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Molecular fingerprint of hepatic steatosis

Hepatic steatosis associates with adverse molecular signatures in subjects without diabetes

Maik Pietzner[1,2], Kathrin Budde[1,2], Georg Homuth[3], Gabi Kastenmüller[4], Ann-Kristin Henning[1], Anna Artati[5], Jan Krumsiek[6], Henry Völzke[2,7,8], Jerzy Adamski[5,9,10], Markus M. Lerch[11], Jens P. Kühn[12,13], Matthias Nauck[1,2], Nele Friedrich[1,2]

[1]*Institute of Clinical Chemistry and Laboratory Medicine, University Medicine Greifswald, Greifswald, Germany*

[2]*DZHK (German Center for Cardiovascular Research), partner site Greifswald, Greifswald, Germany*

[3]*Interfaculty Institute for Genetics and Functional Genomics, University Medicine and Ernst-Moritz Arndt-University Greifswald, Greifswald, Germany*

[4]*Institute of Bioinformatics and Systems Biology, Helmholtz Zentrum München, Neuherberg, Germany*

[5]*Institute of Experimental Genetics, Genome Analysis Center, Helmholtz Zentrum München, Neuherberg, Germany*

[6]*ICB (Institute of Computational Biology), Helmholtz Zentrum München, Neuherberg, 85764, Germany*

[7]*Institute for Community Medicine, University Medicine Greifswald, Greifswald, 17475, Germany*

[8] *DZD (German Center for Diabetes Research), site Greifswald, Greifswald, 17475, Germany*

[9]*Lehrstuhl für Experimentelle Genetik, Technische Universität München, Freising-Weihenstephan, Germany*

[10]*DZD (German Center for Diabetes Research), München-Neuherberg, Germany*

[11]*Department of Medicine A, University Medicine Greifswald, Greifswald, Germany*

[12]*Institute of Diagnostic Radiology and Neuroradiology, University Medicine Greifswald, Germany*

[13]*Institute of Diagnostic Radiology, University Medicine, Carl Gustav Carus University Dresden, Germany*

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Background & Aims: Exaggerated hepatic triglyceride accumulation, i.e. hepatic steatosis, represents a strong risk factor for type 2 diabetes mellitus and cardiovascular disease. Despite a clear association of hepatic steatosis with impaired insulin signaling the precise molecular mechanisms involved are still under debate. We combined data from several metabolomics techniques to gain a comprehensive picture of molecular alterations related to the presence of hepatic steatosis in a diabetes-free sample (N=769) of the population-based Study of Health in Pomerania (SHIP).

Methods: Liver fat content (LFC) was assessed using MRI. Metabolome measurements of plasma and urine samples were done by mass spectrometry and nuclear magnetic resonance spectroscopy. Linear regression analyses were used to detect significant associations with either LFC or markers of hepatic damage. Possible mediations through insulin resistance, hypertriglyceridemia and inflammation were tested. A predictive molecular signature of hepatic steatosis was established using regularized logistic regression.

Results: The LFC-associated atherogenic lipid profile, tightly connected to shifts in the phospholipid content, and a pre-diabetic amino acid cluster were mediated by insulin resistance. Molecular surrogates of oxidative stress and multiple associations with urine metabolites, e.g., indicating altered cortisol metabolism or phase II detoxification products, were unaffected in mediation analyses. Incorporation of urine metabolites slightly improved classification of hepatic steatosis.

Conclusions: Comprehensive metabolic profiling allowed us to reveal molecular patterns accompanying hepatic steatosis independent of the known hallmarks. Novel biomarkers from urine, e.g. cortisol glucuronide, are worthwhile for follow-up in patients suffering from more severe liver impairment compared to our merely healthy population-based sample.

Liver fat content was strongly associated with a wealth of metabolites in plasma and urine independent of known hallmarks. A prominent example was the urine excretion of adrenal steroids.

BACKGROUND

The inflated hepatic accumulation of triglycerides (TG), typically above 5%, represents a pathophysiological condition defined as hepatic steatosis which might further proceed to steatohepatitis and even to cirrhosis. The latter is associated with an increased risk for hepatocellular carcinoma [1].

Obesity represents a major contributor to the development of hepatic steatosis [2]. Estimates of its prevalence greatly vary between 10 and 35% depending on the definition used (based on ultrasound examination, liver biopsy, magnetic resonance imaging (MRI), or/ and serum markers) and ethnicity [3]. Furthermore, in 70% of patients with type 2 diabetes mellitus hepatic steatosis could be found [4]. Despite its high correlation with obesity, hepatic steatosis represents an independent predictor for insulin resistance [5] and cardiovascular risk and hence mortality [6, 7].

The pathophysiological mechanisms underlying hepatic steatosis are still incompletely understood. In general, development is thought to be caused by increased release of free fatty acids from adipose tissue as a result of nutritional overload and possibly impaired insulin sensitivity [8]. As the amount of fatty acids which are subsequently taken up by the liver exceeds the hepatic metabolic capacities for oxidation, excess TG are stored as lipid droplets in the hepatocyte cytoplasm.

Apart from the classical hallmarks of hepatic steatosis, i.e., hypertriglyceridemia, insulin resistance and inflammation, a number of metabolome analysis (for review see [9]) have greatly broaden our understanding of the underlying pathology and suggested novel biomarkers. Briefly, metabolomics approaches primarily done in a case-control setting have revealed alterations in simple [10, 11] and complex lipids [12, 13], amino acid metabolism [14-16], amides [17], and shifts in metabolites produced by microbiota [18]. Exemplarily, surrogate markers of oxidative stress, namely γ-glutamyl dipeptides, have been shown to discriminate between different stages of liver disease [19]. More recently, Alonso *et al.* were able to describe three distinct molecular profiles of fatty liver disease based on the combination of an animal model and patient data [20]. However, up to now such studies were mostly restricted to matched case-control designs including (morbidly) obese subjects [9] and are thus of only limited generalizability. Therefore, in the present study, we analyzed the association between liver fat content (LFC) determined by MRI and metabolites present in fasting plasma as well as urine samples from 769 selected non-diabetic subjects from the population-based Study of Health in Pomerania (SHIP). By means of statistical mediation analyses we were able to separate between those molecular signatures assignable to the classical hallmarks accompanying hepatic steatosis and putative novel ones.

METHODS

Study Population

The Study of Health in Pomerania (SHIP-TREND) is a population-based study conducted in West Pomerania, a rural region in north-east Germany and a detailed description of the sampling

procedure and the study population can be found elsewhere [21]. In total, 4420 subjects chose to participate (50.1% response). All participants gave written informed consent before taking part in the study. The study was approved by the ethics committee of the University of Greifswald and conformed to the principles of the declaration of Helsinki. SHIP data are publicly available for scientific and quality control purposes by application at www.community-medicine.de.

For a subsample of 1000 subjects, plasma as well as urine metabolome data based on mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR) were available. Of these, 203 had to be excluded due to missing exposure or confounder data. Two and 28 participants were excluded because of a reported history of liver disease and diagnosed diabetes, respectively. Finally, a total sample of 769 subjects was included in the analyses. Figure 1 summarizes sample compilation and statistical analyses.

Participants' characteristics and medical histories were recorded using computer-aided personal interviews. Smoking status was categorized as current, former or never smokers. Daily alcohol consumption was calculated using beverage-specific pure ethanol volume proportions averaged across 30 days prior the interview. Subjects exercising for at least two hours a week were classified as physically active. Waist circumference (WC) was measured midway between the lower rib margin and the iliac crest in the horizontal plane. Body-mass-index (BMI) was calculated as weight (kg) / height² (m²).

Standard Laboratory Assays

Fasting blood samples (≥ 8 hours) were collected between 6:00 am and 12:00 pm from the cubital vein of subjects in the supine position and analyzed immediately or stored by -80°C in the Integrated Research Biobank (Liconic, Liechtenstein). Serum cystatin C, lipids (total cholesterol, high-density (HDL) and low-density lipoprotein (LDL) cholesterol, TG), high-sensitivity C-reactive protein (hsCRP), albumin and serum activities of ALT, AST and GGT were measured by standard methods (Dimension VISTA, Siemens Healthcare Diagnostics, Eschborn, Germany). Plasma insulin levels were measured (Centaur XP by Siemens Healthcare Diagnostics) and the homeostatic model assessment of insulin resistance (HOMA-IR) index was calculated as insulin (µU/ml) × glucose (mmol/l)/22.5 [22]. We calculated the AST/ALT ratio, the NAFLD-score [23] and the FIB4-score [24] to include surrogates of liver fibrosis. Cystatin C-based estimated glomerular filtration rate (eGFR) was calculated using the CKD-EPI equation [25].

MRI examinations were performed on a 1.5-Tesla MR system (Magnetom Avanto, Siemens Healthcare AG, Germany; software version Syngo MR B15) using a body phased array coil. Assessment of LFC was performed using calculation of proton density fat fraction based on chemical shift encoded MRI as previously described in detail [26].

Metabolome Analyses

A detailed description of all applied measurement techniques is given in the Supplemental Information. Briefly, four different approaches were combined: 1) non-targeted MS-based profiling of plasma and urine samples as reported previously [27] 2) targeted MS-based profiling of plasma samples using the AbsoluteIDQ p180 Kit (BIOCRATES LifeSciences AG, Innsbruck, Austria) 3) NMR-based profiling of urine samples as reported previously [28] and 4) NMR-based profiling of plasma samples to derive lipoprotein particles.

After quality control and pre-processing (see Supplemental Information) 613 plasma (Tab. S1) and 587 urine (Tab. S2) metabolites were available for statistical analyses. Note that some of these could not be unambiguously assigned to a chemical identity and are referred to hereafter with the notation "X" followed by a unique number. Data on lipoprotein particles comprise 117

measures describing the gradient from Very-low-density lipoprotein (VLDL) particles to HDL particles, including their triglycerides, cholesterol, free cholesterol, phospholipid as well as apolipoprotein B (ApoB), A1 (Apo-A1) and A2 (Apo-A2) content.

Statistical Analysis

Linear regression models were performed to assess the associations of LFC as well as serum activities of ALT, AST, and GGT (independent variables) with plasma (including lipoprotein particles) and urine metabolites (dependent variables). To fulfill requirements of linear regression ALT, AST, LFC and metabolite levels were log-transformed. Serum activities of GGT were transformed to -1/GGT. All models were adjusted for age, sex, BMI, alcohol consumption and physical activity. Notably, we combined men and women in the present analyses as no strong evidence for an interaction between sex and one of the liver traits became obvious. The same analyses were done for the fibrosis scores. In a second step, a possible mediation of significant associations by HOMA-IR, serum glucose, total TG (not for lipoproteins) and hsCRP was performed. Analyses were implemented using the R package *mediate* to obtain bootstrap p-values (N=2000 samples) for the mediation effect as well as confidence intervals for the proportion mediated. We defined a significant mediation if the p-value was <0.01 and at least 10% of the association was mediated through one of the four variables. Sensitivity analyses were done by excluding subjects reporting heavy drinking (n=53; men >30g/day and women >20g/day). To combine the metabolome data with lipoproteins, linear regression models were run with the lipoprotein as exposure and the metabolite as outcome controlling for age, sex, and BMI. To account for multiple testing, we adjusted the p-values from regression analyses by controlling the false discovery rate (FDR) at 5% using the Benjamini-Hochberg procedure.

Integration of multi-fluid data was achieved by computation of metabolic networks using Gaussian graphical modelling (GGM). The procedure is outlined in the Supplemental information.

A signature predictive for hepatic steatosis (LFC > 5%) using least absolute shrinkage and selection operator (LASSO) for variable selection was compiled. Using a two-staged cross-validation procedure allowed us for testing robustness of selected features across random subsets of the population as well as to assess generalizability of the results (see Supplemental Information). Briefly, a score was calculated by counting each time a feature survived the feature selection using the LASSO in the test set and weighted by the discriminative ability (area under the ROC-curve) on the independent validation set. The score could be seen as the mean discriminative ability of the final sparse model to predict presence of hepatic steatosis if the specific variable was included. Three types of variable set ups were used to perform this classification. First, considering only clinical variables as presented in Table 1. Second, only metabolites significantly associated with LFC and third, a combination of both. Finally, three sparse logistic regression models were built to predict hepatic steatosis. The latter ones were further assessed for generalization in a Monte-Carlo cross-validation procedure. Statistical analyses were done using R 3.3.2 (R Foundation for statistical computing, Vienna, Austria).

RESULTS

General characteristics of the study population are displayed in Table 1. Briefly, 34.7% of the participants presented with hepatic steatosis. These participants were characterized by an adverse metabolic profile, comprising higher concentrations of glycemic parameters (e.g., fasting glucose or HOMA-IR), higher LDL and lower HDL-cholesterol concentrations, higher hsCRP

concentrations as well as higher serum activities of liver enzymes and higher values of fibrosis scores (with the exception of the AST/ALT ratio).

Lipoprotein particles and mediation by HOMA-IR

An atherogenic lipoprotein particle profile was revealed to be associated with LFC in linear regression analyses (Fig. 2). In detail, LFC was positively associated with total TG levels, as well as with VLDL and small LDL particles. In contrast, LFC associated inversely with large LDL particles and HDL particles measures. ALT, AST, and, GGT serum activities partially mirrored these associations but with less pronounced association strengths. Small HDL particles were uniquely positively associated with ALT, GGT, or AST activities.

The vast majority of the associations with respect to LFC, ALT, and GGT were mediated by HOMA-IR (Fig. 2 and Tab. S3). The highest proportion of mediation was observed for LDL_2, LDL_3 and large VLDL particle measures (all above 50%). The associations between LFC and large LDL particles as well as between ALT and small-dense HDL particles were unaffected.

Small molecules and mediation by HOMA-IR and total TG

Linear regression analyses revealed 179 and 103 metabolites in plasma and urine, respectively, to be associated with at least one of the measures of liver function (Fig. 3A; Tab. S4/S5). LFC was the most prominent trait with 129 and 93 significantly associated metabolites in plasma and urine, respectively.

About half of the associations in plasma (N=79) and about one third in urine (N=26) were at least partially mediated, thereby total TG and HOMA-IR were the most important mediators (Fig. 3B).

With respect to plasma, total TG accounted in part for positive associations between LFC and lipid species, e.g. lysolipids, diacyl PCs as well as inverse associations with sphingolipids or serine. Positive associations between LFC and branched-chain amino acid (BCAA) catabolites (e.g. 3-methyl-2-oxobutyrate), alanine, or carbohydrates as well as the inverse association with sphingolipids or glycine were mediated by HOMA-IR. The inverse association between LFC and lysoPC C18:2 was mediated by HOMA-IR and hsCRP. Similar mediations became apparent with respect to ALT and GGT whereas associations with AST were far less affected (Fig. 3, Tab S6).

The positive association between LFC/ALT and urine 3-sialylactose was affected by all mediators to a degree of up to 53%. Similar strong mediating effects in urine were noted for the unknown X-02249 (inversely with LFC) and X-17340, tetrahydrocortisone or alanine (positively with LFC).

After discarding mediated associations, only plasma xanthine levels remained significantly positively associated with all traits. However, even plasma levels of adrenate, docosapentaenoate, γ-glutamylleucine and γ-glutamylphenylalanine were positively associated with all traits but were slightly mediated (max. 15%) by serum glucose (LFC) or HOMA-IR (ALT).

Metabolic fingerprint of LFC

The largest number of non-mediated associations remained for LFC with a comparable amount of significantly associated metabolites in plasma (N=58) and urine (N=68) (Fig. 3C). In plasma, two prominent metabolite signatures were detected: 1) decreased levels of ether-PCs (thereafter referred as PC ae CXX:Y) and 2) increased levels of BCAAs and aromatic amino acids as well as dipeptide derivatives (Fig. 2). Inverse associations with LFC were restricted to these lipid species, with the additional exceptions of 3-phenylpropionate, stachydrine and some unknown

compounds. Further positive associations with LFC were detected for the levels of proline, tryptophan, indolelacetate, urate, piperine and 7-α-hydroxy-3-oxo-4-cholestenoate (Tab. S3).

Associations with urine metabolites were almost exclusively detected for LFC (Fig. 3C). In line with the increased plasma levels mentioned above, the urine levels of BCAA-derivatives as well as lactate were also positively associated with LFC. In contrast, several glycine conjugates like isovaleryl- and isobutyrylglycine as well as γ-glutamylthreonine exhibited an inverse association (Tab. S2). Additionally, LFC was inversely associated with the urine levels of several xenobiotic species, e.g., 4-vinylpheno sulfate, hippurate or cinnamoylglycine. Almost one-third of the LFC-associated metabolites in urine were of unknown identity.

Metabolite associations with serum markers of hepatic damage

Only a few plasma metabolites were solely associated with one of the liver enzymes but not with LFC. Briefly, serum AST activities were positively associated with several acylcarnitine species and monounsaturated fatty acids. ALT was uniquely positively associated with two ether-PCs (PC ae C36:0 and PC ae C38:6). Inverse associations with 2-aminoheptanoate and citrate were specific for GGT. Only few weak metabolite associations with liver enzyme serum activities were observed in urine (Fig. 3C).

Fibrosis scores and exclusion of heavy drinkers

Similarities between the NAFLD and the FIB4-score with LFC were restricted to inverse associations with large LDL-particle measures (Fig. S2). Both scores were in general associated with lower concentrations of almost all LDL measures. Compared to LFC, only few metabolites in plasma (Fig. S3) or urine (Fig. S4) associated with either the NAFLD or the FIB4-score, partially being residual from either ALT or AST.

Excluding participants who reported heavy drinking changed the association strength between the traits under investigation and lipoproteins or metabolites only minor (Fig. S5 and S6). Only associations between AST and lipoprotein measures changed markedly.

Interrelation between lipoproteins and lipid species

Figure S1 summarizes the association results for lipoprotein measures and lipid species for those obvious from linear regression analyses for the amount of liver fat. Briefly, strong positive associations became obvious between ether-PCs and large LDL as well as small dense HDL particles. The TG-content of small VLDL particles ($VLDL_6$) was positively associated with various free fatty acid species.

A metabolite signature with predictive value for hepatic steatosis

Our procedure to classify hepatic steatosis (LFC > 5%) exclusively based on metabolites exhibited a performance that was comparable to that of clinical variables (ROC-AUC ~ 0.89; Fig. 4 and Tab 2). A combined feature selection approach using metabolome and clinical data led to a moderate but significant improvement in the ROC-AUC (p=0.002) from 0.89 to 0.91 (Fig. 4). These results were confirmed using a Monte-Carlo cross-validation procedure (Fig. S8). Even the net reclassification index improved significantly (0.62; 95%-CI [0.47 - 0.76]; p<0.001). Urine levels of X-20643, X-12407 and uracil as well as plasma levels of glycine were associated with decreased odds for hepatic steatosis whereas HOMA-IR, age, waist circumference, ALT serum activities and hsCRP levels were associated with increased odds (Tab. 2).

DISCUSSION

The present study aimed to characterize early (i.e. subclinical) molecular signatures of hepatic fat accumulation in a sample from the general population. The broad panel of detected metabolites

that were associated with LFC partly reflects physiological aspects of hepatic fat accumulation apart from established comorbidities, i.e. insulin resistance, hypertriglyceridemia, or inflammation. In particular, several urine metabolites were exclusively associated with LFC. The corresponding signature that indicates among others altered cortisol degradation enabled moderate improvement for the classification of hepatic steatosis.

Insulin sensitivity partially mediates an atherogenic lipoprotein profile

The most obvious hallmark of hepatic steatosis is an impaired TG metabolism manifested by dyslipidemia which is likely accompanied by insulin resistance and hepatic inflammation [8]. Indeed, more than half of the detected associations with lipoproteins were mediated to a significant amount by HOMA-IR. Possible responsible mechanisms include increased hepatic uptake of fatty acids, either released from adipose tissue or from remnant VLDL particles, and hepatic *de novo* lipogenesis finally increasing VLDL secretion as a consequence of peripheral insulin resistance [29]. Increased availability of plasma fatty acids from remnants is further supported by our cross-metabolomics analyses linking an increase in the TG-content of small, i.e. remnant VLDL particles ($VLDL_6$), to increased levels of a broad range of plasma fatty acids (Fig. S1). Of note, the associations between the TG-content of $VLDL_6$ or plasma levels of monounsaturated fatty acids with LFC were not mediated by HOMA-IR. The latter nicely aligns with a recent report on an insulin-independent positive correlation between plasma levels of palmitoleate and hepatic phosphorus metabolism in metabolically healthy individuals [30].

TG-rich LDL particles are either taken up by hepatocytes *via* LDL-receptors or further hydrolyzed by hepatic lipase (HL) yielding small dense LDL particles [31]. Our observation of a LFC-associated decrease in large LDL particles together with an increase in small dense LDL particles argues for a prolonged dwelling time of LDL-particles in the circulation, including shrinkage by HL activity [31], making them prone to oxidation. In line with this hypothesis hepatic steatosis was characterized by diminished LDL-receptor expression [32]. Oxidized LDL particles in turn mediate the adverse, pro-inflammatory setting implicated in the onset of cardiovascular disease [33]. Despite this LDL-signature was mediated to a significant amount by HOMA-IR (Fig. 2), the inverse association between LFC and large LDL-particles (LDL_1) was unaffected and might hence represent an early event in the adverse relation between hepatic steatosis, insulin resistance, and cardiovascular disease. Of note, the same mechanism likely accounts for the inverse associations between LFC and ether-PCs, as both strongly associate (Fig. S1) and PCs are integral for the monolayer surrounding lipoprotein particles. This observation emphasizes the particular value of multi-platform metabolomics approaches to contextualize findings.

Altered small-dense HDL composition as hint towards progression to steatohepatitis

The transition from hepatic steatosis to steatohepatitis constitutes a continuum rather than a discrete event and hence we could also identify molecular signatures described in more advanced stages of liver impairment, e.g., an accumulation of PCs in the circulation [11, 34]. With respect to ALT activities but not LFC our results partially confirm these observations and our multi-platform metabolomics approach once more allows us to link these findings to lipoprotein metabolism. Briefly, we observed consistent positive associations between PCs and small-dense HDL particles (HDL_3) both uniquely associated with ALT (Fig. 3). Enriched PC content of HDL particles has been shown to increase efflux of free cholesterol from scavenger receptor BI (SR-BI) expressing cells [35]. As SR-BI, the HDL-receptor is highly expressed on hepatocytes this might indicate altered reverse cholesterol transport in relation to hepatic steatosis or steatohepatitis given the unique association with elevated liver enzyme activities.

BCAA catabolites are linked by insulin sensitivity with liver fat

A frequently published link between obesity, hepatic steatosis, and impaired glucose homeostasis comprises an accumulation of BCAAs and aromatic acids [36-40]. Consistently, we observed a BMI-independent association with all these amino acid species and LFC. Besides an increased proteolysis, responsible mechanism for BCAA accumulation might include decreased catabolism in adipose tissue [41] or skeletal muscle [42] as the first step of BCAA-catabolism is facilitated by branched-chain ketoacid dehydrogenase (BCKDH) in non-hepatic tissues. Accumulation of such catabolites, including 3-methyl-2-oxobutyrate, is considered to mediate the adverse effects of BCAAs [43] E.g., suppressing pyruvate dehydrogenase activity which catalyzes the formation of acetyl-CoA from pyruvate [44]. Notably, a subsequent shift in pyruvate utilization towards lactate and alanine formation aligns well with our observation of positive associations between LFC and these metabolites. Further, increased activity of the BCKDH kinase, and hence diminished BCKDH activity due to hyper-phosphorylation, has been shown to integrate BCAA-catabolism with stimulated hepatic *de novo* lipogenesis due to phosphorylation of ATP-citrate lyase generating substrates for lipogenesis [45]. Importantly, the associations with BCAA-catabolites (but not primary BCAAs), lactate, and alanine were all significantly mediated by HOMA-IR (Tab. S4). In conclusion, our BMI-independent observations as well as confirmative results from previous studies [15, 46] strongly argue for hepatic (or at least ectopic) fat accumulation as key mechanism for impaired BCAA-catabolism. However, even if diminished BCAA-metabolism seems to integrate hepatic lipogenesis the pathophysiological event linking it to insulin resistance remains elusive but a solitary increase in BCAAs in plasma seems to be not a sufficient criterion. The latter aligns with a recent study by Gaggini and colleagues who reported less prominent alterations in plasma BCAA concentrations among hepatic steatosis patients without type 2 diabetes [40].

A signature of increased oxidative stress is a hallmark of hepatic steatosis

Surrogates of oxidative stress in respect to hepatic steatosis have been noted by several previous studies with γ-glutamyl amino acids and glutamate [precursors of the antioxidant glutathione (GSH)] being the most prominent examples [19, 40]. Notably, those markers were able to indicate progressive liver disease, i.e. fibrosis [40]. Consistently, intensified mitochondrial respiration causing accumulation of reactive oxygen species has been described for liver biopsies from patients with hepatic steatosis and steatohepatitis [47]. Besides GSH synthesis, residual serum activity of GGT might also account for these observations, as the latter association was not attributable to any of the tested mediators. Beyond biomarker research, the application of genome-scale metabolic modelling revealed the crucial importance of the further upstream metabolites glycine and serine [48]. In general, these observations well align with our findings but we observed a strong dependence of these associations on insulin sensitivity, i.e. those were mediated to a great extent by HOMA-IR. Interestingly, among patients with hepatic steatosis plasma glycine concentrations strongly correlated with hepatic but not peripheral insulin resistance [40]. Hence, it would be of particular interest to define if diminished insulin sensitivity causes (hepatic) oxidative stress or *vice versa*.

In contrast, the strong positive association with plasma xanthine with all liver traits was unaffected by HOMA-IR adjustment but also points towards augmented defense against systemic/hepatic oxidative stress. Xanthine is an intermediate in purine degradation finally resulting in the formation of urate one of the most important antioxidants in human blood. The reaction is catalyzed by xanthine oxidase (XO) and recent cellular and mouse models [49] showed increased activity of XO in hepatic steatosis which is supported by observational studies

[50, 51]. The far less pronounced association with plasma urate compared to xanthine levels in the present study might be due to the oxidation of urate in a state of high oxidative stress accompanying hepatic steatosis. Subsequently, the consistent positive association with xanthine might be a surrogate for increased XO activity to provide urate as antioxidant.

Urinary fingerprint of LFC

The significant associations with LFC and urine metabolites are of particular interest as they were in the majority 1) not mirrored by common markers of liver damage and 2) not mediated by total TG, HOMA-IR, fasting glucose or, hsCRP and hence present besides pathophysiological insights the potential of complementary biomarkers. Some of the metabolites were closely related to processes already described for plasma metabolites, e.g. increased levels of BCAA catabolites or lactate as marker for impaired glucose metabolism.

Altered phase I and phase II detoxification

Apart from that, several steroid species in urine showed an inverse association with LFC. In particular those attributable to be released from the adrenal cortex, e.g. dehydroepiandrosterone sulfate (DHEA-S) or etiocholanone. Interestingly, this contrasts to some extent the positive associations seen in plasma. Considering that most of the observed associations were related to sulphated or glucuronidated compounds this might indicate an altered metabolism/degradation of adrenal derived steroids. While the relation between glucocorticoids and hepatic steatosis was frequently described (see below) data on other adrenal derived hormones is less established. Presence of hepatic steatosis was linked with higher DHEA-S levels among adults [52] and an altered adrenal steroid profile in urine among children [53]. A diminished activity of responsible sulfotransferases in the progressively impaired liver [54] might be one plausible explanation. In general, the urine profile associated with LFC points towards a diminished detoxification capacity of the liver, not only phase II as presented above but also phase I. E.g., the urinary metabolites gentisate and 5-hydroxyindolacetate (inversely associated with LFC) represent degradation products of tyrosine and serotonin, respectively.

A molecular signature predictive for FLD

Extensive feature selection revealed a model which allowed for a slightly better identification of hepatic steatosis cases compared to classical clinical measures. Notably, we designed the classification algorithm to generate a sparse set of variables comprising complementary information and hence not necessarily including all top associated metabolites/clinical features. Despite this improvement might be of limited direct clinical relevance the identified molecules might be of particular value for the discrimination of different subtypes of hepatic steatosis as has been shown recently [20].

Besides known risk factors like abdominal obesity, insulin resistance or already outlined molecular perturbations (glycine), the most consistent parameter was the unknown urinary compound X-20643 which was linked to a decreased odd for hepatic steatosis. A putative annotation of the metabolite might be possible due to its direct neighboring with plasma cortisol in the derived GGM (Fig. 4) [55]. Based on its molecular weight (~539.4 Da) this would fit to a conjugation of cortisol with glucuronic acid yielding the respective glucuronide indicating a diminished degradation of cortisol. Cortisol or more general glucocorticoid excess either caused endogenously (Cushing´s syndrome) or exogenously was consistently linked with the development and presence of hepatic steatosis (for review see [56]). Cortisol is thereby assumed to drive several hallmarks of hepatic steatosis, including increased lipogenesis and VLDL assembly [56, 57]. Notably, no association between LFC and plasma cortisol levels became

apparent even when accounting for blood sampling time. In summary, altered cortisol metabolism, in particular in the liver, was a prominent independent hallmark of hepatic steatosis in the present study and degradation intermediates of cortisol in urine might be a suitable proxy for prolonged hypercortisolism accompanying hepatic steatosis.

Strengths and Limitations

The present study comprised one of the most comprehensive metabolomics approaches in an epidemiological setting to address the metabolic fingerprint of liver function. The assessment of LFC using MRI represents thereby an outstanding feature. However, the absence of liver biopsies restricted the distinction of progressive liver diseases and represents a drawback of the present approach. However, it has to be noted that despite being present in about one-third of our study population the degree of hepatic steatosis was rather mild. Therefore, we could only speculate about a transfer of our findings in more severe states of liver disease. Furthermore, the cross-sectional character of the study provides only observational results and restricts functional insights on the molecular level. This fact also subsumes a possible residual confounding by obesity in linear regression analyses even if accounting for BMI. Despite those limitations the sample size conducted is a clear advantage for the classification assessment as it provides by far more information about the generalizability of the achieved results as in tightly controlled experimental settings which constitutes the gross of research conducted so far with respect to metabolomics and hepatic steatosis.

Conclusion

The present high-quality metabolomics approach among a population-based sample characterized by the absence of diabetes revealed a molecular fingerprint of hepatic steatosis which was characterized by complex alterations in lipid metabolism with lipoprotein particles as key driver, augmented defense against oxidative stress as well as adverse cortisol signaling. References to impaired BCAA-catabolism and accumulation of small dense LDL particles were strongly related to diminished insulin sensitivity accompanying hepatic steatosis. From a clinical perspective the use of urine samples to identify (or stratify) subjects with hepatic steatosis might be of particular interest as the presented markers provide complementary information to those already established.

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Corresponding author and to whom reprint request should be addressed: Maik Pietzner, Institute of Clinical Chemistry and Laboratory Medicine, University Medicine Greifswald, Ferdinand-Sauerbruch-Straße NK, D-17475 Greifswald, Germany, Phone: +49 - 3834 – 8619659, FAX: +49 - 3834 – 865502, E-mail: maik.pietzner@uni-greifswald.de

DISCLOSURE SUMMARY

I certify that neither I nor my co-authors have a conflict of interest as described above that is relevant to the subject matter or materials included in this Work.

REFERENCES

1. Michelotti, G.A., M.V. Machado, and A.M. Diehl, *NAFLD, NASH and liver cancer.* Nat Rev Gastroenterol Hepatol, 2013. **10**(11): p. 656-65.
2. Browning, J.D. and J.D. Horton, *Molecular mediators of hepatic steatosis and liver injury.* J Clin Invest, 2004. **114**(2): p. 147-52.
3. Vernon, G., A. Baranova, and Z.M. Younossi, *Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults.* Aliment Pharmacol Ther, 2011. **34**(3): p. 274-85.
4. Leite, N.C., G.F. Salles, A.L. Araujo, C.A. Villela-Nogueira, and C.R. Cardoso, *Prevalence and associated factors of non-alcoholic fatty liver disease in patients with type-2 diabetes mellitus.* Liver Int, 2009. **29**(1): p. 113-9.
5. Fabbrini, E., F. Magkos, B.S. Mohammed, T. Pietka, N.A. Abumrad, B.W. Patterson, A. Okunade, and S. Klein, *Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity.* Proc Natl Acad Sci U S A, 2009. **106**(36): p. 15430-5.
6. Lonardo, A., S. Sookoian, C.J. Pirola, and G. Targher, *Non-alcoholic fatty liver disease and risk of cardiovascular disease.* Metabolism, 2015.
7. Targher, G. and C.D. Byrne, *Clinical Review: Nonalcoholic fatty liver disease: a novel cardiometabolic risk factor for type 2 diabetes and its complications.* J Clin Endocrinol Metab, 2013. **98**(2): p. 483-95.
8. Neuschwander-Tetri, B.A., *Non-alcoholic fatty liver disease.* BMC Medicine, 2017. **15**(1): p. 45.
9. Dumas, M.E., J. Kinross, and J.K. Nicholson, *Metabolic phenotyping and systems biology approaches to understanding metabolic syndrome and fatty liver disease.* Gastroenterology, 2014. **146**(1): p. 46-62.
10. Puri, P., R.A. Baillie, M.M. Wiest, F. Mirshahi, J. Choudhury, O. Cheung, C. Sargeant, M.J. Contos, and A.J. Sanyal, *A lipidomic analysis of nonalcoholic fatty liver disease.* Hepatology, 2007. **46**(4): p. 1081-90.
11. Puri, P., M.M. Wiest, O. Cheung, F. Mirshahi, C. Sargeant, H.K. Min, M.J. Contos, R.K. Sterling, M. Fuchs, H. Zhou, S.M. Watkins, and A.J. Sanyal, *The plasma lipidomic signature of nonalcoholic steatohepatitis.* Hepatology, 2009. **50**(6): p. 1827-38.
12. Barr, J., J. Caballeria, I. Martinez-Arranz, A. Dominguez-Diez, C. Alonso, J. Muntane, M. Perez-Cormenzana, C. Garcia-Monzon, R. Mayo, A. Martin-Duce, M. Romero-Gomez, O. Lo Iacono, J. Tordjman, R.J. Andrade, M. Perez-Carreras, Y. Le Marchand-Brustel, A. Tran, C. Fernandez-Escalante, E. Arevalo, M. Garcia-Unzueta, K. Clement, J. Crespo, P. Gual, M. Gomez-Fleitas, M.L. Martinez-Chantar, A. Castro, S.C. Lu, M. Vazquez-Chantada, and J.M.

Mato, *Obesity-dependent metabolic signatures associated with nonalcoholic fatty liver disease progression.* J Proteome Res, 2012. **11**(4): p. 2521-32.
13. Feldman, A., S.K. Eder, T.K. Felder, L. Kedenko, B. Paulweber, A. Stadlmayr, U. Huber-Schonauer, D. Niederseer, F. Stickel, S. Auer, E. Haschke-Becher, W. Patsch, C. Datz, and E. Aigner, *Clinical and Metabolic Characterization of Lean Caucasian Subjects With Non-alcoholic Fatty Liver.* Am J Gastroenterol, 2017. **112**(1): p. 102-110.
14. Kalhan, S.C., L. Guo, J. Edmison, S. Dasarathy, A.J. McCullough, R.W. Hanson, and M. Milburn, *Plasma metabolomic profile in nonalcoholic fatty liver disease.* Metabolism, 2011. **60**(3): p. 404-13.
15. Lake, A.D., P. Novak, P. Shipkova, N. Aranibar, D.G. Robertson, M.D. Reily, L.D. Lehman-McKeeman, R.R. Vaillancourt, and N.J. Cherrington, *Branched chain amino acid metabolism profiles in progressive human nonalcoholic fatty liver disease.* Amino Acids, 2015. **47**(3): p. 603-15.
16. Sookoian, S., P. Puri, G.O. Castano, R. Scian, F. Mirshahi, A.J. Sanyal, and C.J. Pirola, *Nonalcoholic steatohepatitis is associated with a state of betaine-insufficiency.* Liver Int, 2017. **37**(4): p. 611-619.
17. von Schonfels, W., E. Patsenker, R. Fahrner, T. Itzel, H. Hinrichsen, M. Brosch, W. Erhart, A. Gruodyte, B. Vollnberg, K. Richter, A. Landrock, S. Schreiber, S. Bruckner, G. Beldi, B. Sipos, T. Becker, C. Rocken, A. Teufel, F. Stickel, C. Schafmayer, and J. Hampe, *Metabolomic tissue signature in human non-alcoholic fatty liver disease identifies protective candidate metabolites.* Liver Int, 2015. **35**(1): p. 207-14.
18. Dumas, M.E., R.H. Barton, A. Toye, O. Cloarec, C. Blancher, A. Rothwell, J. Fearnside, R. Tatoud, V. Blanc, J.C. Lindon, S.C. Mitchell, E. Holmes, M.I. McCarthy, J. Scott, D. Gauguier, and J.K. Nicholson, *Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice.* Proc Natl Acad Sci U S A, 2006. **103**(33): p. 12511-6.
19. Soga, T., M. Sugimoto, M. Honma, M. Mori, K. Igarashi, K. Kashikura, S. Ikeda, A. Hirayama, T. Yamamoto, H. Yoshida, M. Otsuka, S. Tsuji, Y. Yatomi, T. Sakuragawa, H. Watanabe, K. Nihei, T. Saito, S. Kawata, H. Suzuki, M. Tomita, and M. Suematsu, *Serum metabolomics reveals gamma-glutamyl dipeptides as biomarkers for discrimination among different forms of liver disease.* J Hepatol, 2011. **55**(4): p. 896-905.
20. Alonso, C., D. Fernandez-Ramos, M. Varela-Rey, I. Martinez-Arranz, N. Navasa, S.M. Van Liempd, J.L. Lavin Trueba, R. Mayo, C.P. Ilisso, V.G. de Juan, M. Iruarrizaga-Lejarreta, L. delaCruz-Villar, I. Minchole, A. Robinson, J. Crespo, A. Martin-Duce, M. Romero-Gomez, H. Sann, J. Platon, J. Van Eyk, P. Aspichueta, M. Noureddin, J.M. Falcon-Perez, J. Anguita, A.M. Aransay, M.L. Martinez-Chantar, S.C. Lu, and J.M. Mato, *Metabolomic Identification of Subtypes of Nonalcoholic Steatohepatitis.* Gastroenterology, 2017. **152**(6): p. 1449-1461.e7.
21. Volzke, H., D. Alte, C.O. Schmidt, D. Radke, R. Lorbeer, N. Friedrich, N. Aumann, K. Lau, M. Piontek, G. Born, C. Havemann, T. Ittermann, S. Schipf, R. Haring, S.E. Baumeister, H. Wallaschofski, M. Nauck, S. Frick, A. Arnold, M. Junger, J. Mayerle, M. Kraft, M.M. Lerch, M. Dorr, T. Reffelmann, K. Empen, S.B. Felix, A. Obst, B. Koch, S. Glaser, R. Ewert, I. Fietze, T. Penzel, M. Doren, W. Rathmann, J. Haerting, M. Hannemann, J. Ropcke, U. Schminke, C. Jurgens, F. Tost, R. Rettig, J.A. Kors, S. Ungerer, K. Hegenscheid, J.P. Kuhn, J. Kuhn, N. Hosten, R. Puls, J. Henke, O. Gloger, A. Teumer, G. Homuth, U. Volker, C. Schwahn, B. Holtfreter, I. Polzer, T. Kohlmann, H.J. Grabe, D. Rosskopf, H.K. Kroemer, T. Kocher, R.

Biffar, U. John, and W. Hoffmann, *Cohort profile: the study of health in Pomerania.* Int J Epidemiol, 2011. **40**(2): p. 294-307.
22. Matthews, D.R., J.P. Hosker, A.S. Rudenski, B.A. Naylor, D.F. Treacher, and R.C. Turner, *Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man.* Diabetologia, 1985. **28**(7): p. 412-419.
23. Angulo, P., J.M. Hui, G. Marchesini, E. Bugianesi, J. George, G.C. Farrell, F. Enders, S. Saksena, A.D. Burt, J.P. Bida, K. Lindor, S.O. Sanderson, M. Lenzi, L.A. Adams, J. Kench, T.M. Therneau, and C.P. Day, *The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD.* Hepatology, 2007. **45**(4): p. 846-54.
24. Sterling, R.K., E. Lissen, N. Clumeck, R. Sola, M.C. Correa, J. Montaner, S.S. M, F.J. Torriani, D.T. Dieterich, D.L. Thomas, D. Messinger, and M. Nelson, *Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection.* Hepatology, 2006. **43**(6): p. 1317-25.
25. Inker, L.A., C.H. Schmid, H. Tighiouart, J.H. Eckfeldt, H.I. Feldman, T. Greene, J.W. Kusek, J. Manzi, F. Van Lente, Y.L. Zhang, J. Coresh, A.S. Levey, and C.-E. Investigators, *Estimating glomerular filtration rate from serum creatinine and cystatin C.* N Engl J Med, 2012. **367**(1): p. 20-9.
26. Kuhn, J.P., D. Hernando, B. Mensel, P.C. Kruger, T. Ittermann, J. Mayerle, N. Hosten, and S.B. Reeder, *Quantitative chemical shift-encoded MRI is an accurate method to quantify hepatic steatosis.* J Magn Reson Imaging, 2014. **39**(6): p. 1494-501.
27. Knacke, H., M. Pietzner, K.T. Do, W. Romisch-Margl, G. Kastenmuller, U. Volker, H. Volzke, J. Krumsiek, A. Artati, H. Wallaschofski, M. Nauck, K. Suhre, J. Adamski, and N. Friedrich, *Metabolic Fingerprints of Circulating IGF-1 and the IGF-1/IGFBP-3 Ratio: A Multifluid Metabolomics Study.* J Clin Endocrinol Metab, 2016. **101**(12): p. 4730-4742.
28. Pietzner, M., G. Homuth, K. Budde, I. Lehmphul, U. Volker, H. Volzke, M. Nauck, J. Kohrle, and N. Friedrich, *Urine Metabolomics by (1)H-NMR Spectroscopy Indicates Associations between Serum 3,5-T2 Concentrations and Intermediary Metabolism in Euthyroid Humans.* Eur Thyroid J, 2015. **4**(Suppl 1): p. 92-100.
29. Choi, S.H. and H.N. Ginsberg, *Increased very low density lipoprotein (VLDL) secretion, hepatic steatosis, and insulin resistance.* Trends Endocrinol Metab, 2011. **22**(9): p. 353-63.
30. Kahl, S., B. Nowotny, K. Strassburger, A. Bierwagen, B. Kluppelholz, B. Hoffmann, G. Giani, P.J. Nowotny, F. Wallscheid, E. Hatziagelaki, G. Pacini, J.H. Hwang, and M. Roden, *Amino Acid and Fatty Acid Levels Affect Hepatic Phosphorus Metabolite Content in Metabolically Healthy Humans.* J Clin Endocrinol Metab, 2018. **103**(2): p. 460-468.
31. Fon Tacer, K. and D. Rozman, *Nonalcoholic Fatty liver disease: focus on lipoprotein and lipid deregulation.* J Lipids, 2011. **2011**: p. 783976.
32. Min, H.K., A. Kapoor, M. Fuchs, F. Mirshahi, H. Zhou, J. Maher, J. Kellum, R. Warnick, M.J. Contos, and A.J. Sanyal, *Increased hepatic synthesis and dysregulation of cholesterol metabolism is associated with the severity of nonalcoholic fatty liver disease.* Cell Metab, 2012. **15**(5): p. 665-74.
33. Griffin, B.A., D.J. Freeman, G.W. Tait, J. Thomson, M.J. Caslake, C.J. Packard, and J. Shepherd, *Role of plasma triglyceride in the regulation of plasma low density lipoprotein (LDL) subfractions: relative contribution of small, dense LDL to coronary heart disease risk.* Atherosclerosis, 1994. **106**(2): p. 241-53.
34. Anjani, K., M. Lhomme, N. Sokolovska, C. Poitou, J. Aron-Wisnewsky, J.L. Bouillot, P. Lesnik, P. Bedossa, A. Kontush, K. Clement, I. Dugail, and J. Tordjman, *Circulating*

phospholipid profiling identifies portal contribution to NASH signature in obesity. J Hepatol, 2015. **62**(4): p. 905-12.
35. Yancey, P.G., M. de la Llera-Moya, S. Swarnakar, P. Monzo, S.M. Klein, M.A. Connelly, W.J. Johnson, D.L. Williams, and G.H. Rothblat, *High density lipoprotein phospholipid composition is a major determinant of the bi-directional flux and net movement of cellular free cholesterol mediated by scavenger receptor BI.* J Biol Chem, 2000. **275**(47): p. 36596-604.
36. Wang, T.J., M.G. Larson, R.S. Vasan, S. Cheng, E.P. Rhee, E. McCabe, G.D. Lewis, C.S. Fox, P.F. Jacques, C. Fernandez, C.J. O'Donnell, S.A. Carr, V.K. Mootha, J.C. Florez, A. Souza, O. Melander, C.B. Clish, and R.E. Gerszten, *Metabolite profiles and the risk of developing diabetes.* Nat Med, 2011. **17**(4): p. 448-53.
37. Floegel, A., N. Stefan, Z. Yu, K. Muhlenbruch, D. Drogan, H.G. Joost, A. Fritsche, H.U. Haring, M. Hrabe de Angelis, A. Peters, M. Roden, C. Prehn, R. Wang-Sattler, T. Illig, M.B. Schulze, J. Adamski, H. Boeing, and T. Pischon, *Identification of serum metabolites associated with risk of type 2 diabetes using a targeted metabolomic approach.* Diabetes, 2013. **62**(2): p. 639-48.
38. Menni, C., E. Fauman, I. Erte, J.R. Perry, G. Kastenmuller, S.Y. Shin, A.K. Petersen, C. Hyde, M. Psatha, K.J. Ward, W. Yuan, M. Milburn, C.N. Palmer, T.M. Frayling, J. Trimmer, J.T. Bell, C. Gieger, R.P. Mohney, M.J. Brosnan, K. Suhre, N. Soranzo, and T.D. Spector, *Biomarkers for type 2 diabetes and impaired fasting glucose using a nontargeted metabolomics approach.* Diabetes, 2013. **62**(12): p. 4270-6.
39. Newgard, C.B., J. An, J.R. Bain, M.J. Muehlbauer, R.D. Stevens, L.F. Lien, A.M. Haqq, S.H. Shah, M. Arlotto, C.A. Slentz, J. Rochon, D. Gallup, O. Ilkayeva, B.R. Wenner, W.S. Yancy, Jr., H. Eisenson, G. Musante, R.S. Surwit, D.S. Millington, M.D. Butler, and L.P. Svetkey, *A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance.* Cell Metab, 2009. **9**(4): p. 311-26.
40. Gaggini, M., F. Carli, C. Rosso, E. Buzzigoli, M. Marietti, V. Della Latta, D. Ciociaro, M.L. Abate, R. Gambino, M. Cassader, E. Bugianesi, and A. Gastaldelli, *Altered amino acid concentrations in NAFLD: Impact of obesity and insulin resistance.* Hepatology, 2018. **67**(1): p. 145-158.
41. Herman, M.A., P. She, O.D. Peroni, C.J. Lynch, and B.B. Kahn, *Adipose tissue branched chain amino acid (BCAA) metabolism modulates circulating BCAA levels.* J Biol Chem, 2010. **285**(15): p. 11348-56.
42. Lerin, C., A.B. Goldfine, T. Boes, M. Liu, S. Kasif, J.M. Dreyfuss, A.L. De Sousa-Coelho, G. Daher, I. Manoli, J.R. Sysol, E. Isganaitis, N. Jessen, L.J. Goodyear, K. Beebe, W. Gall, C.P. Venditti, and M.E. Patti, *Defects in muscle branched-chain amino acid oxidation contribute to impaired lipid metabolism.* Mol Metab, 2016. **5**(10): p. 926-36.
43. Lynch, C.J. and S.H. Adams, *Branched-chain amino acids in metabolic signalling and insulin resistance.* Nat Rev Endocrinol, 2014. **10**(12): p. 723-36.
44. Walajtys-Rode, E. and J.R. Williamson, *Effects of branched chain alpha-ketoacids on the metabolism of isolated rat liver cells. III. Interactions with pyruvate dehydrogenase.* J Biol Chem, 1980. **255**(2): p. 413-8.
45. White, P.J., R.W. McGarrah, P.A. Grimsrud, S.C. Tso, W.H. Yang, J.M. Haldeman, T. Grenier-Larouche, J. An, A.L. Lapworth, I. Astapova, S.A. Hannou, T. George, M. Arlotto, L.B. Olson, M. Lai, G.F. Zhang, O. Ilkayeva, M.A. Herman, R.M. Wynn, D.T. Chuang, and C.B.

Newgard, *The BCKDH Kinase and Phosphatase Integrate BCAA and Lipid Metabolism via Regulation of ATP-Citrate Lyase.* Cell Metab, 2018. **27**(6): p. 1281-1293.e7.
46. Haufe, S., H. Witt, S. Engeli, J. Kaminski, W. Utz, J.C. Fuhrmann, D. Rein, J. Schulz-Menger, F.C. Luft, M. Boschmann, and J. Jordan, *Branched-chain and aromatic amino acids, insulin resistance and liver specific ectopic fat storage in overweight to obese subjects.* Nutr Metab Cardiovasc Dis, 2016. **26**(7): p. 637-42.
47. Koliaki, C., J. Szendroedi, K. Kaul, T. Jelenik, P. Nowotny, F. Jankowiak, C. Herder, M. Carstensen, M. Krausch, W.T. Knoefel, M. Schlensak, and M. Roden, *Adaptation of hepatic mitochondrial function in humans with non-alcoholic fatty liver is lost in steatohepatitis.* Cell Metab, 2015. **21**(5): p. 739-46.
48. Mardinoglu, A., R. Agren, C. Kampf, A. Asplund, M. Uhlen, and J. Nielsen, *Genome-scale metabolic modelling of hepatocytes reveals serine deficiency in patients with non-alcoholic fatty liver disease.* Nat Commun, 2014. **5**: p. 3083.
49. Xu, C., X. Wan, L. Xu, H. Weng, M. Yan, M. Miao, Y. Sun, G. Xu, S. Dooley, Y. Li, and C. Yu, *Xanthine oxidase in non-alcoholic fatty liver disease and hyperuricemia: One stone hits two birds.* J Hepatol, 2015. **62**(6): p. 1412-9.
50. Sirota, J.C., K. McFann, G. Targher, R.J. Johnson, M. Chonchol, and D.I. Jalal, *Elevated serum uric acid levels are associated with non-alcoholic fatty liver disease independently of metabolic syndrome features in the United States: Liver ultrasound data from the National Health and Nutrition Examination Survey.* Metabolism, 2013. **62**(3): p. 392-9.
51. Liu, C.Q., C.M. He, N. Chen, D. Wang, X. Shi, Y. Liu, X. Zeng, B. Yan, S. Liu, S. Yang, X. Li, X. Li, and Z. Li, *Serum uric acid is independently and linearly associated with risk of nonalcoholic fatty liver disease in obese Chinese adults.* Sci Rep, 2016. **6**: p. 38605.
52. Koga, M., H. Saito, M. Mukai, T. Saibara, and S. Kasayama, *Serum dehydroepiandrosterone sulphate levels in patients with non-alcoholic fatty liver disease.* Intern Med, 2011. **50**(16): p. 1657-61.
53. Gawlik, A., M. Shmoish, M.F. Hartmann, E. Malecka-Tendera, S.A. Wudy, and Z. Hochberg, *Steroid Metabolomic Disease Signature of Nonsyndromic Childhood Obesity.* J Clin Endocrinol Metab, 2016. **101**(11): p. 4329-4337.
54. Yalcin, E.B., V. More, K.L. Neira, Z.J. Lu, N.J. Cherrington, A.L. Slitt, and R.S. King, *Downregulation of sulfotransferase expression and activity in diseased human livers.* Drug Metab Dispos, 2013. **41**(9): p. 1642-50.
55. Krumsiek, J., K. Suhre, A.M. Evans, M.W. Mitchell, R.P. Mohney, M.V. Milburn, B. Wagele, W. Romisch-Margl, T. Illig, J. Adamski, C. Gieger, F.J. Theis, and G. Kastenmuller, *Mining the unknown: a systems approach to metabolite identification combining genetic and metabolic information.* PLoS Genet, 2012. **8**(10): p. e1003005.
56. Rose, A.J. and S. Herzig, *Metabolic control through glucocorticoid hormones: an update.* Mol Cell Endocrinol, 2013. **380**(1-2): p. 65-78.
57. Vegiopoulos, A. and S. Herzig, *Glucocorticoids, metabolism and metabolic diseases.* Mol Cell Endocrinol, 2007. **275**(1-2): p. 43-61.

Figure 1 Flowchart of the analyses procedure.

Figure 2 Color coded corrected p-values (controlling the false discovery rate (FDR) at 0.05; dotted lines) from linear regression analyses using liver fat content (LFC), alanine transaminase (ALT), aspartate transaminase (AST) or γ-glutamyl transpeptidase (GGT) as explanatory variables and lipoprotein particles as outcome. Models were adjusted for age, sex, body mass

index, smoking, alcohol consumption and physical activity. Orange shadings indicate positive associations whereas blue the opposite direction. Hatched boxes indicate mediation of the association either by a measure of insulin resistance (HOMA-IR; homeostatic model assessment of insulin resistance), high-sensitivity C-reactive protein (hsCRP) or serum glucose. VLDL = very low-density lipoprotein; LDL = low-density lipoprotein; IDL = intermediate-density lipoproteine; HDL = high-density lipoprotein; Apo = apolipoprotein

Figure 3 A) Corrected p-values (controlling the false discovery rate (FDR) at 0.05; dotted lines) from linear regression analyses using liver fat content (orange), alanine transaminase (ALT; purple), aspartate transaminase (AST; green) or γ-glutamyl transpeptidase (GGT; blue) as explanatory variables and plasma (upper panel) or urine metabolites (lower panel) as outcome. Results were separated by association direction - positive (β>0) or negative (β<0). Corresponding beta estimates and FDR values are given in table S1 and S2. Metabolites marked with a triangle exceeded the plotting range. **B)** Boxplots for the estimated proportion mediated between the exposure and metabolites by serum glucose, high-sensitivity C-reactive protein, a measure of insulin resistance (HOMA-IR; homeostatic model assessment of insulin resistance) and total triglycerides. **C)** Color coded FDR-values from linear regression analyses using liver fat content (LFC), ALT, AST or GGT as explanatory variables and plasma or urine metabolites as outcome, limiting to non-mediated metabolites. Significant associations (FDR < 0.05) are framed black. Orange shading indicates positive and blue shading indicates negative associations, respectively. Hatched boxes indicate mediation of the association either by a measure of insulin resistance (HOMA-IR; homeostatic model assessment of insulin resistance), total triglycerides (TG), high-sensitivity C-reactive protein or serum glucose.

Figure 4 *left panel* ROC curves and AUC with 95%-confidence interval (CI) for the three different models to predict fatty liver disease: Clinical variables – green; Metabolites – purple; Combination of both – orange. *right panel* Subnetwork of the derived GGM with emphasize on the unknown urinary predictors X – 20643 and X - 16774. On each node the results from linear regression analysis for liver fat content (LFC, orange), serum alanine aminotransferase (ALT; purple), asparagine aminotransferase (AST; green) and γ-glutamyl transpeptidase (GGT; blue) were mapped as portion of the associations strength given as –log10(FDR-value). Significant results in at least one trait, false discovery rate (FDR) below 5%, were highlighted by colors. Node sizes were chosen as maximum association strength of the single traits. The prefix *P* denotes plasma metabolites whereas *U* indicates urine metabolites. Edges represent significant partial correlations (par. cor.) between metabolites. Type and color represent metabolite and fluid dependencies.

Table 1 General Characteristics of the study population.

Characteristic	whole sample (n=769)	Liver fat content <5% (n=502)	Liver fat content >5% (n=267)	P-value*
Age (years)	51 (41; 61)	47 (38; 57)	57 (48; 64)	<0.01
Females (%)	56.0%	62.3%	44.2%	<0.01
Smoking (%)				<0.01
never smoker	42.1%	44.0%	38.6%	
former smoker	36.4%	31.6%	45.3%	
current smoker	21.4%	24.3%	16.1%	
Physically active (%)	73.6%	73.7%	73.4%	0.99
Alcohol consumption (g/day)	4.03 (1.30; 10.36)	3.70 (1.14; 8.65)	4.91 (1.40; 14.69)	<0.01
Waist circumference (cm)	86 (78; 96)	82 (74; 89)	97 (88; 105)	<0.01
Body mass index (kg/m²)	26.7 (23.9; 29.6)	25.3 (22.9; 27.8)	29.5 (27.2; 32.4)	<0.01

Glucose (mmol/l)	5.3 (4.9; 5.7)	5.2 (4.9; 5.5)	5.5 (5.1; 6.0)	<0.01
HOMA-IR	2.04 (1.41; 3.20)	1.68 (1.22; 2.33)	3.33 (2.28; 4.90)	<0.01
Insulin (mU/l)	8.6 (6.0; 12.9)	7.2 (5.4; 10.0)	13.5 (9.8; 18.4)	<0.01
Triglycerides (mmol/l)	1.21 (0.86; 1.70)	1.04 (0.76; 1.41)	1.56 (1.17; 2.12)	<0.01
LDL-cholesterol (mmol/l)	3.36 (2.76; 3.98)	3.24 (2.63; 3.83)	3.60 (3.04; 4.11)	<0.01
HDL-cholesterol (mmol/l)	1.44 (1.22; 1.72)	1.53 (1.30; 1.78)	1.30 (1.11; 1.55)	<0.01
Total cholesterol (mmol/l)	5.4 (4.8; 6.2)	5.3 (4.7; 6.1)	5.6 (4.9; 6.3)	<0.01
hsCRP, mg/l	1.13 (0.61; 2.21)	0.94 (0.54; 1.77)	1.55 (0.90; 2.91)	<0.01
Liver fat content (%)	3.43 (2.16; 6.59)	2.45 (1.89; 3.35)	9.2 (6.4; 14.9)	-
Hepatic steatosis† (%)	34.7%	0%	100%	-
ALT (µkatal/l)	0.37 (0.27; 0.52)	0.32 (0.25; 0.43)	0.49 (0.37; 0.67)	<0.01
AST (µkatal/l)	0.29 (0.23; 0.36)	0.27 (0.21; 0.33)	0.33 (0.27; 0.41)	<0.01
GGT (µkatal/l)	0.48 (0.38; 0.66)	0.43 (0.36; 0.56)	0.62 (0.48; 0.88)	<0.01
AST/ALT	0.74 (0.59; 0.94)	0.80 (0.64; 1.00)	0.66 (0.53; 0.79)	<0.01
NAFLD-Score	-2.11 (-2.86; -1.34)	-2.38 (-3.03; -1.58)	-1.62 (-2.29; -0.89)	<0.01
FIB4-Score	0.81 (0.56; 1.08)	0.76 (0.51; 1.02)	0.90 (0.67; 1.19)	<0.01
eGFRcys (ml/min/1.72m²)	114 (105; 122)	117 (108; 124)	109 (100; 118)	<0.01

HOMA-IR = homeostatic model of insulin resistance; hsCRP = high-sensitivity C-reactive protein; HDL = high density lipoprotein LUS = liver ultra sound pattern; ALT = alanine transaminase; AST = aspartate transaminase; GGT = γ-glutamyl transpeptidase; eGFR = estimated glomerular filtration rate based on cystatin C measurements, Continuous data are expressed as median (25th percentile; 75th percentile); nominal data are given as percentages. *χ2-test (nominal data) or Mann-Whitney-U test (interval data) were performed to test for difference by liver fat content. †defined as liver fat content greater than five percent;

Table 2 Summary on predictors selected in at least one-third of the loops in the classification scheme for fatty liver disease.

Clinical Traits				**Metabolites**				**Combined**			
Variable	**Score**	**OR (95%-CI)**	**Selected**	**Variable**	**Score**	**OR (95%-CI)**	**Selected**	**Variable**	**Score**	**OR (95%-CI)**	**Selected**
ALT	0.87	2.79 (2.31;3.40)	30	**P::Glycine**	0.86	0.65 (0.55;0.76)	30	**HOMA-IR**	0.88	4.38 (3.48;5.60)	30
HOMA-IR	0.87	4.38 (3.48;5.60)	30	**U::X - 20643**	0.80	0.58 (0.49;0.68)	28	**U::X - 20643**	0.88	0.58 (0.49;0.68)	30
Waist circumference	0.84	4.58 (3.66;5.83)	29	**P::butyrylcarnitine**	0.75	2.27 (1.89;2.76)	26	**Waist circumference**	0.85	4.58 (3.66;5.83)	29
Age	0.67	2.04 (1.72;2.43)	23	**P::Tyrosine**	0.72	2.69 (2.20;3.32)	25	**ALT**	0.76	2.79 (2.31;3.40)	26
Total triglycerides	0.64	2.90 (2.39;3.57)	22	**U::X - 15472**	0.66	2.33 (1.95;2.81)	23	**Age**	0.73	2.04 (1.72;2.43)	25
hsCRP	0.61	1.75 (1.49;2.07)	21	**U::uracil**	0.66	0.60 (0.51;0.71)	23	**P::Glycine**	0.68	0.65 (0.55;0.76)	23
Alcohol intake	0.47	1.36 (1.17;1.58)	16	**U::X - 16774**	0.58	2.33 (1.94;2.82)	20	**U::X - 12407**	0.56	0.80 (0.68;0.93)	19
				P::lysoPC a C18:2	0.58	0.65 (0.55;0.76)	20	**U::uracil**	0.56	0.60 (0.51;0.71)	19
				P::PC ae C42:5	0.55	0.54 (0.45;0.63)	19	**hsCRP**	0.53	1.75 (1.49;2.07)	18
				P::glutamate	0.49	2.54 (2.10;3.10)	17	P::butyrylcarnitine	0.44	2.27 (1.89;2.76)	15
				P::γ-glutamylphenylalanine	0.46	2.62 (2.17;3.20)	16	P::PC aa C32:1	0.41	1.55 (1.32;1.82)	14
				P::PC aa C32:1	0.40	1.55 (1.32;1.82)	14	P::PC ae C42:5	0.35	0.54 (0.45;0.63)	12
				P::PC aa C40:6	0.40	1.59 (1.36;1.88)	14	U::X - 16581	0.30	0.71 (0.61;0.83)	10
				U::X - 16581	0.38	0.71 (0.61;0.83)	13	P::glutamate	0.29	2.54 (2.10;3.10)	10
				P::X - 01911	0.31	1.98 (1.67;2.37)	11				
				P::Valine	0.31	2.37	11				

						(1.97;2.88)					
				U::X - 12407	0.29	0.80 (0.68;0.93)	10				

OR (95%-CI) = crude odds ratio per standard deviation increase for hepatic steatosis with 95%-confidence interval; Score = defined as average area under the curve in the final classification loop in case the variable was included (see Methods); Selected = number of times the variable was selected for the final classifier (max = 30); Metabolites depicted in bold were used to build the final classifier. ALT = alanine aminotransferase; HOMA-IR = homeostatic model assessment of insulin resistance; hsCRP = high-sensitivity C-reactive protein;