Digestion

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Lipoprotein and Metabolic Profiles Indicate Similar Cardiovascular Risk of Liver Steatosis and NASH

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Keywords
Nonalcoholic fatty liver disease · Cholesterol · NMR analysis · Amino acids · Noninvasive test

Abstract
Background and Aim: Nonalcoholic fatty liver disease (NAFLD) affects about 25% of the global population, with no reliable noninvasive tests to diagnose nonalcoholic steatohepatitis (NASH) and to differentiate between NASH and nonalcoholic fatty liver (NAFL) (steatosis alone). It is unclear if NAFL and NASH differ in cardiovascular risk for patients. Here, we compared obese NAFLD patients with a healthy cohort to test whether cholesterol compounds could represent potential noninvasive markers and to estimate associated risks. ***Method:*** Serum samples of 46 patients with histologically confirmed NAFLD (17 NAFL, 29 NASH) who underwent bariatric surgery were compared to 32 (9 males, 21 females) healthy controls (HCs). We analyzed epidemiological data, liver enzymes, cholesterol and lipid profile, and amino acids. The latter were analyzed by nuclear magnetic resonance spectroscopy. ***Results:*** Total serum and high-density lipoprotein (HDL) cholesterol were significantly lower in the NAFLD group than in HCs, with a stronger reduction in NASH. Similar observations were made for sub-specification of HDL-p, HDL-s, SHDL-p, and LHDL-p cholesterols. Low-density lipoprotein (LDL)-s and LLDL-p cholesterol were significantly reduced in NAFLD groups. Interestingly, SLDL-p cholesterol was significantly higher in the NAFL group with a stronger elevation in NASH than in HCs. The amino acids alanine, leucin, and isoleucine were significantly higher in the NAFL and NASH groups than in HCs. ***Conclusion:*** We show in this study that cholesterol profiles, apolipoproteins, and amino acids could function as a potential noninvasive test to screen for NAFLD or even NASH in larger populations. However, few differences in cholesterol profiles were identified between the NAFL and NASH groups, indicating similar cardiovascular risk profiles.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) including nonalcoholic steatohepatitis (NASH) represents a widespread and continuously increasing liver disease entity and is a rising burden on societies and health-care systems [1, 2]. With a suspected quarter of the world population being affected by NAFLD by now [3], it is of vital importance to find affordable and noninvasive markers that will help identify patients suffering from NAFLD or NASH [4].

Up to date, the only way to diagnose nonalcoholic fatty liver (NAFL, without inflammation) or NASH with certainty is a liver biopsy. There are 2 scoring systems to histologically diagnose patients with either NAFL or NASH: one is the NAFLD activity score (NAS) [5] and the other is the steatosis, activity, and fibrosis score [6]. Since liver biopsy is an invasive method with potential risks, it cannot be applied to a large population. Therefore, efforts are ongoing to find noninvasive testing methods to distinguish NAFL from NASH [7–9].

By now, several noninvasive algorithms have been suggested, trying to diagnose patients with either NAFL or NASH, such as the APRI Score (ratio of aspartate aminotransferase (AST)/thrombocytes) [10], BARD score (AST/alanine aminotransferase (ALT) ratio, BMI, and diabetes) [11], or the NAFLD fibrosis score (age, BMI, insulin resistance/diabetes, thrombocytes amount, and albumin) [12]. Most of these scores achieve only a moderate area under the curve, which hampers an effective differential diagnosis between NAFL and NASH. Recently, we introduced a new score based on age, γGT, M30, adiponectin, and HbA1c to address this unsatisfactory performance [13]. Unlike the other scores, it is performing better in distinguishing NAFL from NASH independent of fibrosis stage, but it still has limitations. Further improvement in noninvasive diagnosis in NAFLD is required, that is, by identification of novel serum markers.

One of the major challenges in NAFLD is mortality from cardiovascular complications such as heart disease or stroke, rather than from the actual liver disease [14, 15]. Multiple studies have shown that liver enzymes are not representative of the severity of neither the liver disease nor the actual health burden [16–18]. Regarding liver-related outcomes, NASH indeed represents a more severe condition with a higher risk of liver disease progression and mortality from liver disease. However, it is open to debate if cardiovascular risk is different between NAFL and NASH, with many studies indicating only incrementally higher risk or mortality for NASH [19].

Lipoproteins are supramolecular lipid transport particles that are commonly used to evaluate cardiovascular and metabolic diseases. The general cholesterol test, however, is not a very precise method [20]. Therefore, a more specific measurement using nuclear magnetic resonance (NMR) spectroscopy has been developed [21, 22]. The standard classification is according to their decreasing density and hence increasing size into high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very LDL particles. NMR spectroscopy allows for subdivision of these categories into smaller groups according to the particle size [20].

In the present study, we aimed to compare obese patients with histologically confirmed NAFL or NASH to healthy controls (HCs) regarding demographic data, serum biomarkers, and detailed cholesterol profile gathered by NMR spectroscopy. In detail, we analyzed whether there are distinctly different characteristics that (1) would help identify people suffering from NAFLD and (2) either differ between NAFL and NASH patients based on non-nvasive markers or help assess cardiovascular risk of NAFL and NASH.

Methods

Patients

The patient cohort consisted of 46 patients (17 NAFL, 29 NASH) undergoing bariatric surgery at the Alfried Krupp Hospital in Essen, Germany, between 2010 and 2017. Patients were grouped into NAFL and NASH according to histological assessment by NAS. Serum samples, liver biopsies (intraoperative wedge biopsies), and epidemiological data (height, weight, and BMI) were collected on the date of surgery. Standard laboratory parameters and liver enzymes were measured at the same hospital. Medication at the time of surgery was collected from medical records.

Healthy Controls

Serum samples and epidemiological data (height, weight, BMI, and medication) were collected from 32 healthy volunteers at the University Hospital in Magdeburg, Germany, between October and December 2018. Liver biopsies were not performed in these participants. Their medication record was documented during anamnesis via interview. All procedures adhered to the Declaration of Helsinki and the requirements of the IRB.

Laboratory Parameters

Serum enzyme concentrations of AST, ALT, alkaline phosphatase, bilirubin, creatinine, glutamate dehydrogenase, and gamma-glutamyl transferase were determined on a cobas® 8000 modular analyzer series c701 (Roche Diagnostics, Mannheim, Germany), in the Institute of Laboratory Medicine, Otto-von-Guericke University Magdeburg. INR was determined on a STA R Max3 coagulometer (Stago Deutschland GmbH) in the same institute.

Table 1. Blood count and clinical chemistry of HCs and NAFLD subgroups

Parameter	HCs	NAFL	NASH
AST, U/L	25.4±12.6	22.9±9.4	41.7±39.1**, ##
ALT, U/L	19.7±9.7	25.8±21.9	37.1±29.3***, #
GGT, U/L	16.5±6.8	26.7±20.0	48.1±47.3***
Total bilirubin, mg/dL	0.47±0.23	0.59±0.42	0.54±0.23
Quick, %[1]	97 (90.5, 101)	97 (93.5, 100)	100 (91.5, 100)
INR	1.01±0.05	1.03±0.09	1.01±0.08
PTT, s	30.6±2.0	30.1±3.4	28.4±3.2*
Hb, g/dL	14.1±1.5	13.5±1.1	14.0±1.3
Hkt, %	42.2±3.8	29.3±19.7	33.1±19.0
Erythrocyte count, Gpt/L	4.8±0.5	4.8±0.5	4.9±0.4
Thrombocyte count, Gpt/L	276.7±64.5	306.1±65.3	311.2±74.8
Leukocyte count, Gpt/L	7.3±1.9	9.1±2.3*	51.1±194.8
Sodium, mmol/L	140.3±2.3	138.3±2.1§§	138.1±2.4§§
Potassium, mmol/L	4.2±0.44	4.2±0.25	4.3±0.31
Cr, mg/dL	0.86±0.15	0.79±0.26*	0.70±0.13**
Urea, mg/dL	9.5±2.8	12.9±13.1*	9.7±9.3***

HCs, healthy controls; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase. NAFLD, nonalcoholic fatty liver disease; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis. Data are given as mean and standard deviation. Significance versus HCs (Dunn's multiple test after Kruskal-Wallis): * <0.05; ** <0.01; ** <0.0001; versus NAFL: # <0.05; ## <0.01. Significance versus controls (Tukey's multiple comparison test after 1-way ANOVA): § <0.05; §§ <0.01. [1] For Quick, median and 25/75 percentiles are given.

Quantification of Cholesterol Profiles, Lipid Profiles, and Metabolites via NMR Spectroscopy

Lipid particle profiles, amino acids (alanine, valine, leucine, and isoleucine), lactate, and glucose concentrations in sera were determined at the INFAI GmbH for all the patients and HCs via NMR spectrometry using a Bruker Avance III 600 MHz NMR spectrometer. The resulting data were analyzed with AXINON® lipoFIT®-S100 (numares AG, Regensburg, Germany) software according to the manufacturer's protocol. In short, ^{1}H-magnetic resonance spectroscopy was coupled with specialized evaluation algorithms for metabolic profiles. Using the above-mentioned software, the factors listed in Table 1 were standardized, measured, and evaluated.

Statistical Analysis

Statistical analysis was performed using Prism, version 8 (GraphPad Software Inc., La Jolla, CA, USA). Unless otherwise stated, data are shown as mean and standard deviation. Continuous variables were tested for significance by 1-way ANOVA with Dunn's multiple comparison test to correct for multiple testing.

Results

Patients with NAFL and NASH Are Significantly Older and Have a Higher BMI than the HC Group

The 46 NAFLD patients (17 NAFL patients and 29 NASH patients according to NAS) had a median age of 40.7 (±10.9) years and a median BMI of 52.2 (±9.0) (Fig. 1). There was no significant difference between NAFL and NASH patients. Clinical data of all included participants are presented in Table 1.

To have a strong effect for a possible separation between NAFLD patients and HCs, a higher proportion of young and normal weight individuals were recruited into the HC group. This resulted in a significant difference in age and BMI between the NAFLD group and HCs. The median age of the HC group was 30.3 (±10.9; $p = 0.0003$ vs. NAFL; $p = 0.02$ vs. NASH) years. The HC group had a median BMI of 22.84 (±3.5; $p < 0.0001$) and consisted of 21 females and 9 males. In the NAFL group, there were 3 males and 14 females, and in the NASH group, 10 males and 15 females. No significant differences in demographic factors were found between the NAFL and NASH groups. Distribution of fibrosis scores in the NAFL and the NASH patients was not significantly different (see online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000510600).

Total Cholesterol and HDL Cholesterol Are Significantly Lower in NASH Patients than HCs

The mean total serum cholesterol concentration was lower in NAFL and NASH patients than in HCs. How-

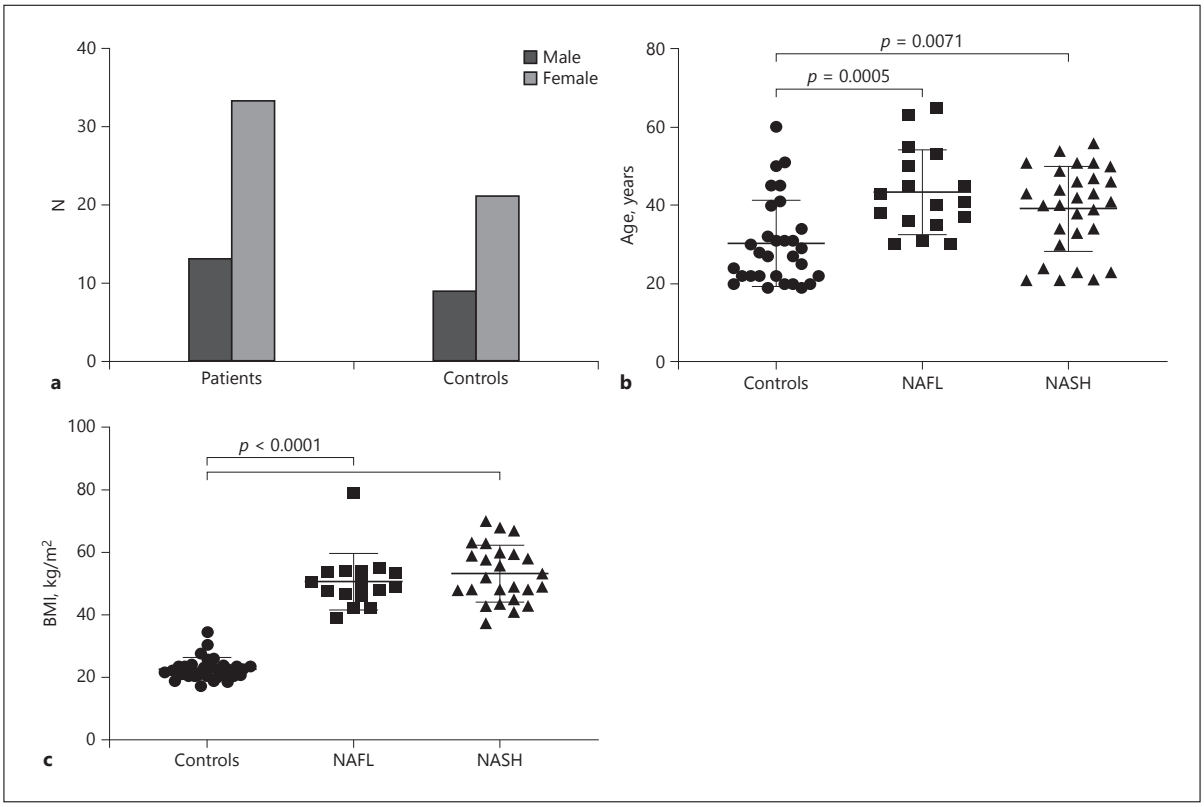

Fig. 1. Comparison of the study group based on gender, age, and BMI. The patient cohort is more female (**a**), significantly older (**b**), and has a significantly higher BMI (**c**) than the control group. Shown are scatter dot plots with mean and SD. Statistical significance was determined by 1-way ANOVA using GraphPad software, version 8. NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis.

ever, only the difference between HCs and NASH patients was significant ($p = 0.0206$; Fig. 2a). Serum concentrations of HDL cholesterol were significantly lower in NAFL and NASH patients than in HCs ($p < 0.0001$; Fig. 2c). No significant difference was observed for LDL cholesterol concentrations (Fig. 2b). The LDL/HDL ratio, which has been applied for cardiovascular risk assessment, was significantly higher in NAFL and NASH patients than in HCs but did not differ between NAFL and NASH patients (online Suppl. Fig. 1).

To exclude a potential effect of treatment with lipid-lowering drugs in obese NAFLD patients, which would cause an overall improved cholesterol profile, discharge letters were checked for medication. Medication at discharge for each individual patient is given in online suppl. Table 2. Only 1 patient was identified, who was discharged with a statin. However, there were some patients with medication for thyroid hormones and those under antidiabetic medication. There were 3/31 HCs, 2/17 NAFL patients, and 4/29 NASH patients treated for hypothyroidism in our cohort, with no significant difference in the proportion of patients taking this type of medication. In the NAFL and NASH groups, 2 patients each were on antidiabetic medication; however, this did not affect the available results on HbA1c (NAFL: $n = 11$, HbA1c 5.715%; NASH: $n = 22$, HbA1c 5.894%; p value = 0.7687), which were within normal range. For HCs, no HbA1c measurements were available.

Subclasses of HDL and LDL Show Significant Differences between NAFL and NASH Patients and HCs

The NMR-based cholesterol profile allows for the analysis of subclasses for HDL and LDL. The HDL-A-c subclass was found to be significantly reduced ($p < 0001$)

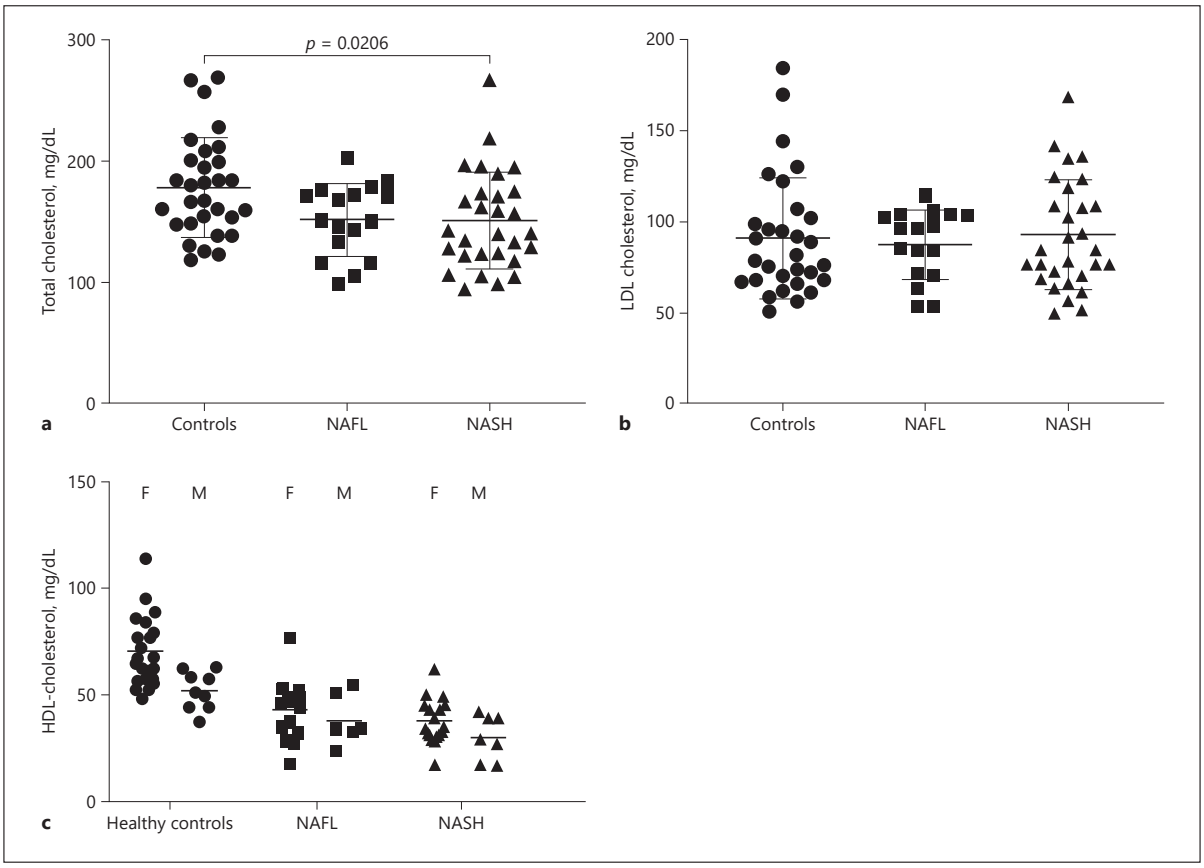

Fig. 2. Significant decrease in the cholesterol profile between HCs and NASH patients. There is a significant decrease in total cholesterol levels in the serum of NASH patients when compared with HCs (**a**). The decrease in total cholesterols is not impacted by a change in LDL cholesterol (**b**), but by a significant decrease in HDL cholesterol (**c**). For HDL, a sex-specific distribution is known. HDL concentrations of female HCs were significantly higher than those of all other groups ($p < 0.0001$), including male HCs ($p < 0.01$). Within male participants, HDL concentrations in NASH were significantly lower than those of HCs ($p = 0.02$). Shown are scattered dot plots with mean and SD. Statistical significance was determined by 1-way ANOVA using Prism software. HCs, healthy controls; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

for NASH patients compared to HCs. The serum levels for the HDL-C-c subclass in comparison to HCs were significantly lower for NAFL patients ($p = 0.009$) and NASH patients ($p < 0.001$). Even though serum levels of HDL-C-c in NASH were nominally lower than those of NAFL patients, significance was not reached.

Although overall LDL levels did not differ among HCs and patient groups, LDL subclasses exhibited significant differences between NASH patients and HCs. LDL-A-c was significantly lower in NASH patients than the control group ($p = 0.0478$; Fig. 3). In contrast, LDL-Bc levels were significantly higher in NASH patients than HCs ($p = 0.0348$; Fig. 3). Very low-density lipoprotein particles did not differ in size or particle concentration between HC and NAFLD groups (online Suppl. Fig. 2).

The Amino Acid Profile Is Significantly Altered in NASH Patients

In addition to the cholesterol profile, a selection of amino acids, namely, alanine, leucine, isoleucine, and valine, was detected by NMR in sera of HCs and NAFLD patients. Serum concentrations of alanine ($p = 0.01$;

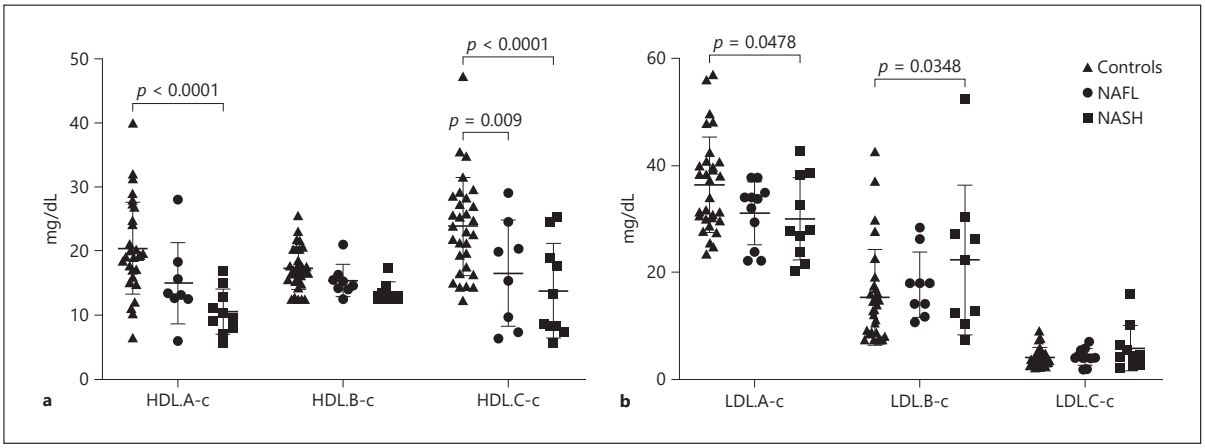

Fig. 3. Analysis of subclasses of HDL and LDL cholesterol. Significant difference between HCs and NASH patients in HDL-A-c, as well as HDL-C-c levels for HCs and NAFL, as well as NASH patients (**a**). LDL-A-c levels are significantly lower in NASH patients than in HCs, LDL-c levels are significantly elevated compared to that in the HCs (**b**). HDL and LDL-A represent large particles, and LDL-B and LDL-C medium and small particles, respectively. Shown are scattered dot plots with mean and SD. Statistical significance was determined by 2-way ANOVA using Prism software. HCs, healthy controls; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Fig. 4a), leucine ($p = 0.0415$; Fig. 4b), and isoleucine ($p = 0.0264$; Fig. 4c) were significantly higher in NASH patients than in HCs. Valine levels were not significantly different between the groups (not shown). Amino acid serum levels differed neither between HCs and NAFL patients, nor between NAFL and NASH patients.

Glucose and Lactate Levels Are Significantly Increased in NAFL and NASH Patients

As expected, glucose serum levels were significantly higher in NAFL patients (mean = 117.2 mg/dL; $p = 0.003$) and NASH patients (mean = 117.4 mg/dL; $p < 0.0001$) than in HCs (mean 82.13 mg/dL) (Fig. 5a). In addition, serum lactate concentrations were significantly higher in NAFL (mean = 32.76 mg/dL; $p = 0.0288$) and NASH patients (mean = 31.23 mg/dL; $p = 0.0372$), respectively, than in the HC group (mean = 20.19 mg/dL) (Fig. 5b). However, there was no difference between NAFL and NASH patients for glucose or lactate concentrations in serum.

Discussion

Noninvasive assessment and monitoring of NAFLD remains a complex and challenging task [23–25]. Markers to effectively and reliably discern steatosis from NASH are not available, making assessment of the liver risk in patients with obesity or components of the metabolic syndrome difficult.

In this study, we describe a cohort of obese NAFLD patients, who were grouped into NAFL and NASH according to histological assessment by NAS. A group of younger, mostly normal weight controls was used as a reference. The central findings were lower HDL cholesterol and HDL-C-c subclass concentrations in NAFLD than in HCs, lower LDL-A-c subclass and higher LDL-C-c subclass as well as higher concentrations of branched chain amino acids in NASH patients than in HCs, and higher glucose and lactate serum concentrations in NAFLD. None of the serum markers analyzed by NMR showed significant differences between NAFL and NASH patients.

The HC group and both the NAFL and the NASH group differed significantly in age and BMI. This was intended to maximize a possible effect size for detection of NAFLD as a whole and to avoid inclusion of "healthy" participants with undiagnosed NAFLD. In addition, NAFL patients were slightly older than NASH patients, suggesting a longer duration of the underlying obesity. If progression from NAFL to NASH was linear and based on duration of obesity/excess calorie consumption alone, the inverse situation would be expected. However, the BMI was slightly higher in NASH patients than NAFL

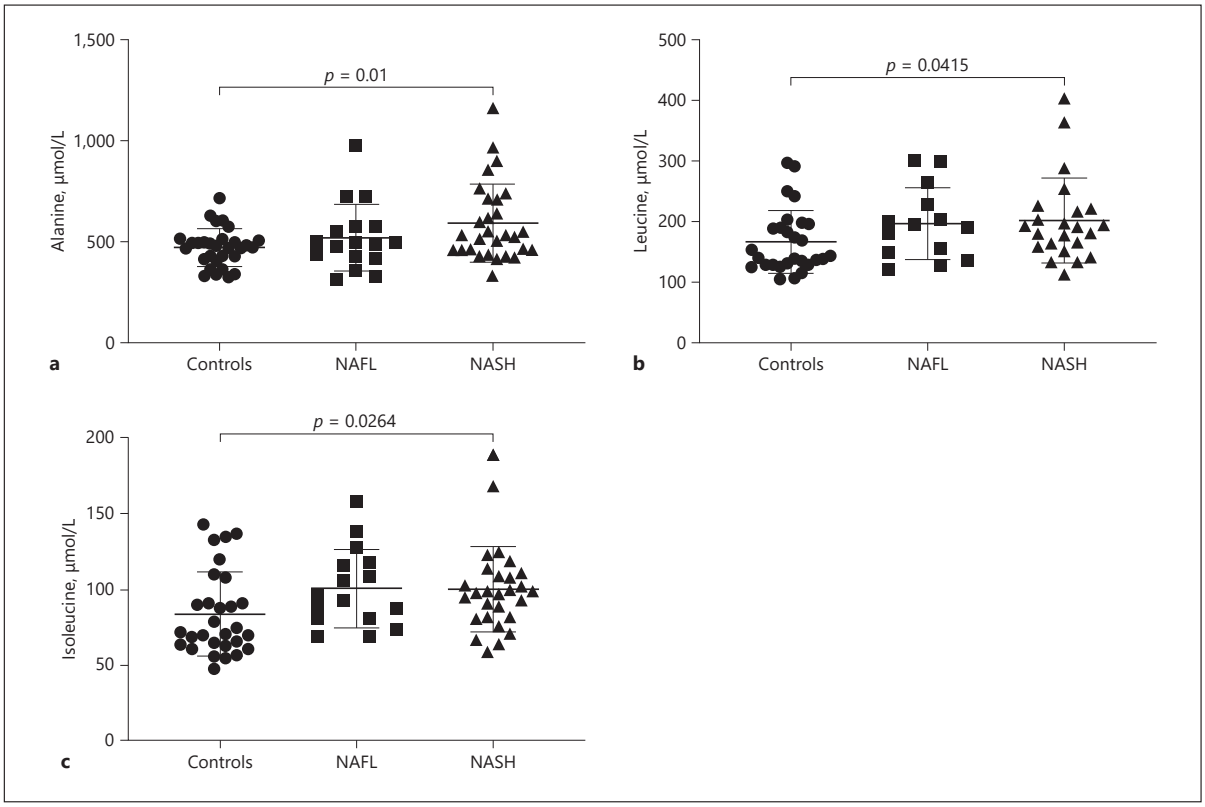

Fig. 4. Significant changes in amino acids between NASH patients and HCs. A steady increase from healthy people over NAFL to NASH patients can be seen. In NASH patients, significantly increased levels can be seen for alanine (**a**), leucine (**b**), and isoleucine (**c**) compared to that in HCs. Shown are scattered dot plots with mean and SD. Statistical significance was determined by 2-way ANOVA using Prism software. HCs, healthy controls; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis.

patients, which might be attributable to the higher proportion of male NASH patients. There was no further correlation between BMI and severity of the disease, as previously described in other studies [26, 27]. Several other publications, however, show a correlation between BMI and NAFLD/NASH or at least the hepatic fat content [8, 28–30]. Our own and previous results of other groups show that BMI is not a feasible, noninvasive marker to separate NAFL and NASH.

The focus of our initial analysis was to identify novel noninvasive biomarkers for the separation of NAFL and NASH by NMR subclass detection of cholesterol/lipoprotein particles. Generally, one would have expected an increase in total cholesterol levels, with a decrease in HDL levels with a consecutive increase in LDL cholesterol in NAFLD patients compared to HCs [31]. While we indeed observed decreased HDL and increased total cholesterol levels in NAFLD patients, LDL levels were not changed in NAFLD patients compared to HCs, which has been previously noted [32, 33]. These unchanged LDL serum concentrations are not associated with widespread statin use in our cohort (only 1 patient received statin treatment). Lipoprotein subclasses in serum exhibited an incremental increase or decrease from HCs over NAFL to NASH patients. This has previously been described in a different analysis [34] with similar overall results, including unchanged LDL serum concentrations, but with a lower resolution of lipoprotein distribution. The cholesterol content of HDL subclass HDL-C-c, which represents small HDL particles, was significantly lower in NAFL and NASH patients than in HCs. The largest subclasses HDL-A-c and LDL-A-c were significantly lower and LDL-B-c

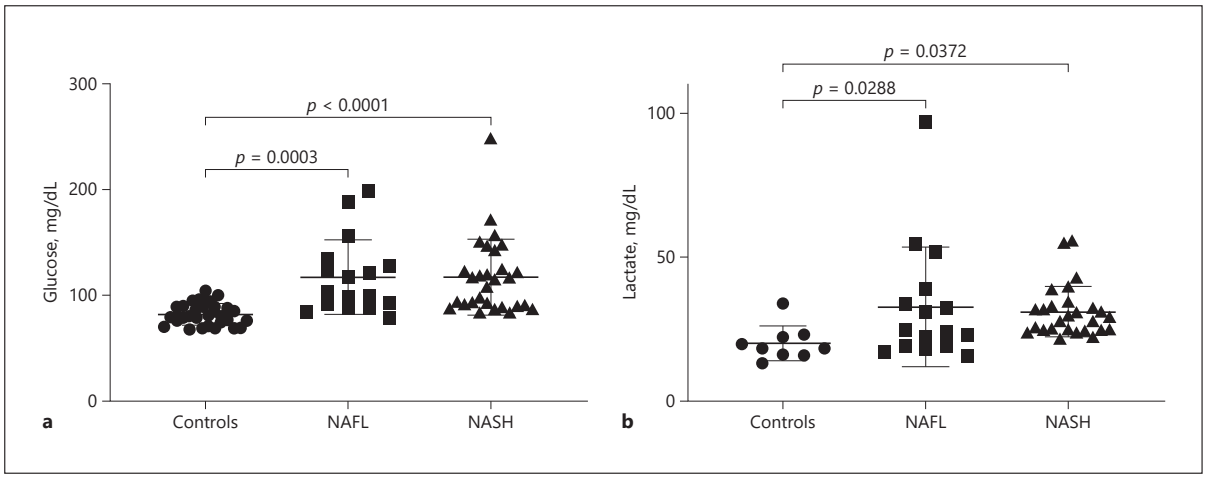

Fig. 5. Significant increase in glucose and lactate levels between NAFL/NASH patients and HCs. A steady increase from healthy people over NAFL to NASH patients can be seen. NAFL and NASH patients show significanly increased levels for glucose (**a**) and lactate (**b**) in our analyzed cohort. Shown are scattered dot plots with mean and SD. Statistical significance was determined by 2-way ANOVA using Prism software. HCs, healthy controls; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis.

higher only in NASH patients than in HCs. The findings on HDL and LDL subclasses support two assumptions: one is that changes in lipoprotein subclasses might be useful to detect NAFLD independent of histological severity in populations, and the second is that lipoprotein and cholesterol profiles do not differ between NAFL and NASH patients in an extent useful for noninvasive separation of these groups. There are several possible explanations why LDL-A (larger particles) are not increased in NAFLD patients.

Hepatic lipase activity is increased in NAFLD patients [35] and hydrolyzes triglycerides and phospholipids of large LDL particles, leading to smaller particles that may correspond to the LDL-B and partly also to the LDL-C fractions that are inversely correlated to the distribution of the LDL-A fraction among HCs and NAFL/NASH patients. The diminished number of large particles is concomitantly associated with lower cholesterol concentrations in the respective particle fractions.

Further, either the LDL degradation is increased for larger particles (as by Kupffer cells or [to a lesser degree] hepatocytes) [36], or the medium and small-sized particles are not as affected by the degradation. Small LDL particles have a decreased affinity for the LDL receptor, resulting in a prolonged circulation time in the blood. Because of their smaller size, they get trapped more easily in arterial walls, preventing them from degradation [37]. Additionally, smaller LDL particles are more pro-atherogenic than large LDL particles [38], which would support our findings that cardiovascular risk profiles are comparable because we are not able to see a significant difference between NAFL and NASH groups in either lipoprotein subclassification.

Both large and small LDL particles have been reported to contribute by different pathomechanisms to patients' overall cardiovascular risk profile. There is growing evidence that both the absolute number of LDL particles and the ratio of LDL/HDL particles determined by NMR can independently improve CVD risk assessment [39]. In our study, the higher cardiovascular risk of patients with NAFL and NASH was particularly due to the less favorable ratio between the number of LDL and HDL particles (online Suppl. Fig. 1). However, the very low effects observed in this study might be due to relatively small group sizes.

Cardiovascular events are the major cause of death in NAFLD patients, which has been described multiple times in past years [40, 41]. Serum HDL concentrations are commonly applied to assess cardiovascular risk [42]. In the present study, the serum HDL and the HDL-C-c concentrations in NAFL and NASH patients were significantly lower than in HCs. Despite nominally lower concentrations of both factors in NASH than NAFL patients, no significant difference was observed. This would imply

a similar cardiovascular risk independent of the severity of NAFLD. While some studies showed increased cardiovascular risk for NASH compared to NAFL [14], other groups found similar cardiovascular risk in NAFL and NASH patients [43]. It is undisputed that NASH confers a higher risk for liver-related disease progression and morbidity as well as a higher risk for diabetes. However, our data, as well as previous studies of other groups [44, 45], suggest that separation of NAFL and NASH regarding the cardiovascular risk may not be of clinical relevance. Unless this issue has been cleared in sufficiently powered prospective studies, our recommendation would be to more closely monitor all NAFLD patients for cardiovascular risk and associated comorbidities.

In addition to the above-discussed detailed lipoprotein and cholesterol profiles, NMR-based serum analyses allowed us to detect select (branched chain) amino acids. In accordance with our results, a recent study by Goffredo [46] described elevated levels for valine, leucine, and isoleucine in obese adolescents. Higher serum concentrations of these amino acids negatively correlated with insulin sensitivity in this group. In this study, higher baseline levels of valine were predictive of a progression of the hepatic fat content. Similar findings were made by van den Berg et al. [47], showing a positive correlation between the amount of branched-chain amino acids, type 2 diabetes, and NAFLD development. This is in line with our findings of significantly increased branched-chain amino acids in NASH patients, indicating a stronger insulin resistance than in HCs. Branched-chain amino acid concentrations of NAFL patients were between those found in HCs and NASH patients, without reaching significance. This could be associated to greater variability in insulin resistance within this group of patients [48]. Patients with liver disease usually show wide modification of essential amino acids and common branched-chain amino acids (such as valine, leucine, and isoleucine) [49]. Isoleucine, leucine, and valine might mediate or regulate the activation of multiple important hepatic metabolic signaling pathways ranging from glucose regulation to insulin signaling [50]. As branched-chain amino acid concentrations varied between NAFL and NASH patients, larger studies might be able to identify an effect size, which would allow for separation and thus noninvasive diagnosis of either NAFL or NASH.

Another interesting finding in our cohort was the elevated level of serum glucose and lactate with stepwise increases from HCs over NAFL to NASH. This also supports previous data in mice fed a high fat diet, showing elevated levels of alanine, glucose, lactate, and pyruvate. This has been previously confirmed in NASH patients [51], when using metabolomics data. Unfortunately, in that data set, it was not possible to make a distinction between steatohepatitis and hepatic steatosis. While again neither lactate nor glucose allows for a significant distinction between NAFL and NASH, both factors are significantly altered compared to that in HCs. This demonstrates, on the one hand, that NAFLD as a whole could be identified noninvasively and, on the other hand, that factors associated to carbohydrate and lipid metabolism do not differ between NAFL and NASH patients, indicating a similar risk profile for metabolic changes within the liver.

The present study has some limitations. The overall cohort size is relatively small with only 46 patients and 30 HCs in comparison. Since detailed medication was retrospectively taken from clinical records, we cannot exclude with certainty what kind of treatments obese patients may have received prior to their bariatric surgery. Additionally, there is understandably no histological data available for the HC group; thus, the presence of NAFL/NASH cannot be ruled out with absolute certainty. However, the control group was deliberately chosen with younger age and lower mean BMI to allow for a strong separation between "healthy" and obese NAFLD patients.

In summary, this study demonstrates that patients with NAFLD, including NAFL and NASH, exhibit changes in serum markers of lipid, amino acid, and carbohydrate metabolism. In particular, HDL and possibly LDL profiles demonstrate a clear cardiovascular risk in NAFLD patients, which hardly differs between NAFL and NASH. Serum amino acid concentrations suggest a stronger insulin resistance in NASH than in NAFL patients and might allow separation of these groups. Larger prospective studies will have to demonstrate feasibility of this approach.

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Statement of Ethics

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the IRB (Ethik-Kommission der Medizinischen Fakultät der Universität Duisburg-Essen; Germany; 15-6356-BO). All procedures adhered to the Declaration of Helsinki and the requirements of the IRB. Enrolled patients gave written informed consent to the study.

Conflict of Interest Statement

The authors declare no conflict of interest.

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Author Contributions

Study concept and design: A.C. Acquisition of data: A.S.L., A.G., E.Y., S.A., and A.L. Analysis and interpretation of data: A.G. and J.P.S. Drafting of the manuscript: A.G. Critical revision of the manuscript for important intellectual content: J.P.S., A.G., A.C. and M.P.Ö. Statistical analysis: A.G., J.P.S. and M.P.Ö. Administrative, technical, or material support: S.A., E.Y., A.C., G.G., and K.B. Study supervision: A.C. and M.P.Ö.

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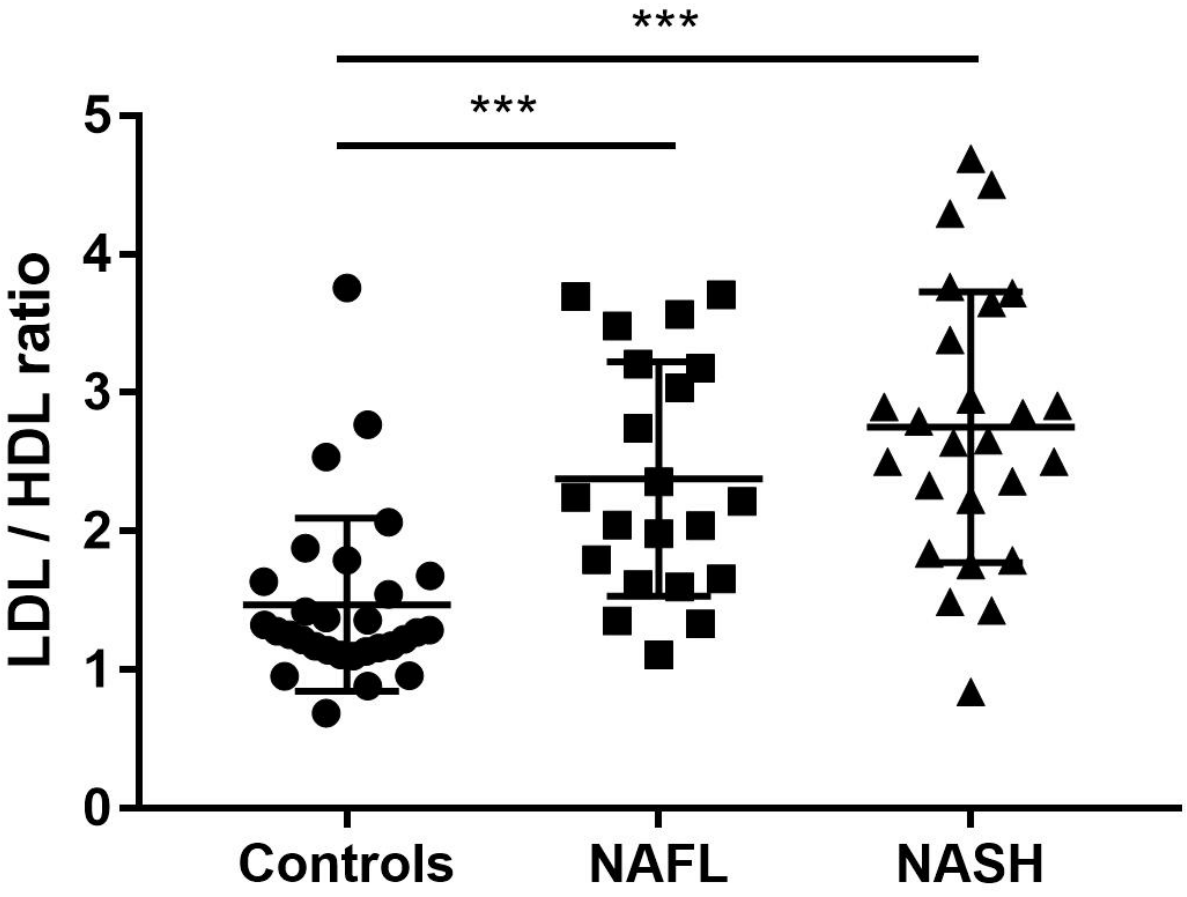
Supplementary Table 1: Distribution of fibrosis stages among NAFL and NASH patients.

Fibrosis stage	**NAFL patients**	**NASH patients**
1	6	4
2	12	16
3	2	4
4	1	0
Mild Fibrosis (1-2)	18	20
Advanced Fibrosis (3-4)	3	4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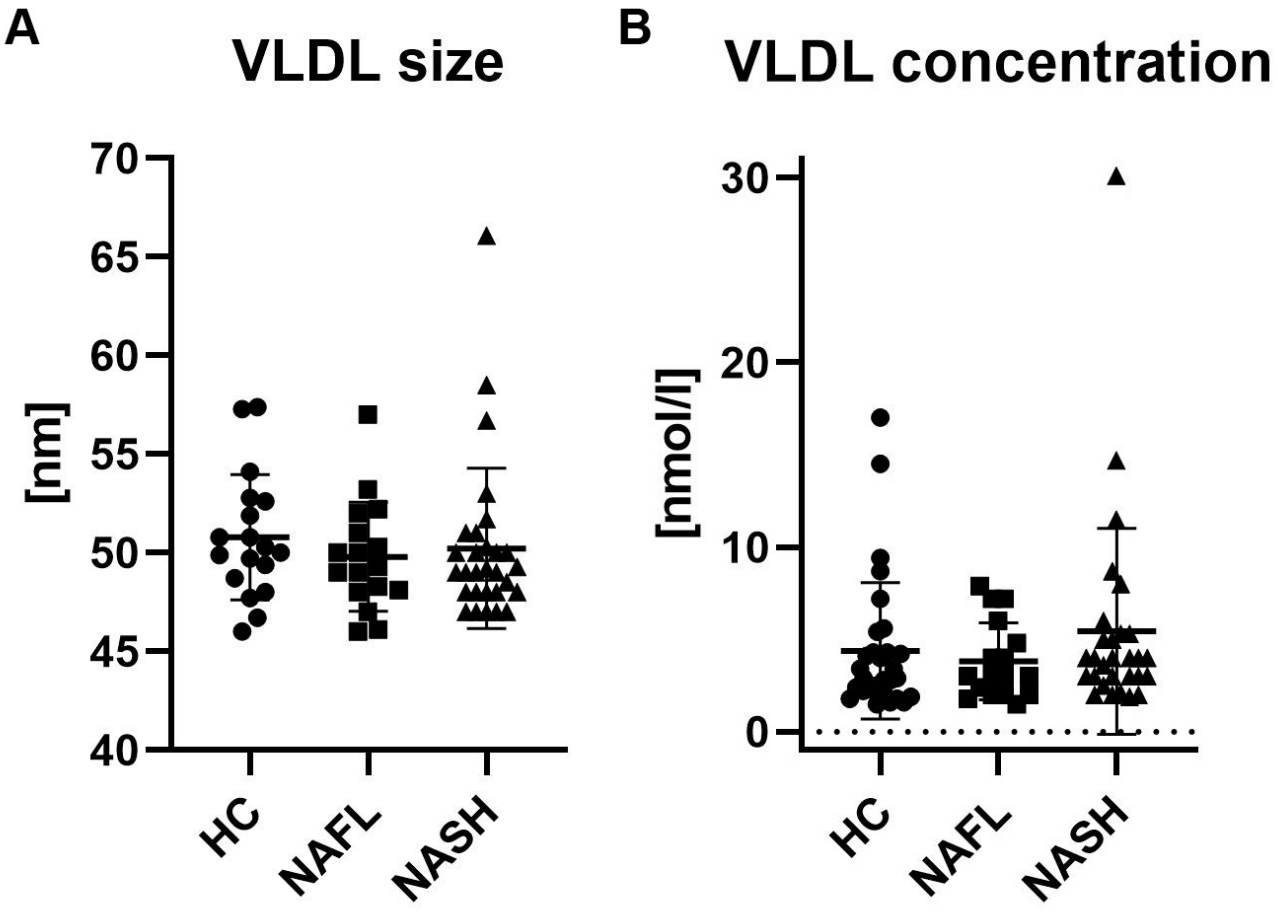
***
LDL / HDL ratio
5
4
3
2
1
0
Controls
NAFL
NASH

Supplementary Table S2: Medication of recruited patients at the time of bariatric surgery.

Internal patient no.	NASH	Medication
1	No	Omeprazole
2	No	L-Thyroxinee
3	No	no medication
4	No	no medication
5	No	Fluoxetine, acetylsalicylic acid, Pantoprazolee
6	No	no medication
7	No	no medication
8	No	Oxycodone ret., Metamizole, Carbamazepine, Nortriptyline, Pantoprazole, L-Thyroxine, Cryproterone, Rituximab
9	No	no medication
10	No	no medication
11	No	no medication
12	No	Metoprolol, Pantoprazole
13	No	no medication
14	No	Pantoprazole
15	No	Fluoxetine, Trimipramine
16	No	Pantoprazolee
17	No	no medication
18	No	no medication
19	No	unknown
20	No	no medication
21	No	Bisoprolol, Febuxostat, Candesartan, Rivaroxaban, Insulin, Liraglutide, Simvastatin, Omeprazole
22	Yes	no medication
23	Yes	Venlafaxine, Torasemide, Thyroxine, Pantoprazole
24	Yes	no medication
25	Yes	Esomeprazole
26	Yes	Pantoprazole
27	Yes	Metformin, Insulin, Ramipril
28	Yes	no medication
29	Yes	no medication
30	Yes	Torasemide, L-Thyroxine, Pantoprazole, oral contraceptives
31	Yes	no medication
32	Yes	no medication
33	Yes	no medication
34	Yes	no medication
35	Yes	no medication
36	Yes	L-Thyroxine, Metoprolol, Pantoprazole, Alendronic acid
37	Yes	no medication
38	Yes	no medication
39	Yes	L-Thyroxine, Paroxetine, Esomeprazole
40	Yes	no medication
41	Yes	no medication
42	Yes	no medication
43	Yes	Torasemide, Omeprazole, Metoprolol, Ramipril, Formoterol, Glimepiride, Salbutamol
44	Yes	Pantoprazole
45	Yes	no medication
46	Yes	Pantoprazole, Bisoprolol


A
VLDL size
[nm]
70
65
60
55
50
45
40
HC
NAFL
NASH
B
VLDL concentration
[nmol/l]
30
20
10
0
HC
NAFL
NASH

Supplementary Figure S1: NAFLD patients exhibit an increased LDL/HDL ratio compared to healthy controls. The LDL/HDL ratio, which has been applied for cardiovascular risk assessment was significantly higher in NAFL and NASH than in HC but did not differ between NAFL and NASH. Statistical significance was tested by Mann Whitney U test (non-normal distributed data). ***: $p < 0.0001$.

Supplementary Figure S2: No significant changes in VLDL among HC and NAFLD groups. Neither VLDL particle size (A) nor VLDL particle concentration (B) differs among the three groups. Shown are scattered dot plots with mean and SD. Statistical significance was determined by one-way anova using Prism Software. HC: healthy control, NAFL: non-alcoholic fatty liver, NASH: non-alcoholic steatohepatitis.