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Non-alcoholic fatty liver disease is associated with lower hepatic and erythrocyte ratios of phosphatidylcholine to phosphatidylethanolamine

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is associated with altered hepatic lipid composition. Animal studies suggest that the hepatic ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE) contributes to steatogenesis and inflammation. This ratio may be influenced by dysregulation of PE N-methyltransferase (PEMT) pathway or low choline diet. Alterations in the liver may also influence lipid composition in circulation such as in erythrocytes, which therefore may have utility as a biomarker of hepatic disease. Currently, no study has assessed both liver and erythrocyte PC/PE ratios in NAFLD. Aim was to compare PC/PE ratio in liver and erythrocytes of patients with simple steatosis (SS) or steatohepatitis (NASH) to healthy controls. PC and PE were measured by mass spectrometry in 28 patients with biopsy proven NAFLD (14 SS, 14 NASH) and 9 healthy living liver donors as controls. The hepatic PC/PE ratio was lower in SS (median [range]) (1.23 [0.27-3.40]) and NASH (1.29 [0.77-3.22]) compared to controls (3.14 [2.20-3.73]); both $P < 0.001$), but it was not different between SS and NASH. PC was lower and PE higher in the liver of SS patients compared to controls, whereas in NASH only PE was higher. The PC/PE ratio in erythrocytes was also lower in SS and NASH compared to controls, due to lower PC in both patient groups. PE in erythrocytes was not different among the groups. In conclusion, NAFLD patients have lower PC/PE ratio in liver and erythrocytes than healthy controls, which may play a role in the pathogenesis. Underlying mechanisms require further investigation.

Keywords: non-alcoholic fatty liver disease, steatohepatitis, phosphatidylcholine, phosphatidylethanolamine, lipid composition, healthy controls, mass spectrometry

Introduction

Non-alcoholic fatty liver disease (NAFLD) includes benign simple hepatic steatosis (SS) and non-alcoholic steatohepatitis (NASH), which can progress to fibrosis and cirrhosis (Dowman et al. 2010). NAFLD is considered to be the hepatic manifestation of the metabolic syndrome (Musso et al. 2008) and is associated with profound changes in lipid metabolism (Musso et al. 2009, Tessari et al. 2009). Several cross-sectional studies have shown that the hepatic and plasma fatty acid composition is altered in patients with NAFLD compared to controls (Araya et al. 2004, Puri et al. 2007, Allard et al. 2008, Kotronen et al. 2009, Puri et al. 2009), with a reduction in long-chain polyunsaturated fatty acids (PUFA), and increased monounsaturated fatty acids being the most consistent findings across these studies. Detailed lipidomic analyses have recently also revealed altered distribution of the lipid classes in the liver (Puri et al. 2007, Kotronen et al. 2009). Higher amounts of diacylglycerols, triacylglycerols, and free cholesterol, as well as differences in phospholipid subfractions were found in subjects with NAFLD compared to controls (Puri et al. 2007, Kotronen et al. 2009). Lipids are important structural components of the cell membrane and participate in signal transduction, e.g. as substrates for the formation of inflammatory mediators (Eyster 2007). Therefore, the observed changes in lipid profiles might play a role in NAFLD pathogenesis.

The amount and balance of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the two major cell membrane phospholipids, may be of special importance. PC is produced in most mammalian cells from dietary choline, and to a lesser extent in the liver from PE via the PE N-methyltransferase (PEMT) pathway (Cui and Houweling 2002, Li et al. 2006). Dysregulation of these pathways may lead to perturbations of PC and PE homeostasis resulting in cell damage and cell death (Cui and Houweling 2002). PC biosynthesis is necessary for normal very low density lipoprotein secretion from hepatocytes (Zeisel 2006), hence, a reduction in PC, e.g. through choline deficient diets, might lead to hepatic steatosis (Zeisel 2006, Li and Vance 2008).

Mechanistic studies in animal models have yielded insight as to why PEMT function and the hepatic PC/PE ratio may be important in the development of NAFLD (Li et al. 2006). In PEMT knock-out ($Pemt^{-/-}$) mice, a choline deficient diet leads to hepatic steatosis, steatohepatitis and death from liver failure (Walkey et al. 1998, Li et al. 2006), with a concurrent reduction in the hepatic PC/PE ratio from ~1.8 to ~0.8 (Li et al. 2006). The authors suggest that the lower PC/PE

ratio leads to a disruption in membrane integrity resulting in hepatocyte damage and inflammation, similar to NASH, whereas an absolute reduction of both PC and PE but maintenance of a normal PC/PE ratio manifests in benign SS (Li et al. 2006). The authors also presented data from a small pilot study suggesting that hepatic PC/PE ratio might be reduced in patients with NASH compared to controls (Li et al. 2006), but the authors give no details about the patients and the nature of the control group, and no other human studies have subsequently extended these results. It is not well understood why some patients show only benign hepatic SS, whereas other develop progressive NASH (Dowman et al. 2010). Comparing PC/PE ratio between SS and NASH, could elucidate, whether disturbed phospholipid balance may be one of the factors distinguishing the two phenotypes. Erythrocyte lipid composition is commonly used to reflect fatty acid composition from the diet and hepatic metabolism (Rodríguez-Cruz et al. 2012), thus erythrocyte PC/PE ratio may have utility as a biomarker of perturbations in liver fatty acid and phospholipid metabolism. Overall, the aim of the current study was therefore to compare the PC/PE ratio in liver tissue and erythrocytes among patients with SS, NASH, and healthy controls.

Materials and methods

Patients

This was a cross-sectional study. Between July 2007 and February 2009, male and female ambulatory patients (age ≥ 18 y) referred to the liver clinic for elevated transaminases (aspartate transaminase (AST) and/or alanine transaminase (ALT) at least 1.5 times normal) and diagnosed with either SS or NASH on liver biopsy were recruited for the study as well as healthy subjects from the Living Donor Program for liver transplant recipients. *Exclusion criteria:* Alcohol consumption >20 g/d; liver disease of any etiology other than NAFLD (e.g. viral hepatitis, autoimmune hepatitis, Wilson's disease); anticipated need for liver transplantation within one year or complications of liver disease; any reasons contraindicating a liver biopsy; medications known to precipitate steatohepatitis, supplementation of antioxidant vitamins, ursodeoxycholic acid or any experimental drug in the 6 months prior to entry; pregnancy or lactation. The healthy living donors were those meeting the criteria from the transplant protocol for the Living Donor Program and the same exclusion criteria as for NAFLD. Donors were recruited at one of their

clinic visits shortly before the planned transplantation. For liver biopsy, patients with suspected NAFLD had one part of the liver sample taken for routine pathological assessment and another part for study purposes. For healthy controls, a wedge biopsy was obtained at the time of the donor's hepatectomy, to be used for the same parameters. Fasting blood work, anthropometric measurements and food-protocols were obtained either at the follow-up visit (patients) or at enrolment (healthy controls). The study was performed according to the guidelines of the 1975 Declaration of Helsinki, was approved by the Research Ethics Board, University Health Network, Toronto, Ontario, Canada. All subjects gave their informed written consent.

Sample Collection and Preparation

Liver samples for histologic evaluation were stored in 10% formalin; a second portion of the liver tissue for lipid analysis was immediately placed on dry ice and stored at -80°C. Tissue samples were later thawed, weighed, and homogenized for 1 min in ice-cold 20 mmol/l phosphate buffered saline, pH 7.3, with 5mmol/l butylated hydroxytoluene to prevent *ex-vivo* lipid peroxidation. The suspension was aliquoted and stored at -80°C until analysis. Blood for measurement of PC/PE ratio in erythrocytes was collected after overnight fast in ethylenediaminetetraacetic acid containing tubes and centrifuged (910 × g, 10 min). Plasma and buffy coat were removed and packed erythrocytes were aliquoted and frozen at -80°C. Plasma and serum samples for standard blood biochemistry tests were collected and analyzed by the Laboratory Medicine Program at the University Health Network.

Liver Histology

A single pathologist (MG) reviewed biopsy specimens with two hematoxylin-eosin–stained sections for necroinflammatory grading and two Masson trichrome–stained sections for assessment of fibrosis.

The Brunt's system was used to assess the degree of steatosis, inflammation and fibrosis in NAFLD (Brunt et al. 1999). Presence of steatosis was defined as having at least 5% of hepatocytes containing large-droplet fat. To distinguish between cases with simple steatosis and steatohepatitis, a separate category for zone 3 fibrosis characteristic of steatohepatitis as well as hepatocellular ballooning were evaluated. Inflammation and NAFLD activity score were also assessed (Kleiner et al. 2005).

Ratio of Phosphatidylcholine to Phosphatidylethanolamine

Lipids from liver tissue and erythrocytes were extracted according to Folch method (Folch et al. 1957). For the extraction, 150 μ l liver homogenate (containing 0.15 – 2.49 mg of liver tissue) or 250 μ l erythrocytes were mixed with 4 ml of chloroform: methanol (2:1) (v/v) with 0.02% butylated hydroxytoluene as antioxidant. Samples were mixed vigorously for 10 sec (vortex) and kept overnight on a shaker. After addition of 1 mL saline (0.85% NaCl), samples were left on the shaker for 1 h and then centrifuged (132 x g, 10 min). The bottom organic phase was carefully aspirated with a pasteur pipette, transferred into a pre-weighed tube and dried under a gentle stream of nitrogen gas. The dried lipid was weighed and reconstituted in chloroform. Lipids were diluted with chloroform to 0.08 mg/mL final concentration and diheptadecanoyl PC and PE were added as internal standards at 0.15 μ mol/L final concentration. Samples were further diluted 1:1 with chloroform:methanol (1:2, v/v) with 5 mM ammonium acetate and analyzed by nanoelectrospray infusion tandem mass spectrometry using the Triversa Nanomate System (Advion Biosciences, Ithica, NY, USA) interfaced onto a QTRAP 5500 QqQ hybrid Mass Spectrometer (AB SCIEX, Concord, ON, Canada) (Ekroos et al. 2003, Ejlsing et al. 2006). Multiplex precursor ion and neutral loss scanning was carried out in both positive and negative polarities. For the glycerophospholipid fragments and scanning parameters listed in the supplementary Material (Supplementary Tables S1 and S2) online. This technique is especially suited for the *shotgun* analysis of complex lipid extracts from the combination of up to 60 experiments carried out in parallel in a single acquisition method using both polarities for full glycerophosphatidyl choline and glycerophosphatidyl ethanolamine characterization and quantitation using the lipid class-specific internal standards (Stahlman et al. 2009). For data analysis, LipidView™ Software 1.0 (AB SCIEX) was used for lipid fragment library searching for the identification and quantification of the detected PC and PE lipids (Ejlsing et al. 2006). All datasets were subjected to spectral peak extractions using MS tolerance of 0.5 Da with a minimum signal/noise ratio over 10, isotope corrected, and then searched against the lipid fragments database for confirmed lipid species identities. The identified PC and PE lipids were then normalized by the appropriate internal standard and reported as a corrected ratio to quantify across all samples. For more details on the methods see the Supplementary Material online.

Blood Biochemistry

Plasma ALT, AST, alkaline phosphatase and total bilirubin as well as serum triglycerides, total and high density lipoprotein cholesterol were measured using the Architect c8000 system (Abbot Laboratories, Abbot Park, IL, USA). Low density lipoprotein cholesterol was calculated as total – high density lipoprotein cholesterol. Fasting plasma glucose was measured by the enzymatic hexokinase method on an Architect c8000 System (Abbot Laboratories). Serum insulin was determined by radioimmunoassay (Immulite 2500, Siemens Diagnostics, Los Angeles, CA, USA). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated (Matthews et al. 1985).

Nutritional Assessment

Body mass index (BMI) was calculated as weight/height^2 [kg/m^2] and waist circumference was measured to assess central obesity (men >102 cm; women >88 cm) (2001). Each participant completed one 7-day food record using the 2D Food Portion Visual chart (Nutrition Consulting Enterprises, Framingham, MA, USA) to estimate portion sizes (Millen and Morgan 1996). Protocols were analyzed using Diet Analysis Plus Version 7.0.1 (Thomson Wadsworth, Stamford, CT, USA).

Clinical Data

Medical history, including presence of diabetes, dyslipidemia, and cardiovascular disease were assessed. Use of medication, dietary supplements and smoking habits were documented. These data were asked from the patients and confirmed from the clinical charts.

Statistical Analysis

The three groups were compared using ANOVA with Scheffe's post-hoc test (continuous, normally distributed variables) or Kruskal-Wallis followed by Mann-Whitney test (continuous, not normally distributed variables). Two group comparisons were done by t-test or Mann-Whitney U test as appropriate. Chi-square tests and z-tests were performed for categorical variables and Spearman correlations were calculated using IBM SPSS V20.0 (IBM, Somers, NY, USA). Values reported are mean $\pm$ SEM (normal distributed), median (range) (skewed distribution) or % of valid cases (categorical variables) unless stated otherwise. $P < 0.05$ was

considered significant.

Results

Thirty-five patients and 12 healthy donors consented. In the NAFLD group, four patients dropped out before biopsy, one was excluded, as NAFLD was not confirmed on histology, and two did not have enough liver tissue available for analysis, resulting in 28 patients included in the analysis. Nineteen of these patients provided a blood sample for erythrocyte collection. Liver tissue and erythrocytes were available for nine healthy controls.

Patient Characteristics

In the NAFLD group, 14 subjects were diagnosed with SS, and 14 had NASH (Table 1). The amount of steatosis (% of hepatocytes), fibrosis stage, inflammation, ballooning score and NAFLD activity score but not steatosis grading, were higher in NASH than in SS. Age and gender distribution were not significantly different among the three groups. Patients with NASH had higher BMI and waist circumference, and more central obesity compared to SS and controls (Table 2). Waist circumference was also higher in SS than in controls. Some patients with SS and NASH but none of the controls had hypertension and were taking medication related to cardiovascular disease ($P < 0.05$) (Table 2). Other medication use and frequency of diabetes or dyslipidemia were not different. Plasma transaminases were higher in SS and NASH compared to controls, and AST was also higher in NASH than in SS (Table 3). The NASH group had higher fasting insulin compared to both other groups and higher HOMA-IR than controls. Triacylglycerols were higher in both patient groups than in controls. Dietary intake (data not shown), including total fat, cholesterol, polyunsaturated and saturated fatty acids and folate, was not different between groups. Choline intake could not be assessed from our food analysis program. Physical activity was also not different among the three groups (data not shown).

PC/PE Ratio, PC and PE in Liver and Erythrocytes

The PC/PE ratio in liver (Figure 1a) and erythrocytes (Figure 1b) was significantly lower in both patient groups compared to controls, but there was no difference between SS and NASH. The total amount of PC in the liver was lower in SS than in controls (Figure 2a), whereas the

difference between NASH and controls was not significant ($P=0.072$). PE was higher in both SS and in NASH compared to healthy subjects (Figure 2a). In erythrocytes, the amount of PC was significantly lower in SS and NASH than in controls, but there was no difference for PE among the three groups (Figure 2b). We then determined, whether there was any association between PC/PE ratio in liver and erythrocytes. In patients and controls combined, PC/PE ratios in liver and erythrocytes were not significantly correlated (Spearman's $r=0.276$; $P=0.203$) (Figure 3). However, we observed that healthy controls were characterized by high PC/PE ratios both in liver and in erythrocytes, whereas the NALFD patients had a lower PC/PE ratio in at least one of these tissues. The plot shows no distinction between SS and NASH (Figure 3).

Discussion

Our results provide evidence that patients with NAFLD, both NASH and SS, have reduced PC/PE ratio in liver and erythrocytes compared to healthy controls. The lower hepatic PC/PE ratio corresponds to previous observations in mice and human pilot data (Li et al. 2006). This is the first study confirming altered hepatic PC/PE ratio in humans including a true healthy control group and two well defined groups of patients with SS and NASH, respectively. Findings from the present study are also consistent with results reported by Puri et al. (2007) observing lower amounts of PC and PE in the liver of patients with SS and a non-significant reduction of PC in NASH compared to controls, but PC/PE ratio was not calculated. Furthermore, controls underwent non-related abdominal surgery and might therefore not represent truly healthy individuals.

Several factors linked to phospholipid metabolism and diet may affect PC, PE and PC/PE ratio. PC homeostasis depends on dietary choline and production of PC from PE in the liver and both pathways are regulated through a complex network intersecting with methionine and folate metabolism (Zeisel and da Costa 2009). Therefore the lower PC/PE ratio in liver and erythrocytes in patients with NAFLD may have several causes. First, a suboptimal choline intake may play a role. In humans, choline is an essential nutrient (Zeisel and da Costa 2009), and deficiency can lead to reversible hepatic steatosis (Buchman et al. 2001, Sha et al. 2010) and apoptosis of hepatocytes (Sha et al. 2010). Even though choline is present in many foods, it is possible that a large proportion of the population does not meet their dietary requirements (Zeisel

and da Costa 2009). A lack of other nutrients involved in PC metabolism, like betaine, methionine, or folate, may also play a role (Miglio et al. 2000, Abdelmalek et al. 2009). In our study, dietary intake, including micronutrients, was similar between groups but data on choline, betaine and methionine intake are not available. In future studies on phospholipid metabolism in NAFLD, these nutrients should be assessed. Secondly, dysregulation of PC metabolism may be present. Hepatic phospholipids showed a higher amount of PE and lower PC in both patient groups, even though the latter was statistically significant only in SS. This suggests that changes in PC/PE ratio could be explained through a reduction in PEMT activity, which catalyzes the production of PC from PE in the liver, e.g. through a functional mutation of the PEMT gene (Song et al. 2005). This would lead indirectly to a build-up of PE substrate or suggest that hepatic cells use more PE in membrane to compensate for the loss in PC (Li et al. 2006). This is in accordance with the literature showing that individuals with a functional single nucleotide polymorphism (SNP) of PEMT (Val175Met) are more likely to develop NAFLD, as the enzyme is important for about 30% of the PC synthesis in the liver (Song et al. 2005). The importance of the PEMT pathway in NAFLD is further supported by human intervention trials, showing that supplementation of betaine - a compound that appears to stimulate PC biosynthesis via the PEMT pathway (Ji and Kaplowitz 2003, Abdelmalek et al. 2009) - can reduce steatosis and liver transaminases in NASH (Miglio et al. 2000, Abdelmalek et al. 2009). PC may also be depleted through other mechanism, e.g. increased conversion to sphingomyelin and resultant production of diacylglycerol (Puri et al. 2007). However, we can only speculate about this, and the activity of enzymes involved in PC and PE metabolism, thus are avenues to pursue in future studies to elucidate underlying mechanisms. In mouse models, a choline deficient diet can induce benign hepatic fat accumulation without inflammation and with normal liver enzymes when both PC and PE are reduced but a normal PC/PE ratio is maintained (Li et al. 2006). In contrast, a reduction of the PC/PE ratio presents with more steatosis, significant ballooning and inflammation, and animals die of liver failure (Li et al. 2006). In contrast, in this human study, we did not see a difference in PC/PE ratio between SS and NASH. One possible explanation is the less dramatic difference between the two phenotypes in our patients compared to the mouse model. Our patients with NASH had relatively mild disease: most had no or mild fibrosis and no or only few ballooned cells; half of the patients had grade 1 lobular inflammation, and the median NAFLD activity score was 5 (3-8). Subjects with SS also had lower PC/PE ratio. Those

patients had only liver steatosis on histology but they also had elevated liver enzymes. Due to ethical reasons, liver biopsies are usually not performed on patients with SS on ultrasound but normal liver transaminases. In addition, the mouse model used by Li et al. was based on choline deficient diet alone, whereas in humans many genetic and environmental factors contribute to the development and progression of NAFLD (Abu-Shanab and Quigley 2010, Daly et al. 2011).

Among these factors, dietary nutrients other than choline, such as the type of fat, may have played a role (Araya et al. 2004, da Costa et al. 2006, Allard et al. 2008) although, based on food records, we did not detect a difference in diet.

PC/PE ratios in liver and erythrocytes were not significantly correlated, but as shown in Figure 3, healthy controls had higher PC/PE ratios in both liver and erythrocytes, whereas patients have lower ratios either in one or both of the tissues. This suggests that patients with NAFLD in general show somedysregulation of phospholipid balance, but this can be differently pronounced in lipogenic liver tissue compared to peripheral red blood cells. We could speculate that this is linked to PEMT function, as PEMT contributes to 30% of PC synthesis in the liver, but the enzyme is not expressed in erythrocytes (Li et al. 2006).

Study strenghts and limitations

The major strenght of our study is the inclusion of a true healthy control group from the living liver donor program. This is exceptional for human studies, as liver tissue can usually only be obtained through an invasive liver biopsy, which cannot be justified in healthy controls. Control groups in other studies underwent e.g. elective antireflux or unspecified abdominal surgeries (Araya et al. 2004, Puri et al. 2007) or had a liver biopsy for elevated liver enzymes (Allard et al. 2008) and therefore their tissue might not reflect completely healthy conditions.

We are aware of certain limitations, including the small sample size, which did not allow to control for potential confounders like higher body mass index in the NASH group or ethnic background of the subjects (Dong et al. 2007). It is also possible that the small sample size prevented us from detecting differences in dietary intake. It would also be important in future studies, to quantify the dietary intake of choline, betaine and other compounds of phospholipid metabolism. Lastly, the cross-sectional nature of our study does not allow us to establish a cause-effect relationship between PC/PE ratio and NAFLD. Our findings will now guide us in the

development of more complex protocols to examine the underlying mechanisms including PEMT and other enzymatic activity.

In summary, hepatic PC/PE ratio is lower in patients with SS and NASH compared to healthy controls, due to a combination of lower PC and higher PE. PC/PE ratio in erythrocytes was also lower in both NAFLD groups compared to controls, due to lower PC content. Thus, evidence is provided demonstrating that liver and circulating phospholipids are implicated in NAFLD. Further human studies warranted to assess the role of PC and PE in the pathogenesis of NAFLD and underlying mechanisms.

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Tables

Table 1. Liver histology of study participants with a diagnosis of non-alcoholic fatty liver disease

	SS	NASH
<i>n</i>	14	14
Steatosis (% of hepatocytes)	29.6±6.4	58.2±6.5 **
Steatosis grading ^a (n)		
1 / 2 / 3	9 / 3 / 2	3 / 4 / 7
Fibrosis stage ^{□ b} (n)		
0 / 1 / 2 / 3 / 4	14 / 0 / 0 / 0	5 / 4 / 1 / 2 / 2 *
Inflammation ^c (n)		
0 / 1 / 2 / 3	14 / 0 / 0 / 0	0 / 7 / 5 / 2 *
Ballooning score ^d (n)		
0 / 1 / 2	14 / 0 / 0	1 / 10 / 3 *
NAFLD activity score ^e (n)	1 (1-3)	5 (3-8) ***

SS, simple steatosis; NASH, non-alcoholic steatohepatitis

Values are mean±SEM or number of patients. Unpaired t-test was used to determine differences in steatosis between the groups. Categorical variables were compared using Pearson chi-square test. *, ** indicate significant difference from SS, * P<0.05, ** P<0.01, *** P<0.001.

Assessment of liver biopsies according to Brunt et al. (1999) and Kleiner et al. (2005)

^a Steatosis grading: 0, <5%; 1, 5 – 33%; 2, >33-66%; 3, >66%

^b Fibrosis staging in NASH: 1, Zone 3 perivenular perisinusoidal/pericellular fibrosis, focal or extensive; 2, as grade 1 with focal or extensive periportal fibrosis; 3, bridging fibrosis, focal or extensive; 4, cirrhosis

^c Lobular inflammation: 0, No foci; 1, <2 foci per 200x field; 2, 2-4 foci per 200x field; 3, >4 foci per 200x field

^d Ballooning: 0, None; 1, Few balloon cells; 2, Many cells/prominent ballooning

^e NAFLD activity score calculated as the unweighted sum of the histological scores for steatosis (0-3), lobular inflammation (0-3) and hepatocyte ballooning (0-2)

Table 2. Clinical characteristics of study participants

	Controls	SS	NASH
<i>N</i>	9	14	14
Age (y)	40.4±4.0	40.9±2.2	42.8±3.2
Female gender (% (n/n))	55.6 (5/9)	21.4 (3/14)	64.3 (9/14)
BMI (kg/m ²)	26.1±1.6	27.4±0.9	32.1±1.4 ^{*,#}
Obese (BMI≥30) (% (n/n))	22.2 (2/9)	21.4 (3/14)	53.8 (7/13)
Waist circumference (cm)	85.0 (75.0-115.5)	97.3 (73.5-115.8) [*]	106.3 (83.8-127.5) ^{**}
Central obesity (% (n/n)) ^a	22.2 (2/9)	42.9 (6/14)	92.3 (12/13) ^{**,##}
Race			
Caucasian (% (n/n))	88.9 (8/9)	50.0 (6/12)	70.0 (7/10)
Asian (% (n/n))	0.0 (0/9)	33.3 (4/12)	30.0 (3/10)
Other (% (n/n))	11.1 (1/9)	16.7 (2/12)	0.0 (0/10)
Smokers (% (n/n))	0.0 (0/9)	7.1 (1/14)	21.4 (3/14)
Medical History			
Diabetes (% (n/n))	0.0 (0/9)	28.6 (4/14)	28.6 (4/14)
Dyslipidemia (% (n/n))	0.0 (0/9)	35.7 (5/14)	35.7 (5/14)
Hypertension (% (n/n))	0.0 (0/9)	42.9 (6/14)	50.0 (7/14) [*]
Medications			
Cardiovascular (% (n/n)) ^b	0.0 (0/9)	42.9 (6/14)	50.0 (7/14) [*]
Anti-diabetic (% (n/n))	0.0 (0/9)	21.4 (3/14)	28.6 (4/14)
Lipid lowering (% (n/n))	0.0 (0/9)	14.3 (2/14)	7.1 (1/14)
Antidepressant (% (n/n))	0.0 (0/9)	7.1 (1/14)	42.9 (6/14)

SS, simple steatosis; NASH, non-alcoholic steatohepatitis; BMI, Body mass index; Values are mean±SEM, median (range) or % and number of patients. Groups were compared by one-way ANOVA with Scheffe's Post-hoc test (continuous variables), Kruskal-Wallis and Mann-Whitney test (not normally distributed variables) or z-test (categorical variables).

^{*}, ^{**} indicate significant difference from controls ^{*} P<0.05, ^{**} P<0.01.

[#], ^{##} indicate significant difference from SS, [#] P<0.05, ^{##} P<0.01.

^a Central obesity defined as waist circumference men >102 cm (men) or >88 cm (women) (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults 2001).

^b Includes antihypertensive medication, diuretics, and blood thinners

Table 3. Blood biochemistry of study participants

	<i>n</i>	Controls	<i>n</i>	SS	<i>n</i>	NASH
Aspartate transaminase (U/l)	9	18 (15-36)	14	32 (17-41) **	14	51 (30-88) **,##
Alanine transaminase (U/l)	9	19.67±4.43	14	54.14±4.80 *	14	76.86±9.87 **
Alkaline phosphatase (U/l)	9	61 (44-109)	14	71 (33-275)	14	86 (51-188)
Bilirubin (µmol/l)	9	11 (3-44)	14	10.5 (6-36)	14	12.5 (4-23)
Glucose (mmol/l)	9	4.9 (4.2-5.2)	14	5.2 (4.5-8.0)	13	5.2 (3.9-16.2)
Insulin (pmol/l)	7	22 (15-211)	13	61 (15-133)	11	12.8 (17-674) *,#
HOMA-IR	7	0.85 (0.47-7.5)	13	2.71 (0.60-6.11)	11	5.4 (0.63-24.36) *
Total cholesterol (mmol/l)	7	4.52±0.29	14	4.75±0.24	12	5.23±0.43
High density lipoprotein cholesterol (mmol/l)	6	1.20±0.19	14	1.05±0.56	12	1.14±0.08
Low density lipoprotein cholesterol (mmol/l)	6	2.93±0.15	14	2.98±0.21	11	3.17±0.36
Triacylglycerols (mmol/l)	7	0.68 (0.58-1.21)	14	1.20 (0.53-4.09) *	12	1.73 (0.93-11.32) **

HOMA-IR, Homeostasis model assessment of insulin resistance; NASH, non-alcoholic steatohepatitis; SS, simple steatosis

One-way ANOVA with Scheffe's Post-hoc test was used to determine differences among the groups.

*, ** indicate significant difference from controls, * P<0.05, ** P<0.01.

#, ## indicate significant difference from SS, # P<0.05, ## P<0.01.

Figures

Fig. 1. Ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE) in (a) liver and (b) erythrocyte lipids of patients with simple steatosis (SS) or non-alcoholic steatohepatitis (NASH) compared to healthy controls. Values shown are mean and SEM. Groups were compared using Kruskal-Wallis followed by Mann-Whitney tests.

, * indicate significant difference from controls, ** $P < 0.01$, *** $P < 0.001$.

Fig. 2. Amount of phosphatidylcholine (PC, open bars) and phosphatidylethanolamine (PE, filled bars) in (a) liver and (b) erythrocyte lipids of patients with simple steatosis (SS) or non-alcoholic steatohepatitis (NASH) compared to healthy controls. Values shown are mean and SEM. Groups were compared using one-way ANOVA with Sheffe's post-hoc test (liver PE) or Kruskal-Wallis followed by Mann-Whitney tests (all other variables).

*, ** indicate significant difference from controls, ** $P < 0.01$, *** $P < 0.001$.

Fig. 3. No significant correlation between PC/PE ratio in liver and erythrocytes of healthy controls and patients with simple steatosis (SS) or non-alcoholic steatohepatitis (NASH) (Spearman's $r=0.276$; $P=0.203$).