2. Error marks show standard error of the mean. *$p<0.05$ vs NAFLD−; ***$p<0.001$ vs NAFLD−. For LC7 the two groups are different at the marginal significance level ($p=0.097$)

The correlation coefficient of the lipid triplet and liver-fat content was higher than that between liver-fat content and fasting insulin, AST or fasting total plasma TG concentrations (ESM Figs 5–7). To evaluate whether adding other biochemical or clinical data improved the prediction of liver fat, we also trained a model by adding fasting insulin as an additional term to the lipid triplet, using the artificial-life ridge regression algorithm as above, but this did not improve the prediction of liver fat above that observed using the lipid triplet alone (ESM Fig. 8).

Application of the lipid triplet to characterise the effect of therapeutic intervention To examine whether the lipid triplet identified as a marker of liver fat responded to weight loss, we measured it in serum samples in a recently described 48 week weight-loss intervention study in which patients were treated

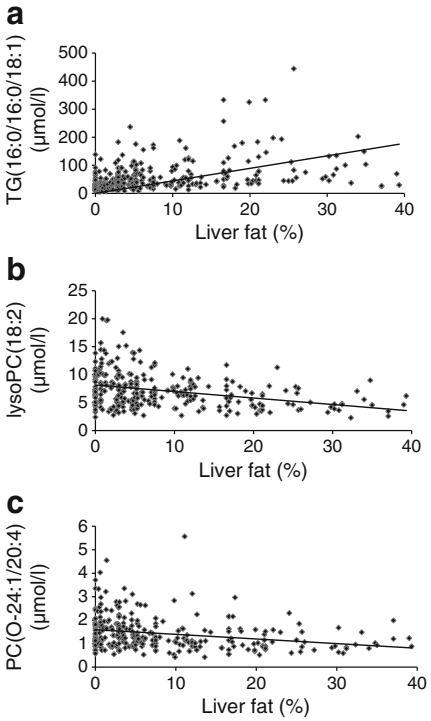

Fig. 2 The relationship between liver-fat content and the selected representative lipids from the clusters that are significantly altered in NAFLD: (**a**) TG(16:0/16:0/18:1) from cluster LC9 (Spearman rank correlation r, 0.45, $p<0.001$); (**b**) lysoPC from LC2 (r, −0.32, $p<0.001$); and (**c**) PC(O-24:1/20:4) from LC4 (r −0.31, $p<0.001$). The regression lines are drawn as guides

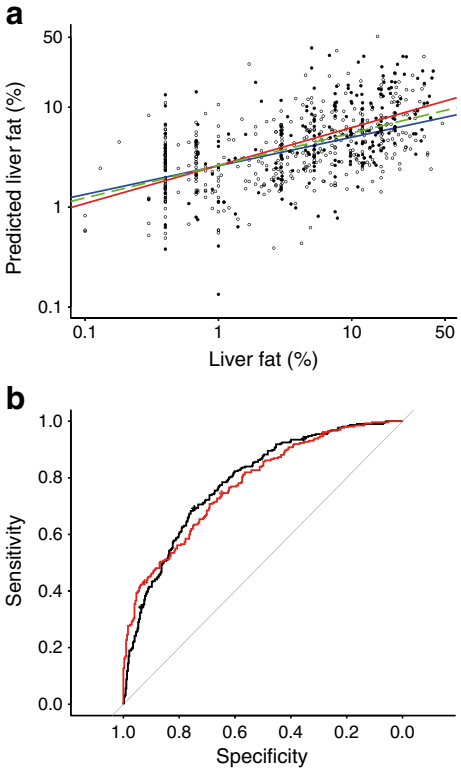

Fig. 3 Prediction of liver-fat content from the model including three lipids, TG(48:0), PC(O-24:1/20:4) and PC(18:1/22:6). (**a**) The relationship between measured and predicted liver fat ($\log_{10}$ scale on both axes). Pearson correlation coefficient r 0.54 ($p<0.001$). Black circles, men; white circles, women. (**b**) ROC curve for NAFLD diagnosis (biomarker-discovery and validation series combined), based on predicted liver fat, and the ROC curves of the reference model [10]. Test lipid combination AUC 0.79 (95% CI 0.75, 0.82), reference AUC 0.78 (95% CI 0.74, 0.82). Optimal cut-off point corresponding to the maximum sum of sensitivity and specificity: test lipid combination 0.648 (95% CI 0.745, 0.693); reference 0.409 (95% CI 0.648, 0.746). Cut-off point for 95% sensitivity: test lipid combination 0.353 (95% CI 0.354, 0.938); reference 0.241 (95% CI 0.286, 0.954). Cut-off point for 95% specificity: test lipid combination 0.918 (95% CI 0.936, 0.343); reference 0.723 (95% CI 0.924, 0.431). Black curve, biomarker-discovery and validation series combined; red curve, reference

with either placebo or the cannabinoid receptor type 1 blocker rimonabant [34]. Liver fat was measured using ^{1}H-MRS.

We analysed serum samples obtained before and after treatment from 12 individuals receiving rimonabant and from eight individuals receiving placebo (ESM Table 3). None of the post-intervention samples was included in the development of the liver-fat marker (discovery or validation series). In agreement with the reported findings [34], liver fat determined using the lipid triplet decreased significantly in the rimonabant group (ESM Table 3). The estimated liver fat correlated closely with the lipid triplet concentration in both intervention arms before, as well as after, the intervention (Fig. 4).

Discussion

Using molecular lipid-profiling platforms based on combining chromatography and MS we identified a lipid triplet that was shown to predict NAFLD. Its diagnostic performance in the biomarker-discovery and validation cohorts was similar to the recently reported NAFLD score [10]. The usefulness of the lipid droplet was demonstrated in a pharmacological intervention study.

Higher liver-fat content was associated with increased serum concentrations of TG containing saturated or monounsaturated fatty acids, and decreased concentrations of PUFA-containing phospholipids and ether lipids. These data are consistent with those in a much smaller group of individuals, which showed that TGs with low carbon number and double-bond content are major constituents of liver fat in human liver biopsies [11] and that these lipids are also enriched in VLDL particles of insulin-resistant individuals [31]. Studies using stable isotope tracers have shown that overproduction of VLDL, rather than a decrease in clearance, accounts for hypertriacylglycerolaemia in NAFLD [35, 36]. The changes in absolute and relative concentrations of TG could reflect increased de novo lipogenesis, which produces saturated fatty acids [14] and is increased in

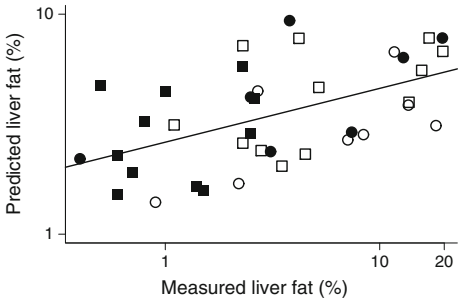

Fig. 4 Comparison of measured and estimated liver fat using the serum molecular signature in participants before and after intervention with rimonabant or placebo ($\log_{10}$ scale on both axes). Pearson correlation coefficient r, 0.53 ($p<0.001$). White circles, placebo at baseline; white squares, rimonabant at baseline; black circles, placebo at 48 weeks; black squares, rimonabant at 48 weeks

NAFLD [13]. Consistent with these data, overfeeding carbohydrates increases both liver fat and de novo lipogenesis [37]. In addition, direct measurement of molecular lipids across the splanchnic bed showed that the fatty liver overproduces saturated and monounsaturated TGs with low double-bond content in NAFLD [12]. The proportional increase of these TGs in the serum of individuals with NAFLD is likely, therefore, to directly reflect their increased amount and production in the liver. This may explain the recent finding from a prospective study that more saturated TGs and those with shorter acyl chains associate with a higher risk of developing type 2 diabetes [38], as NAFLD both precedes and predicts type 2 diabetes [4].

LysoPCs and PUFA-containing phospholipids including ether lipids were negatively associated with liver fat and were, in combination with saturated TGs, included in the lipid signature predictive of NAFLD and liver-fat content. These lipids are abundant in HDL particles [31–33] and their decrease in NAFLD could, therefore, reflect the known negative association between HDL-cholesterol and liver-fat content [39, 40]. However, when combined with specific TGs in the diagnostic model, the specific molecular lipids were better predictors than HDL-cholesterol.

The lipid triplet, when applied to the combined biomarker-discovery and validation series, had sensitivity of 69.3% and specificity 74.5%, while the sensitivity of the reference model [10] was 74.6% and specificity 64.8%. The latter figures were lower than the values of 95% for the NAFLD liver-fat score in the original cohort [10] and those described for the Steatotest (90% and 70%) [41] and the fatty-liver index (87% and 86%, respectively) [42]. The lower sensitivities and specificities of the NAFLD liver-fat score than reported for the original cohort could reflect the following: (1) all predictors work best in the cohort in which they are discovered; and (2) the present cohort included not only Finnish but also Belgian individuals; and (3) the sample size in this study is, to date, the largest used to search for a score predicting steatosis [9].

Although the major usefulness of the present study may be its insight into the pathogenesis of NAFLD, it may in the future be useful in clinical practice for both hepatologists and diabetologists. Such an application would require the development of an assay specifically measuring the lipid triplet. Development of this assay is feasible given that many methods now use targeted UPLC-MS/MS-based methods in routine clinical chemistry laboratories. Once such an assay is set up, the prediction of liver fat using a serum sample would take less time than the assessment of the components included in the reference method (metabolic syndrome, type 2 diabetes, fasting insulin and serum ALT and AST concentrations) or other methods such as the fatty-liver index [9]. Knowledge of the amount of fat in the liver may help to select patients who should undergo further examinations, such as measurement of liver fat by ultrasound methods (e.g. as recently described by Xia et al [43]), or a liver biopsy, as NAFLD predisposes to NASH [44]. In patients with type 2 diabetes, the amount of exogenous insulin required to control hepatic glucose production is directly proportional to liver fat, independent of obesity and other factors [18, 45]. Such patients might particularly benefit from interventions effectively reducing liver fat [46]. A laboratory test measuring the currently described lipid triplet may also help in the follow-up of such individuals.

In conclusion, the lipid triplet identified in the present study as a marker of NAFLD and liver-fat content supports data addressing the pathogenesis of NAFLD. The finding that saturated TG(16:0/18:0/18:1) is the best circulating predictor among TGs suggests that the liver releases TGs that are particularly abundant in it. The other two lipid species that are deficient in serum are the ones most abundant in HDL particles, which, in turn, are decreased in NAFLD.

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Contribution statement MO and HY-J were responsible for the study concept and design. MO, TH, PI, PN, LVG and HY-J supervised the study. TH, HN, JA, IM, AH, RJHB, M-JH, AV, SF, PI, ML, NJ, AJ, PN and LVG acquired the data. MO, TH, AK, PG, SC, TIAS and HY-J analysed and interpreted the data. MO, TH, PG and HY-J drafted the paper. AK, HN, JA, SC, IM, AH, RJHB, M-JH, AV, SF, PI, ML, NJ, AJ, TIAS, PN and LVG critically revised the manuscript for important intellectual content. All authors reviewed and approved the final manuscript.

Duality of interest MO, TH, PG, SC and HY-J are co-inventors on a patent application pertaining to molecular lipid predictors of NAFLD. The other authors declare that there is no duality of interest associated with this manuscript.

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