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Total liver phosphatidylcholine content associates with non-alcoholic steatohepatitis and glycine N-methyltransferase expression.

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List of abbreviations: ALT, alanine aminotransferase; apoB, apolipoprotein B; BMI, body mass index; GNMT, glycine N-methyltransferase; HBV, hepatitis B virus, HCV, hepatitis C virus; PE, phosphatidylethanolamine; PC, phosphatidylcholine; HDL, high density lipoprotein; K OBS, Kuopio Obesity Surgery Study; LDL, low density lipoprotein; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NMR, nuclear magnetic resonance; PEMT, phosphatidylethanolamine N-methyltransferase; PNPLA3, patatin-like phospholipase domain-containing protein 3; SAH, s-adenosylhomocysteine; SAM, S-adenosylmethionine; TG, triglyceride; VLCD, very low-calorie diet; VLDL, very low density lipoprotein

Conflict of interest: MT and PS are shareholders and employees of Nightingale Health Ltd., a company offering NMR based metabolic profiling. Nothing else to declare.

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Abstract:

Background & Aims:

Alterations in liver phosphatidylcholine (PC) metabolism have been implicated in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). Although genetic variation in the phosphatidylethanolamine N-methyltransferase (*PEMT*) enzyme synthesizing PC has been associated with disease, the functional mechanism linking PC metabolism to the pathogenesis of non-alcoholic steatohepatitis (NASH) remains unclear.

Methods:

Serum PC levels and liver PC contents were measured using proton nuclear magnetic resonance (NMR) spectroscopy in 169 obese individuals [age 46.6 ± 10 (mean \pm SD) years, BMI 43.3 ± 6 kg/m², 53 men and 116 women] with histological assessment of NAFLD; 106 of these had a distinct liver phenotype. All subjects were genotyped for *PEMT* rs7946 and liver mRNA expression of *PEMT* and glycine N-methyltransferase (*GNMT*) was analyzed.

Results:

Liver PC content was lower in those with NASH ($P=1.8 \times 10^{-6}$) while serum PC levels did not differ between individuals with NASH and normal liver ($P=0.591$). Interestingly, serum and liver PC did not correlate ($r_s=-0.047$, $P=0.557$). Serum PC and serum cholesterol levels correlated strongly ($r_s=0.866$, $P=7.1 \times 10^{-49}$), while liver PC content did not correlate with serum cholesterol ($r_s=0.065$, $P=0.413$). Neither *PEMT* V175M genotype nor *PEMT* expression explained the association between liver PC content and NASH. Instead, liver *GNMT* mRNA expression was decreased in those with NASH ($P=3.8 \times 10^{-4}$) and correlated with liver PC content ($r_s=0.265$, $P=0.001$).

Conclusions:

Decreased liver PC content in individuals with the NASH is independent of *PEMT* V175M genotype and could be partly linked to decreased *GNMT* expression.

Lay summary:

We demonstrated that liver phosphatidylcholine (PC) content is decreased in those with non-alcoholic steatohepatitis. Although phosphatidylethanolamine N-methyltransferase (*PEMT*) gene regulates PC metabolism, the *PEMT* V175M gene variant did not explain the decreased liver PC content in NASH. Interestingly, glycine N-methyltransferase (*GNMT*) expression in the liver was decreased in those with NASH and it was associated with liver PC content, suggesting a potential role of *GNMT* in altering PC metabolism in NASH.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease affecting approximately 25 percent of individuals worldwide ⁽¹⁾. The disease presents primarily as steatosis of liver, but it can progress to non-alcoholic steatohepatitis (NASH) ⁽²⁾. Steatosis develops when excess fat is stored into liver as triglycerides (TGs) ⁽³⁾. This process is determined by the one-carbon cycle that generates phosphatidylcholine (PC) and S-adenosylmethionine (SAM) ⁽⁴⁾. Accordingly, hepatic PC metabolism has been associated with NAFLD ^(4, 5) and differences in PC subclasses have been reported between individuals with and without NASH ⁽⁶⁾. Furthermore, it has been shown that reduced PC synthesis impairs VLDL secretion ⁽⁷⁾ and may lead to liver

steatosis⁽⁸⁾. In line with these observations liver PC content is also decreased in humans with steatosis^(5,9) and NASH⁽¹⁰⁾.

The regulation of liver PC metabolism is complex and the mechanism how it relates with NASH are unknown. About 30 % of PC synthesis is regulated by phosphatidylethanolamine N-methyltransferase (PEMT), which catalyzes methyl transfer from SAM to phosphatidylethanolamine (PE)^(4,11). Accordingly, *Pemt*^{-/-} mice develop NASH when fed a choline deficient diet⁽¹²⁾. Another important enzyme taking part in the synthesis of PC from SAM and PE is glycine N-methyltransferase (GNMT), which catalyzes the conversion of SAM to S-adenosylhomocysteine (SAH)⁽⁴⁾. In humans, mutations in the *GNMT* gene have been reported to lead to liver disease⁽¹³⁾ and decreased *GNMT* expression in the liver has been associated with NAFLD and advanced fibrosis⁽¹⁴⁾.

Interestingly, *PEMT* V175M G to A substitution at rs7946 has been associated with 1.5 times increased risk of NAFLD in a meta-analysis⁽¹⁵⁾. Based on these findings, it has been suggested that decreased PC synthesis, due to genetic factors or limited dietary choline intake⁽¹⁶⁾, could lead to decreased VLDL export and ultimately to TG accumulation in the liver^(18,19).

Since the mechanism behind disturbed PC homeostasis in humans with NASH are unknown, we decided to analyze the role of *PEMT* V175M genotype and mRNA expression of *PEMT* and *GNMT* as regulators of PC metabolism in humans with normal liver, steatosis and NASH. For this purpose, we assessed serum total PC levels and liver total PC content, *PEMT* V175M genotype and liver mRNA expression of *PEMT* and *GNMT* in 169 individuals who participated in Kuopio Obesity Surgery Study (KOBS).

Methods

Subjects. A total of 169 subjects undergoing bariatric surgery were included into this study from the KOBS study (53 male and 116 female, age 46.6 ± 10 (mean \pm SD) years, BMI 43.3 ± 6 kg/m²; characteristics see Table S1) ⁽¹⁷⁾. Criteria for the surgery were in line with the current guidelines ⁽¹⁸⁾. Study subjects participated in one-day visit, including an interview on the history of previous diseases and current drug treatment, before the surgery. Fasting blood samples were drawn after 12 hours of fasting after 4 weeks of very low-calorie diet (VLCD). All patients with alcohol consumption of more than 20 g per day were excluded from the study. Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) were excluded using serology if alanine aminotransferase (ALT) values were elevated prior to surgery (HCV and HBV infections are rare in Finland). Hemochromatosis was excluded based on histological analysis of liver biopsies, and by normal serum ferritin levels in subjects with elevated serum ALT level. Informed consent was obtained from each participant and the study protocols were approved by the Ethics Committee of Northern Savo Hospital District and were in accordance with the Helsinki Declaration.

Clinical and laboratory measurements. Plasma glucose, insulin, and serum lipids and lipoprotein lipids were determined as previously described ⁽¹⁷⁾.

Quantitative NMR spectroscopy. Fasting concentration of total PC (mmol/L) was analyzed by proton nuclear magnetic resonance (NMR) spectroscopy in native serum samples. In addition, NMR analysis was used to measure cholesterol and TG concentration in different sized lipoproteins in serum. NMR analysis was also used to measure total PC content in the liver (nmol/mg). The details of this methodology have been described previously ^(19, 20). Serum and liver phosphatidylcholine measurements consist mainly phosphatidylcholines. This biomarker contains minor

amounts of some other glycerophospholipids, such as phosphatidylethanolamines and phosphatidylserines, but their concentrations are very low compared to the main glycerophospholipids. Thus, the NMR analysis is basically quantifying only different phosphatidylcholines (i.e. phosphatidylcholines with different fatty acid chains at sn-1 and sn-2 positions of glycerol backbone).^(21, 22)

Mass spectrometry metabolomics. Choline, alanine, betaine and methionine were measured with metabolomics approach with mass spectrometry as described previously⁽²³⁾.

Liver biopsies and histology. Liver biopsies were obtained using Trucut needles (Radiplast AB, Uppsala, Sweden) or as a wedge biopsy during elective gastric bypass operations. Overall histological assessment of liver biopsy samples (n = 169) was performed by one pathologist according to the standard criteria^(24, 25). We divided subjects into three categories based on a liver phenotype based on the EASL Clinical Practice Guidelines⁽²⁶⁾: 1. Normal liver without any steatosis, inflammation, ballooning or fibrosis, 2. Simple steatosis (steatosis > 5%) without evidence of hepatocellular ballooning, inflammation or fibrosis, and 3. NASH. When the subjects were divided into study groups based on the phenotype of their liver histology 60 had normal liver, 22 had simple steatosis and 24 had NASH (Table 1). Sixty-three out of 169 subjects could not be categorized into distinct phenotype (for example those with fatty liver but also mild fibrosis). However, all subjects were included into correlation analyses. NAFLD activity score (NAS) was calculated as an unweighted sum of steatosis (0-3), lobular inflammation (0-3) and hepatocellular ballooning (0-2) scores⁽²⁵⁾.

Liver gene expression and genotyping. Total RNA was extracted and purified using miRNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). Total RNA was converted to cDNA with random primers using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol. The expression of *PEMT* and *GNMT* was determined by the TaqMan Gene Expression Assays (Applied Biosystems) (Hs00540979_m1 and Hs00219089_m1 respectively) according to the protocol, and their expression was normalized to expression of two validated endogenous control genes *RPLP0* and *TBP* (Hs99999902_m1, Hs00427620_m1 respectively).

PEMT V175M (rs7946) and patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) I148M (rs738409) were genotyped using the TaqMan SNP Genotyping Assays (Applied Biosystems) according to the manufacturer's protocol.

Statistical analysis. Data are presented as mean \pm SD. Differences in categorical variables were evaluated by the χ^2 test. Differences between the study groups were compared using Kruskal-Wallis independent samples test. In histological analyses, we primarily focused on subjects, which could be characterized into three study groups (normal liver, steatosis, NASH, n = 106), but when making correlation analyses with individual variables all study subjects were included. Spearman's rank correlation was used for correlation analyses. Analyses were conducted with the SPSS version 24 program (IBM Inc., Armonk, NY). P value of less than 0.05 was considered statistically significant.

Results

Clinical characteristics

A total of 169 individuals were included in this study [53 male and 116 female, age 46.6 ± 10 (mean \pm SD) years, body mass index (BMI) 43.3 ± 6 kg/m²]. Characteristics of the study population based on the liver phenotype (normal liver, simple steatosis and NASH) are shown in Table 1. Age, BMI and ALT did not differ between study groups. In addition, cholesterol and TG levels were similar between the study groups. However, VLDL-TG levels were significantly higher in those with NASH as compared to the ones with normal liver or simple steatosis (0.88 ± 0.5 , 1.01 ± 0.4 and 1.19 ± 0.5 mmol/L, respectively, $P=0.024$) data not shown), as suggested before using detailed serum NMR analysis ⁽²⁷⁾. Fasting glucose and fasting insulin levels were higher in those with NASH when compared to those with normal liver ($P=1.2 \times 10^{-5}$ and $P=0.005$, respectively; Table 1). Type 2 diabetes was more frequent in those with NASH when compared to those with normal liver ($P=3.9 \times 10^{-8}$, χ^2 test). In addition, statin treatment was more frequently used in those with NASH ($P=0.006$). Characteristics of the whole study population are shown in the Table S1.

Serum total phosphatidylcholine level is not altered in those with NASH

Serum total PC level was not different between the study groups (1.65 ± 0.3 , 1.66 ± 0.3 and 1.60 ± 0.4 mmol/L respectively; $P=0.591$, Kruskal-Wallis test for significance, Fig. 1). When we analyzed only those without statin treatment, the results were essentially similar (Table 2).

Liver phosphatidylcholine content is decreased in NASH

Liver total PC content was different between study groups (22.2 ± 4.8 , 18.3 ± 2.6 and 16.6 ± 4.1 nmol/mg, respectively; $P=1.8\times 10^{-6}$). More specifically, liver PC content was lower in those with simple steatosis and NASH when comparing to those with normal liver ($P=4.7\times 10^{-4}$ and $P=6.3\times 10^{-6}$, respectively) (Fig. 1). In addition, when we adjusted the linear model with statin usage, there was still a significant association with NASH ($P=2.3\times 10^{-7}$). In line with this, liver total PC content correlated strongly with grade of steatosis ($r_s=-0.571$, $P=5.3\times 10^{-16}$), lobular inflammation ($r_s=-0.281$, $P=2.0\times 10^{-4}$), ballooning ($r_s=-0.293$, $P=1.1\times 10^{-4}$) and fibrosis ($r_s=-0.294$, $P=1.0\times 10^{-4}$) (Table 2). Because of the observed negative correlation of liver PC content with steatosis, we wanted to estimate if the other correlations with liver PC were independent of the total TG content of the liver. Thus, we calculated the ratio of liver PC to total liver triglycerides. The ratio was significantly different between study groups. Those with steatosis and those with NASH had lower PC/triglyceride ratio compared to those with normal liver ($P=1.4\times 10^{-11}$ and $P=3.4\times 10^{-10}$, respectively, Fig. 1). In fact, correlations of the ratio with histology were stronger than with PC content ($r=-0.790$ $P=8.5\times 10^{-23}$ for NASH). When we excluded those on statin medication from the correlation analysis, the results were similar (Table 2). In contrast to liver PC, serum PC level did not differ between the groups with different liver phenotypes. In fact, serum and liver PC did not correlate with each other, indicating differential regulation ($r_s=-0.047$, $P=0.557$, Fig. 1).

Serum and liver PCs correlated divergently with metabolic disturbances

Serum total PCs correlated strongly with serum total cholesterol ($r_s=0.866$, $P=7.1\times 10^{-49}$), low density lipoprotein (LDL) cholesterol ($r_s=0.731$, $P=1.2\times 10^{-27}$), high density lipoprotein (HDL) cholesterol ($r_s=0.567$, $P=7.6\times 10^{-15}$), triglycerides ($r_s=0.249$, $P=0.002$), very low density (VLDL) triglycerides ($r_s=0.165$, $P=0.038$) and apolipoprotein B (apoB) ($r_s=0.659$, $P=4.6\times 10^{-21}$), but not with BMI, fasting glucose or fasting insulin levels. In contrast, liver PC content correlated negatively with BMI ($r_s=-0.160$, $P=0.039$), serum fasting glucose ($r_s=-0.247$, $P=0.002$) and insulin levels ($r_s=-0.316$, $P=4.8\times 10^{-5}$), but not with serum total, LDL or HDL cholesterol serum triglyceride levels (Fig. 1). Results were essentially similar when analyzing only those with distinct liver phenotype ($n=106$) (Fig S1).

***PEMT* V175M genotype does not explain the decrease of liver PC content in NASH**

Next, we tested if *PEMT* V175M (G to A substitution) explains the detected decrease in liver total PC content in those with NASH. Clinical characteristics based on the *PEMT* V175M genotype are shown in Table 3. First, the *PEMT* V175M allele frequency was not significantly different between liver phenotypes (Table 1). Secondly, serum total PC levels (1.64 ± 0.4 , 1.64 ± 0.3 and 1.69 ± 0.4 mmol/L, respectively; $P=0.593$) or liver total PC content (18.6 ± 4.5 , 18.4 ± 5.1 and 20.6 ± 5.0 nmol/mg, respectively, $P=0.061$) were not different between the individuals with different *PEMT* V175M genotypes. In addition, liver PC to TG ratio was not significantly different between individuals with different *PEMT* genotypes. (Fig. 2).

Serum choline levels or alterations in *PEMT* mRNA expression do not associate with liver PC content

Because *PEMT* V175M was not associated with the decreased PC content in the liver in those with NASH, we analyzed other possible regulators of liver PC content. First, we investigated serum choline levels (reflecting the supply for the primary synthesis pathway of PC⁽¹¹⁾). Most importantly, the choline level did not differ between individuals with different liver phenotypes (Fig. S2) Thus, the association of the *PEMT* V175M genotype with serum choline levels was not likely to explain that those with AA genotype had significantly lower choline levels in serum ($P=0.017$), although they tended to have higher PC content in the liver, compared to those with genotypes GA and GG (Table S2, Fig. 2). Alanine betaine and methionine levels were not different between study groups (Fig. S2).

Next, we investigated if changes in the expression of *PEMT* in the liver associate with liver PC content. *PEMT* mRNA expression in the liver was not different between the liver phenotypes and was not significantly different between the *PEMT* genotypes. In addition, *PEMT* mRNA expression associated neither with serum nor liver PC (Fig. 3, Table S2). Furthermore, there was no correlation between *PEMT* mRNA and serum choline (data not shown).

***GNMT* expression is associated with NASH and with PC metabolism.**

The other potential regulator of liver PC metabolism in addition to *PEMT* is *GNMT*. Interestingly, liver *GNMT* expression was significantly decreased in those with NASH (Fig. 3). Furthermore, *GNMT* correlated negatively with histological grade of steatosis ($r_s=-0.314$, $P=3\times 10^{-5}$) lobular inflammation ($r_s=-0.260$, $P=0.001$), fibrosis ($r_s=-0.249$, $P=0.001$) and with NAFLD activity score ($r_s=-0.306$, $P=5.8\times 10^{-5}$) (Fig. 3).

Importantly, *GNMT* expression was positively correlated with liver PC content ($r_s=0.265$, $P=5\times 10^{-4}$). *GNMT* expression correlated also positively with LDL cholesterol ($r_s=0.193$, $P=0.014$) and negatively with VLDL triglycerides ($r_s=-0.157$, $P=0.047$), but not with total cholesterol ($r_s=0.141$, $P=0.076$), HDL cholesterol ($r_s=0.073$, $P=0.362$), triglycerides ($r_s=-0.130$, $P=0.101$) and apoB ($r_s=0.065$, $P=0.413$) (Fig. 3). Liver *MAT1A* expression correlated negatively with histological grade of steatosis ($r_s=-0.178$, $P=0.021$) lobular inflammation ($r_s=-0.174$, $P=0.024$), fibrosis ($r_s=-0.192$, $P=0.013$), NASH phenotype ($r_s=-0.205$, $P=0.036$) and with NAFLD activity score ($r_s=-0.174$, $P=0.025$). However, there was no significant association with lipids, serum PCs or liver PCs (Fig. 3). Results were similar if we take into analyses only those, who had distinct liver phenotype ($n=106$) (Fig S3).

Discussion

In this study, we analyzed serum and liver total PCs with NMR in a well characterized cohort of 169 severely obese individuals undergoing obesity surgery and having detailed information about liver histology. Interestingly, we found that liver phosphatidylcholines associate with NASH and liver *GNMT* expression but not with circulating phosphatidylcholines.

Our first important finding was that liver total PC content is decreased in those with steatosis and NASH compared to those with normal liver, while serum PC levels were associated neither with NAFLD nor with NASH (Fig. 1). Thus, we replicated in our cohort the previous findings that PC content is decreased in the liver of those with simple steatosis and NASH^(5, 9, 10). Interestingly, lately Kim *et al.* published a study, where they had human liver samples from those with normal liver, steatosis

and NASH, with 15 individuals in each group. They reported that liver PC content is increased in those with steatosis compared to those with normal liver, but that in those with NASH liver PC content is decreased when compared to those with normal liver⁽⁴⁾ which supports our results of decreased PC content in NASH.

Even though it has been hypothesized that decreased PC content in the liver could lead to accumulation of hepatic TGs⁽²⁸⁾ and to NAFLD progression⁽²⁹⁾, by decreasing VLDL secretion, we could not find a correlation between liver PC content and serum VLDL-TG. However, serum total PC level correlated with VLDL-TG indicating that serum PCs reflect VLDL export from the liver. However, it is important to notice that we measured only total PC content. Thus, there might be changes in the fatty acyl-chain composition of PCs in those with NASH. It is known that PCs produced via PEMT pathway (in a process using SAM and generating SAH) are rich in polyunsaturated fatty acids and are preferred to synthesize VLDL particles⁽⁷⁾. Thus, we cannot exclude the possibility that specific PCs associate with VLDL export in those with NASH.

The role of intrahepatic PC metabolism was also supported by the finding that liver PC-TG ratio associated with steatosis and NASH. Importantly, we also observed that serum and liver PCs associated with different metabolic disturbances. Serum PC had a strong positive correlation with serum levels of total cholesterol, LDL cholesterol and HDL cholesterol, which is logical because they are imported in lipoproteins together with PC⁽³⁰⁾ (Fig. 1). In contrast, liver PC content showed a negative correlation with BMI, serum fasting glucose and insulin levels, while liver PC content did not correlate with serum lipids. These differences also strengthen the conclusion that different regulatory mechanism may exist for VLDL secretion and

liver PC metabolism in NASH. Taken together, our findings raised the possibility of a direct link between low liver PC content and NASH.^(7, 9, 15)

We considered the role of genetic regulation behind liver PC metabolism. Liver PC content was not different based on the *PEMT* V175M genotype, although those with AA genotype tended ($P=0.061$) to have higher liver PC content (Fig. 2). However, AA is a risk genotype for NAFLD⁽¹⁵⁾ and thus the notified trend is inverse to what would be expected if the liver PC content would be a mediator for the genetic risk. In any case this indicates that the dysregulation of liver PC in NASH is not caused by the *PEMT* genotype. Interestingly, individuals with *PEMT* AA genotype had lower serum levels of choline (Fig. 2), which may reflect increased synthesis of PCs from the primary (i.e. Kennedy) pathway when *PEMT* pathway is suppressed. Previously, G to A substitution have been reported to lead to 30% decreased *PEMT* expression compared to the wild type⁽³¹⁾. However, in our study *PEMT* expression in the liver was not different between the *PEMT* genotypes. Taken together, our findings indicate that *PEMT* gene is not a major determinant of the observed low total PC content in the liver of those with NASH.

Intriguingly, our novel finding was that *GNMT* mRNA expression correlated negatively with histological grade of steatosis, lobular inflammation, fibrosis and a liver phenotype (Fig. 3). In addition, *GNMT* mRNA expression had a positive correlation with liver total PC content. Our results confirm the earlier findings of low *GNMT* expression in humans with NAFLD^(14, 32). *GNMT* is an enzyme (accounts for 1 % of cytosolic protein), which catalyzes the conversion of SAM to SAH. This conversion is a competitive pathway for phosphatidylcholine synthesis via the *PEMT*

⁽⁴⁾, because SAH can inhibit *PEMT* ⁽³³⁾. Accordingly, increased expression of *Pemt* in mice has been associated with overproduction of SAH ⁽³⁴⁾ and deletion of *GNMT* leads to accumulation of SAM ^(7, 35). Interestingly, deletion of *Pemt* does not regulate liver SAM content in mice ⁽³⁶⁾. Previously, it has been shown that *Gnmt*^{-/-} mice develop steatohepatitis ⁽³²⁾ and HCC ⁽³⁷⁾. Based on these findings it has been suggested that the role of *GNMT* is to optimize the ratio of SAM to SAH ⁽³⁸⁾ and transmethylation flux caused by the absence of *PEMT* is suggested to be compensated by *GNMT* ⁽³⁹⁾. In fact, the balance in the levels of PC and SAM has been suggested to be changed in NAFLD ⁽³⁸⁾. Previously, based on the serum metabolomics profiles, two different subtypes of NASH have been defined and the other one is suggested to be caused by SAM deficiency ⁽⁴⁰⁾. We found that liver *GNMT* expression is lower in those with NASH compared to those with normal liver, and liver *GNMT* expression and liver PC content had a positive correlation. Therefore, our results suggest dysregulation of liver *GNMT* in those with NASH.

Interestingly, liver *GNMT* expression also correlated positively with serum LDL and negatively with serum VLDL TGs, as opposed to e.g. liver *PEMT* expression (Fig. 3). It is known that excess SAM disrupts VLDL assembly in the *GNMT* knockout mice ⁽⁴¹⁾ linking *GNMT* to VLDL export. Furthermore, it has been suggested that *GNMT* may regulate cholesterol metabolism and take part in the development of hyperlipidemia and cholesterol accumulation in the liver ⁽³²⁾. Liver *GNMT* expression showed also correlations with several other liver lipids in the liver (Table S3). Thus, the role of *GNMT* as a regulator of liver lipid homeostasis as well as its role in the pathogenesis of NASH needs further studying.

We also found that liver *MAT1A* expression correlated negatively with NASH (Fig. 3). Previously, it has been published that *MAT1A* expression is decreased in the liver in those with NASH⁽¹⁴⁾. More interestingly, mice deficient in *Mat1a* have been reported to have reduced liver SAM, polyunsaturated fatty acids containing PC, impaired VLDL export and spontaneous development of NASH⁽⁴²⁾. Although we found an association between *MAT1A* expression and NASH, there was no correlation of *MAT1A* expression with total serum and liver PCs or with VLDL triglycerides (Fig. 3). Thus, it seems that *MAT1A* does not fully explain low total liver PCs in those with NASH. However, it is possible that *MAT1A* would have correlation with PC species containing polyunsaturated fatty acids⁽⁴⁰⁾. Taken together, our finding of decreased *GNMT* and *MAT1A* mRNA expression in the liver in those with NASH suggests that directly or indirectly dysregulation of *GNMT* contributes the development of NASH.

This study has some limitations. First, we could not measure different PC species in the serum or in the liver. In addition, we were not able to measure liver PE content to comprehensively evaluate the PE to PC pathway. In addition, measurement of SAM or SAH could have shed more light into the mechanism behind the association between liver PC content and NASH. Thus, further human studies including these measurements are needed to clarify the role of PC metabolism in NASH combining the measurements of different PCs, lyso-PCs and PEs together with the assessment of SAM and SAH levels in the liver and serum.

Based on our results, we suggest that NASH associates with liver total PC content but not with circulating total phosphatidylcholine level. Our results did not support the conclusion that alterations in PC metabolism in NAFLD/NASH could be explained by

variation in the *PEMT* gene. In contrast, the associations of *GMNT* expression with liver PC content and NASH suggest that altered *GMNT* expression may contribute to decreased PC content in those with NASH.

Figure legends:

Figure 1: Phosphatidylcholine (PC) metabolism is altered in individuals with non-alcoholic steatohepatitis (NASH). Level of total phosphatidylcholine in serum was not altered in those with NASH **(A)**, but total phosphatidylcholine content in the liver was lower in those with NASH **(B)**. Scatter plot shows that serum and liver phosphatidylcholines did not correlate, $r =$ Spearman correlation **(C)**. Liver total phosphatidylcholine content to liver total triglyceride (PC to TG) ratio, was lower in those with simple steatosis and NASH when compared to those with normal liver **(D)**. Interestingly, serum and liver phosphatidylcholines had divergent correlations with metabolic phenotypes **(E)**. Asterisk indicates $P < 0.05$ comparing to those with normal liver.

Abbreviations: BMI, Body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; ApoB, apolipoprotein B.

Figure 2: Phosphatidylethanolamine N-methyltransferase (*PEMT*) V175M genotype did not associate with serum total phosphatidylcholine levels **(A)**, but those with genotype AA had higher total phosphatidylcholine content in the liver **(B)**. Liver total phosphatidylcholine to liver total triglyceride (PC to TG) ratio was not different between genotypes **(C)**. Serum choline levels were lower in those with simple

steatosis and non-alcoholic steatohepatitis (NASH) when compared to those with normal liver **(D)**. Asterisk indicates $P < 0.05$ comparing to those with normal liver.

Figure 3: Liver phosphatidylethanolamine N-methyltransferase (*PEMT*) mRNA expression was not different between individuals with different liver histology **(A)**, but liver glycine N-methyltransferase (*GNMT*) mRNA expression was lower in those with simple steatosis and non-alcoholic steatohepatitis (NASH) when compared to those with normal liver. Asterisk indicates $P < 0.05$ **(B)**. Liver *PEMT* mRNA expression did not correlate with liver histology, serum and liver total phosphatidylcholines (PC) or metabolic disturbances. However, *GNMT* mRNA expression had negative correlation with liver steatosis, inflammation and fibrosis. *GNMT* expression also had a positive correlation with low-density lipoprotein (LDL) cholesterol and a negative correlation with very-low-density lipoprotein (VLDL) triglycerides. *GNMT* expression had positive correlation with liver total PC content. *MAT1A* expression correlated negatively with liver steatosis, inflammation and fibrosis **(C)**. Asterisk indicates $P < 0.05$ comparing to those with normal liver.

Abbreviations: ApoB, apolipoprotein B; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NAFLD, non-alcoholic steatohepatitis; VLDL; very-low-density lipoprotein.

References

1. Younossi ZM, Koenig AB, Abdelatif D *et al.* (2016) Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* **64**(1), 73-84.
2. Ibrahim SH, Hirsova P, Gores GJ (2018) Non-alcoholic steatohepatitis pathogenesis: sublethal hepatocyte injury as a driver of liver inflammation. *Gut* **67**(5), 963-972.
3. Yki-Jarvinen H (2014) Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome. *Lancet Diabetes Endocrinol* **2**(11), 901-910.
4. Kim YC, Seok S, Byun S *et al.* (2018) AhR and SHP regulate phosphatidylcholine and S-adenosylmethionine levels in the one-carbon cycle. *Nat Commun* **9**(1), 540-018.
5. Arendt BM, Ma DW, Simons B *et al.* (2013) Nonalcoholic fatty liver disease is associated with lower hepatic and erythrocyte ratios of phosphatidylcholine to phosphatidylethanolamine. *Appl Physiol Nutr Metab* **38**(3), 334-340.
6. Chiappini F, Coilly A, Kadar H *et al.* (2017) Metabolism dysregulation induces a specific lipid signature of nonalcoholic steatohepatitis in patients. *Sci Rep* **7**, 46658.
7. van der Veen, J N, Kennelly JP, Wan S *et al.* (2017) The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. *Biochim Biophys Acta* **1859**(9), 1558-1572.

8. Jacobs RL, Devlin C, Tabas I *et al.* (2004) Targeted deletion of hepatic CTP:phosphocholine cytidyltransferase alpha in mice decreases plasma high density and very low density lipoproteins. *J Biol Chem* **279**(45), 47402-47410.
9. Puri P, Baillie RA, Wiest MM *et al.* (2007) A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology* **46**(4), 1081-1090.
10. Wattacheril J, Seeley EH, Angel P *et al.* (2013) Differential intrahepatic phospholipid zonation in simple steatosis and nonalcoholic steatohepatitis. *PLoS One* **8**(2), e57165.
11. Garcia-Ruiz C, Mato JM, Vance D *et al.* (2015) Acid sphingomyelinase-ceramide system in steatohepatitis: a novel target regulating multiple pathways. *J Hepatol* **62**(1), 219-233.
12. Li Z, Agellon LB, Allen TM *et al.* (2006) The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis. *Cell Metab* **3**(5), 321-331.
13. Luka Z, Capdevila A, Mato JM *et al.* (2006) A glycine N-methyltransferase knockout mouse model for humans with deficiency of this enzyme. *Transgenic Res* **15**(3), 393-397.
14. Moylan CA, Pang H, Dellinger A *et al.* (2014) Hepatic gene expression profiles differentiate presymptomatic patients with mild versus severe nonalcoholic fatty liver disease. *Hepatology* **59**(2), 471-482.
15. Tan HL, Mohamed R, Mohamed Z *et al.* (2016) Phosphatidylethanolamine N-methyltransferase gene rs7946 polymorphism plays a role in risk of nonalcoholic

fatty liver disease: evidence from meta-analysis. *Pharmacogenet Genomics* **26**(2), 88-95.

16. da Costa KA, Kozyreva OG, Song J *et al.* (2006) Common genetic polymorphisms affect the human requirement for the nutrient choline. *FASEB J* **20**(9), 1336-1344.

17. Pihlajamäki J, Grönlund S, Simonen M *et al.* (2010) Cholesterol absorption decreases after Roux-en-Y gastric bypass but not after gastric banding. *Metabolism* **59**(6), 866-872.

18. Fried M, Yumuk V, Oppert JM *et al.* (2014) Interdisciplinary European guidelines on metabolic and bariatric surgery. *Obes Surg* **24**(1), 42-55.

19. Männistö VT, Simonen M, Hyysalo J *et al.* (2015) Ketone body production is differentially altered in steatosis and non-alcoholic steatohepatitis in obese humans. *Liver Int* **35**(7), 1853-1861.

20. Wurtz P, Kangas AJ, Soininen P *et al.* (2017) Quantitative Serum Nuclear Magnetic Resonance Metabolomics in Large-Scale Epidemiology: A Primer on -Omic Technologies. *Am J Epidemiol* **186**(9), 1084-1096.

21. Tukiainen T, Tynkkynen T, Makinen VP *et al.* (2008) A multi-metabolite analysis of serum by ¹H NMR spectroscopy: early systemic signs of Alzheimer's disease. *Biochem Biophys Res Commun* **375**(3), 356-361.

22. Wiesner P, Leidl K, Boettcher A *et al.* (2009) Lipid profiling of FPLC-separated lipoprotein fractions by electrospray ionization tandem mass spectrometry. *J Lipid Res* **50**(3), 574-585.

23. de Mello VD, Paananen J, Lindstrom J *et al.* (2017) Indolepropionic acid and novel lipid metabolites are associated with a lower risk of type 2 diabetes in the Finnish Diabetes Prevention Study. *Sci Rep* **7**, 46337.
24. Brunt EM, Janney CG, Di Bisceglie AM *et al.* (1999) Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol* **94**(9), 2467-2474.
25. Kleiner DE, Brunt EM, Van Natta M *et al.* (2005) Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* **41**(6), 1313-1321.
26. Ratziu V, Bellentani S, Cortez-Pinto H *et al.* (2010) A position statement on NAFLD/NASH based on the EASL 2009 special conference. *J Hepatol* **53**(2), 372-384.
27. Männistö VT, Simonen M, Soininen P *et al.* (2014) Lipoprotein Subclass Metabolism in Non-Alcoholic Steatohepatitis. *J Lipid Res* **55**(12), 2676-2684.
28. Noga AA, Zhao Y, Vance DE (2002) An unexpected requirement for phosphatidylethanolamine N-methyltransferase in the secretion of very low density lipoproteins. *J Biol Chem* **277**(44), 42358-42365.
29. Dongiovanni P, Stender S, Pietrelli A *et al.* (2018) Causal relationship of hepatic fat with liver damage and insulin resistance in nonalcoholic fatty liver. *J Intern Med* **283**(4), 356-370.
30. Dashti M, Kulik W, Hoek F *et al.* (2011) A phospholipidomic analysis of all defined human plasma lipoproteins. *Sci Rep* **1**, 139.

31. Song J, da Costa KA, Fischer LM *et al.* (2005) Polymorphism of the PEMT gene and susceptibility to nonalcoholic fatty liver disease (NAFLD). *FASEB J* **19**(10), 1266-1271.
32. Liao YJ, Chen TL, Lee TS *et al.* (2012) Glycine N-methyltransferase deficiency affects Niemann-Pick type C2 protein stability and regulates hepatic cholesterol homeostasis. *Mol Med* **18**, 412-422.
33. Chen NC, Yang F, Capecchi LM *et al.* (2010) Regulation of homocysteine metabolism and methylation in human and mouse tissues. *FASEB J* **24**(8), 2804-2817.
34. Zeisel SH, Zola T, daCosta KA *et al.* (1989) Effect of choline deficiency on S-adenosylmethionine and methionine concentrations in rat liver. *Biochem J* **259**(3), 725-729.
35. Varela-Rey M, Martinez-Lopez N, Fernandez-Ramos D *et al.* (2010) Fatty liver and fibrosis in glycine N-methyltransferase knockout mice is prevented by nicotinamide. *Hepatology* **52**(1), 105-114.
36. Mudd SH, Brosnan JT, Brosnan ME *et al.* (2007) Methyl balance and transmethylation fluxes in humans. *Am J Clin Nutr* **85**(1), 19-25.
37. Liao YJ, Liu SP, Lee CM *et al.* (2009) Characterization of a glycine N-methyltransferase gene knockout mouse model for hepatocellular carcinoma: Implications of the gender disparity in liver cancer susceptibility. *Int J Cancer* **124**(4), 816-826.

38. Jacobs RL, van der Veen, J N, Vance DE (2013) Finding the balance: the role of S-adenosylmethionine and phosphatidylcholine metabolism in development of nonalcoholic fatty liver disease. *Hepatology* **58**(4), 1207-1209.
39. Mato JM, Martinez-Chantar ML, Lu SC (2013) S-adenosylmethionine metabolism and liver disease. *Ann Hepatol* **12**(2), 183-189.
40. Alonso C, Fernandez-Ramos D, Varela-Rey M *et al.* (2017) Metabolomic Identification of Subtypes of Nonalcoholic Steatohepatitis. *Gastroenterology* **152**(6), 1449-1461.e7.
41. Martinez-Una M, Varela-Rey M, Mestre D *et al.* (2015) S-Adenosylmethionine increases circulating very-low density lipoprotein clearance in non-alcoholic fatty liver disease. *J Hepatol* **62**(3), 673-681.
42. Lu SC, Alvarez L, Huang ZZ *et al.* (2001) Methionine adenosyltransferase 1A knockout mice are predisposed to liver injury and exhibit increased expression of genes involved in proliferation. *Proc Natl Acad Sci U S A* **98**(10), 5560-5565.

Table 1. Characteristics based on the liver phenotype on the whole study cohort.

	Normal liver (n=60)	Simple steatosis (n=22)	NASH (n=24)	P value
Sex (male/female)	17/43	7/15	12/12	0.162
Age (years)	44.5 ± 10.7	46.0 ± 8.8	49.0 ± 10.0	0.076
BMI (kg/m ²)	42.8 ± 5.4	43.4 ± 4.9	44.2 ± 6.1	0.549
Type 2 diabetes (%)	8 (13)	7 (32)	19 (79)	3.9×10⁻⁸ ^{##}
Fasting glucose (mmol/L)	5.8 ± 0.8	6.2 ± 1.0	7.6 ± 2.0	1.2×10⁻⁵ ^{## x}
Fasting insulin (mU/L)	14.3 ± 7.9	17.3 ± 8.9	24.9 ± 17.4	0.005 *
Total cholesterol (mmol/L)	4.17 ± 0.8	4.21 ± 1.0	3.90 ± 0.8	0.313
LDL cholesterol (mmol/L)	2.40 ± 0.7	2.47 ± 1.0	2.10 ± 0.8	0.161
HDL cholesterol (mmol/L)	1.14 ± 0.3	1.06 ± 0.2	1.08 ± 0.3	0.471
Total triglycerides (mmol/L)	1.42 ± 0.6	1.62 ± 0.7	1.61 ± 0.6	0.268
ALT (U/L)	35.0 ± 18.1	46.4 ± 35.6	53.0 ± 36.0	0.080
Statin medication (%)	16 (27)	4 (18)	14 (58)	0.006 ^{##}
Steatosis grade (n)				3.6×10⁻²⁴
<5 %	60	0	0	
5-33 %	0	17	9	
33-66 %	0	3	8	
>66 %	0	2	7	
Lobular inflammation grade (n)				5.2×10⁻²²
None	60	22	0	
<2 foci per 200x field	0	0	14	
2-4 foci per 200x field	0	0	10	
>4 foci per 200x field	0	0	0	
Fibrosis stage (n)				2.1×10⁻¹⁹
0	60	22	1	
1	0	0	17	
2	0	0	3	
3	0	0	3	
4	0	0	0	
PEMT V175M genotype				0.888
GG	4 (6.7%)	2 (9%)	3 (12.5%)	
GA	31 (51.7%)	11 (50%)	10 (41.7%)	
AA	25 (41.7%)	9 (40.9%)	11 (45.8%)	
PNPLA3 I148M genotype				0.867
GG	42 (70.0%)	13 (59.1%)	15 (62.5%)	
GC	15 (25.0 %)	8 (36.4%)	8 (33.3%)	
CC	3 (5.0%)	1 (4.5%)	1 (4.2%)	

Data are given in mean ± standard deviation. Kruskal-Wallis test for significance (Chi-Square for categorical variables).

* P<0.05 between normal liver and NASH

P<0.05 between simple steatosis and NASH

x P<0.05 between normal liver and steatosis

Table 2. Spearman correlations of serum and liver phosphatidylcholines with liver histology.

	Steatosis grade	Lobular inflammation	Portal inflammation	Ballooning	Fibrosis	NASH phenotype	NAFLD activity score
All patients (n=169)							
Serum PC	r=-0.015 P=0.848	r=-0.020 P=0.805	r=-0.141 P=0.077	r=0.019 P=0.814	r=-0.033 P=0.681	r=0.080 P=0.419	r=-0.014 P=0.863
Liver PC	r=-0.571 P=5.3×10⁻¹⁶	r=-0.281 P=0.0002	r=-0.178 P=0.021	r=-0.293 P=0.0001	r=-0.294 P=0.0001	r=-0.500 P=4.7×10⁻⁸	r=-0.523 P=3.7×10⁻¹³
Liver PC to liver TG	r=-0.858 P=5.6×10⁻⁵⁰	r=-0.483 P=2.5×10⁻⁹	r=-0.127 P=0.100	r=-0.455 P=6.1×10⁻¹⁰	r=-0.449 P=9.3×10⁻¹⁰	r=-0.790 P=8.5×10⁻²³	r=-0.799 P=1.5×10⁻³⁸
Without statin (n=116)							
Serum PC	r=-0.008 P=0.936	r=-0.001 P=0.990	r=-0.167 P=0.082	r=0.104 P=0.284	r=-0.011 P=0.906	r=-0.119 P=0.327	r=0.012 P=0.901
Liver PC	r=-0.638 P=1.4×10⁻¹⁴	r=-0.285 P=0.002	r=-0.166 P=0.076	r=-0.244 P=0.009	r=-0.293 P=0.001	r=-0.492 P=1.1×10⁻⁵	r=-0.570 P=3.0×10⁻¹¹
Liver PC to liver TG	r=-0.873 P=2.3×10⁻³⁷	r=-0.463 P=1.6×10⁻⁷	r=-0.152 P=0.104	r=-0.434 P=1.3×10⁻⁶	r=-0.411 P=4.5×10⁻⁶	r=-0.782 P=5.0×10⁻¹⁶	r=-0.834 P=6.8×10⁻³¹

PC = phosphatidylcholine

Table 3. Characteristics of the study cohort based on the *PEMT* V175M genotype.

	GG n=13	GA n=86	AA n=70	P value (K-W)
Sex (male/female)	6/7	31/55	16/54	0.103*
Age (years)	48.4±8.9	45.0±9.6	48.2±9.4	0.111
BMI (kg/m ²)	45.1±7.3	43.3±5.7	42.9±5.1	0.462
Total cholesterol (mmol/L)	4.1±1.1	4.2±1.0	4.1±0.8	0.865
LDL cholesterol (mmol/L)	2.3±1.0	2.4±0.9	2.3±0.7	0.982
HDL cholesterol (mmol/L)	1.1±0.3	1.1±0.3	1.1±0.3	0.353
Triglycerides (mmol/L)	1.8±1.0	1.7±0.8	1.4±0.7	0.018
Fasting glucose (mmol/L)	6.4±1.4	6.6±2.2	6.5±1.6	0.763
Fasting insulin (mU/L)	20.2±21.6	19.5±11.7	17.9±17.1	0.372
ALT (U/L)	34±23	44±25	43±27	0.152
Steatosis grade (n)				0.775*
<5 %	5	36	29	
5-33 %	6	25	24	
33-66 %	1	12	11	
>66 %	1	13	6	
Lobular inflammation grade (n)				0.963*
None	9	61	47	
<2 foci per 200x field	3	21	18	
2-4 foci per 200x field	1	4	5	
>4 foci per 200x field	0	0	0	
Fibrosis stage (n)				0.337*
0	9	47	37	
1	2	32	30	
2	2	3	2	
3	0	3	1	
4	0	1	0	

Data are given in mean ± standard deviation. Kruskal-Wallis test for significance (*Chi-Square for categorical variables).

FIGURE 1

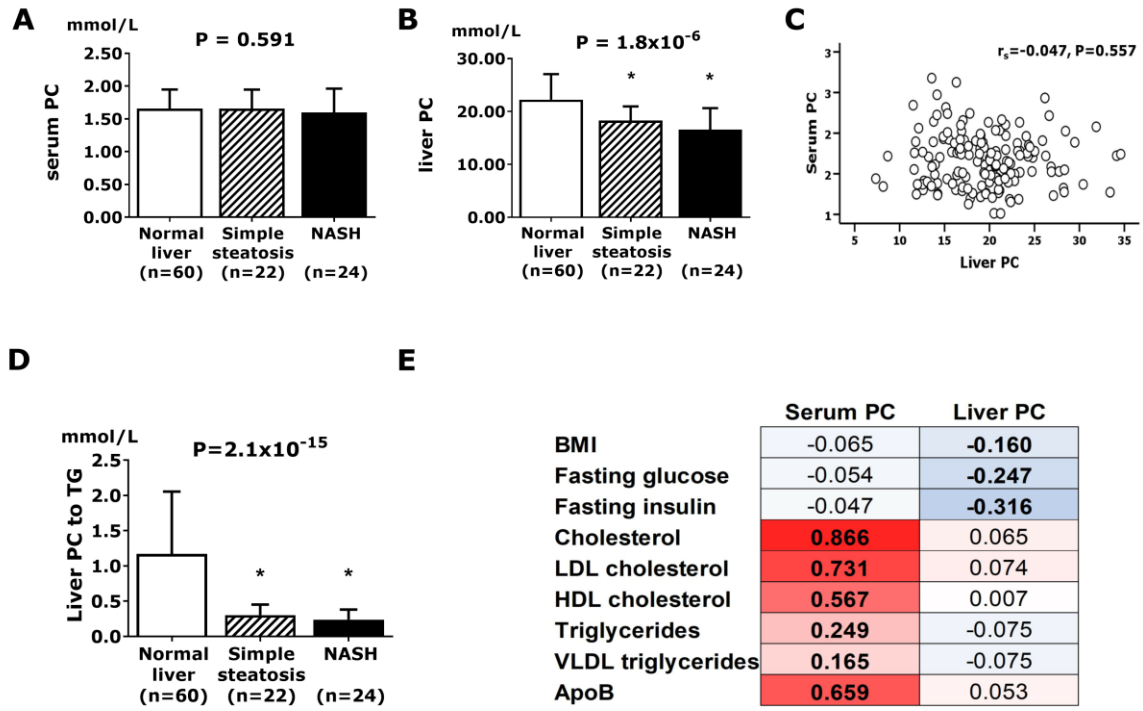


FIGURE 2

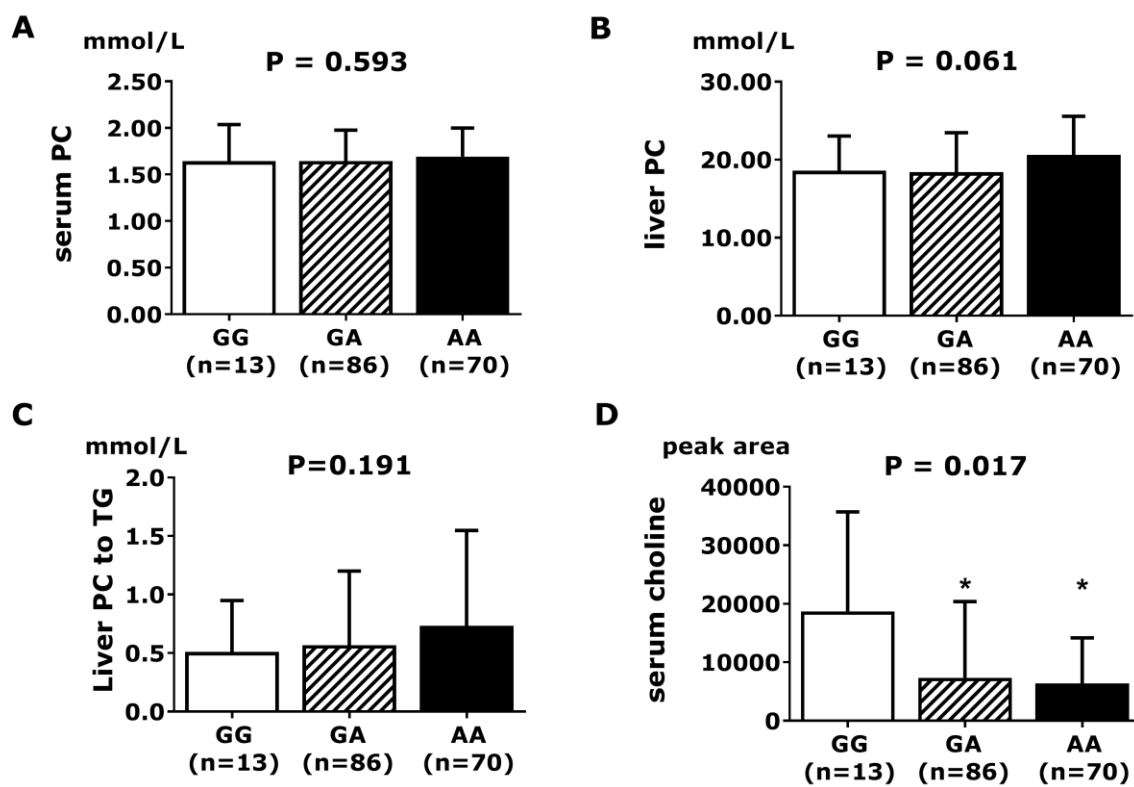
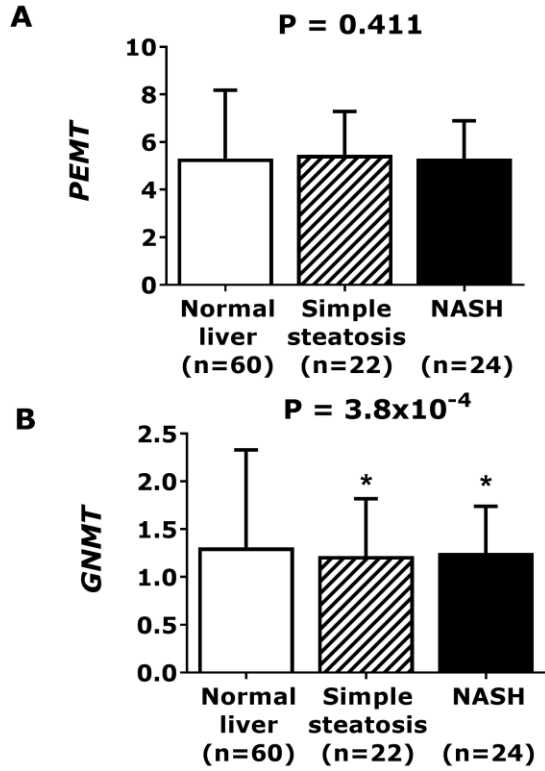


FIGURE 3



C

	<i>PEMT</i>	<i>GNMT</i>	<i>MAT1A</i>
Steatosis grade	0.045	-0.314	-0.178
Lobular inflammation	0.052	-0.260	-0.174
Portal inflammation	-0.040	-0.099	0.032
Ballooning	0.141	-0.103	-0.065
Fibrosis stage	-0.010	-0.249	-0.192
Phenotype	0.120	-0.387	-0.205
NAFLD activity score	0.092	-0.306	-0.174
Cholesterol	0.094	0.141	0.155
LDL cholesterol	0.085	0.193	0.151
HDL cholesterol	0.044	0.073	0.112
Triglycerides	0.016	-0.130	-0.105
VLDL triglycerides	-0.018	-0.157	-0.132
ApoB	0.054	0.065	0.044
Serum PC	0.091	0.078	0.082
Liver PC	0.082	0.265	0.122