S-Adenosylmethionine increases circulating very-low density lipoprotein clearance in non-alcoholic fatty liver disease

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Background & Aims: Very-low-density lipoproteins (VLDLs) export lipids from the liver to peripheral tissues and are the precursors of low-density-lipoproteins. Low levels of hepatic S-adenosylmethionine (SAMe) decrease triglyceride (TG) secretion in VLDLs, contributing to hepatosteatosis in methionine adenosyltransferase 1A knockout mice but nothing is known about the effect of SAMe on the circulating VLDL metabolism.

We wanted to investigate whether excess SAMe could disrupt VLDL plasma metabolism and unravel the mechanisms involved.

Methods: Glycine N-methyltransferase (GNMT) knockout (KO) mice, GNMT and perilipin-2 (PLIN2) double KO (GNMT-PLIN2-KO) and their respective wild type (WT) controls were used. A high fat diet (HFD) or a methionine deficient diet (MDD) was administrated to exacerbate or recover VLDL metabolism, respectively. Finally, 33 patients with non-alcoholic fatty-liver disease (NAFLD); 11 with hypertriglyceridemia and 22 with normal lipidemia were used in this study.

Results: We found that excess SAMe increases the turnover of hepatic TG stores for secretion in VLDL in GNMT-KO mice, a model of NAFLD with high SAMe levels. The disrupted VLDL assembly resulted in the secretion of enlarged, phosphatidylethanolamine-poor, TG- and apoE-enriched VLDL-particles; special features that lead to increased VLDL clearance and decreased serum TG levels. Re-establishing normal SAMe levels restored VLDL secretion, features and metabolism. In NAFLD patients, serum TG levels were lower when hepatic GNMT-protein expression was decreased.

Conclusions: Excess hepatic SAMe levels disrupt VLDL assembly and features and increase circulating VLDL clearance, which will cause increased VLDL-lipid supply to tissues and might contribute to the extrahepatic complications of NAFLD.

Introduction

The liver plays a central role in whole body metabolic homeostasis. It can obtain lipids from the circulation, synthesize them and secrete them in lipoproteins into the blood stream. Very-low-density lipoproteins (VLDL) transport triglycerides (TG) from the liver to peripheral tissues, providing an energy source. Plasma levels of VLDL are defined by the rate of clearance from plasma and the rate of hepatic secretion, so an imbalance between these two processes will lead to dyslipidemia, highly associated with increased risk of cardiovascular disease [1,2], which is one of the extrahepatic complications of non-alcoholic fatty-liver disease (NAFLD) [3].

Keywords: S-Adenosylmethionine; Very-low density-lipoproteins; Glycine N-methyltransferase; Non-alcoholic fatty liver disease.

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Abbreviations: apo, apolipoprotein; CD, control diet; DGAT, diacylglycerol O-acyltransferase; DG, diglyceride; DZA, 3-deazaadenosine; FA, fatty acid; FC, free cholesterol; GNMT, glycine N-methyltransferase; KB, ketone bodies; KO, knockout; MAT, methionine adenosyltransferase; MDD, methionine deficient diet; MTP, microsomal triglyceride transfer protein; NAFLD, non-alcoholic fatty-liver disease; NASH, non-alcoholic steatohepatitis; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine N-methyltransferase; PLIN, perilipin; qRT-PCR, real-time polymerase chain reaction; SAH, S-adenosylhomocysteine; SAMe, S-adenosylmethionine; TG, triglyceride; TGL, triglyceride lipase; VLDL, very-low-density lipoprotein; WT, wild type.
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Individuals with NAFLD exhibit disrupted VLDL metabolism [4,5]. In fact, abnormalities in the hepatic uptake of lipoproteins and/or secretion of VLDL can lead to hepatosteatosis [6,7]. The hepatic VLDL secretion rate is regulated by a variety of factors that must guarantee release of adequate amounts of TGs from the liver. To ensure VLDL secretion, apolipoprotein (apo) B should be translocated in the lumen of the endoplasmic reticulum (ER) during its translation [8], where it interacts with the microsomal TG transfer protein (MTP), whose lipid transfer activity is one of the major determinants in VLDL secretion [9]. ApoE facilitates ApoB maturation and VLDL assembly and secretion [10,11], and it mediates cellular uptake of several lipoproteins from the circulation [12,13].

Most of VLDL-TG (60–70%) is derived from intracellular stores [14,15]; therefore, the mobilization of lipids from cytosolic lipid droplets towards the ER represents a potentially regulated step in VLDL production and secretion [15,16].

Low liver S-adenosylmethionine (SAMe) in methionine adenosyltransferase 1A (MAT1A)-KO mice decreases TG secretion into VLDL particles, which contributes to hepatosteatosis [6]. Glycine N-methyltransferase (GNMT) drives the catabolism of SAMe and its deficiency in mice results in a marked increase in hepatic SAMe content and rapid NAFLD development [17]. SAMe is the methyl donor required for the methylation of phosphatidylethanolamine (PE). In hepatocytes, around 30% of phosphatidylcholine (PC) is synthesized by the sequential methylation of PE, in a reaction catalysed by the enzyme PE N-methyltransferase (PEMT) [18]. We have found that the flux from PE to PC and its catabolism into diglycerides (DGs) and conversion into TGs is stimulated in the liver of GNMT-KO mice [19]. There is a requirement for PEMT in the liver to ensure normal VLDL secretion [18,20]. Thus, we have evaluated if high levels of hepatic SAMe will disrupt VLDL assembly and as a consequence VLDL features and plasma metabolism.

Materials and methods

Human samples

This study comprised 33 non-diabetic patients with cholelithiasis and a clinical diagnosis of NAFLD without necroinflammation or fibrosis (Supplementary Tables 1 and 2) and 36 patients with asymptomatic cholelithiasis in whom a liver biopsy was taken during programme laparoscopic cholecystectomy (Supplementary Table 1). Inclusion criteria for patients are detailed in the Supplementary data section. The study was performed in agreement with the Declaration of Helsinki and with local and national laws. The Human Ethics Committee of the University Hospital Santa Cristina and the University of Basque Country approved the study procedures and written informed consent was obtained from all patients before inclusion in the study.

Animals

3-month-old male GNMT-KO mice, GNMT-PLIN2-KO mice, PLIN2-KO mice and their WT littermates were produced in the animal facility of CIC bioGUNE. They were maintained on different diets detailed in supplemental information. Animal procedures were approved by the University of the Basque Country and CIC bioGUNE Animal Care and Use Committees.

Preparation of labelled VLDL particles and in vivo clearance

Labelled human VLDL particles were obtained by a method previously described [21,22] and as detailed in the Supplementary Materials and methods.

Quantification of lipids

Lipids were extracted and quantified as described before [23]. TGs were quantified using a commercially available kit (A. Menarini Diagnostics). PE, PC and DG were separated by thin layer chromatography and quantified as detailed elsewhere [24].

Statistical analysis

Data were represented as means ± SEM. Differences between groups were tested using the Student’s t-test and two way ANOVA. Significance was defined as p <0.05. The baseline characteristics of the patients studied were compared using the unpaired t test or Mann-Whitney U test. These analyses were performed using SPSS version 15.0 software and GraphPad Version 5.03. Additional methods are detailed in the Supplementary Materials and methods section.

Results

Deletion of GNMT disrupts VLDL assembly, VLDL features and decreases circulating VLDL levels in serum

GNMT-KO mice show steatosis and fibrosis with increased serum aminotransferases [17] and no signs of insulin resistance [19]. In GNMT-KO mice VLDL-TG secretion was increased whereas VLDL-PE was decreased as compared to their wild-type (WT) controls (Fig. 1A). No changes were observed with regard to VLDL-PC secretion (Fig. 1A), while the VLDL size was augmented in GNMT-KO mice (Fig. 1A). All these compositional and physical VLDL features restored after feeding a methionine deficient diet (MDD) for 3 weeks (Fig. 1A). Consistent with the increased VLDL-TG secretion, we found increased turnover of the hepatocyte TG lipid stores (Fig. 1B) and increased MTP and diacylglycerol O-acyltransferase (DGAT) activity while no changes in TG lipase activity (Supplementary Fig. 1A) were observed. The increased hepatocyte TG secretion was re-established after inhibition of PEMT with 3-deazaadenosine (DZA) (Fig. 1C).

One of the factors that define the levels of circulating TGs in blood is the hepatic secretion rate. To our surprise, GNMT deletion in mice resulted in a decrease of serum TG (Fig. 1D), due to most of all VLDL and some LDL subfractions (Fig. 1D). The decrease in VLDL and LDL was also evident when ApoB levels were analysed in serum (Supplementary Fig. 1B). Re-establishing SAMe hepatic levels, by feeding MDD, restored serum TG and ApoB levels in GNMT-KO mice (Fig. 1D and Supplementary Fig. 1B).

In patients with NAFLD (n = 33) serum TG levels ranged from 54 to 317 mg/dl showing a high heterogeneity among subjects (Supplementary Table 1). In order to investigate whether altered SAMe metabolism could be linked with increased VLDL clearance in patients with NAFLD, we classified the individuals into two groups depending on serum TG levels (Supplementary Table 2). In a first group, (NAFLD-1), we introduced those subjects with serum TG levels higher than the mean of TGs in NAFLD patients (130.1 mg/dl) (n = 11) and in a second group (NAFLD-2) we included those with TG levels below 130.1 mg/dl (n = 22). We quantified TG levels in serum and analysed TG distribution in lipoproteins in the serum of the two groups (Fig. 1E) and found that decreased TG levels corresponded to most of all VLDL and some LDL subfractions (Fig. 1E). We also found that in NAFLD-2 patients there is a shift to the left of the maximum peak of VLDL, indicating that VLDL particles are enlarged (Fig. 1E). High hepatic SAMe levels in GNMT-KO mice were linked with enlarged VLDL.
particles (Fig. 1A), while decreased hepatic SAMe levels have been reported to be linked with smaller VLDL particle size [6], so we hypothesized that the SAMe content could also be increased in NAFLD-2 patients as a consequence of decreased GNMT expression. It has recently been described [25,26] that expression of the GNMT and MAT1A genes is decreased in patients with severe NAFLD. Thus, we measured GNMT protein levels in 17 NAFLD liver samples (6 from NAFLD-1 and 11 from NAFLD-2) and observed that in NAFLD-2 liver samples GNMT protein levels were lower than in livers from NAFLD-1 patients.

Fig. 1. Deletion of GNMT disrupts VLDL features and serum VLDL clearance. Wild type (WT), GNMT-KO (KO) and GNMT-KO mice fed a MDD (KO MDD) were fasted for 2 h. (A) Before the injection of 1 g/kg poloxamer (P-407) and 6 h later, VLDLs were isolated from the serum and characterized for triglyceride (TG), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) content and VLDL size. (B) Hepatocytes were incubated with 0.4 mM [3H] oleic acid (5 μCi/dish) and [14C] glycerol (0.5 μCi/dish) (pulse). The percentage of total [3H]-TG and [14C]-TG secreted into the media after both the pulse (4 h) and chase periods (4 h) was calculated. (C) Hepatocytes were incubated with 3-deazaadenosine (DZA, 10 μM) for 4 h and TG secretion into the medium was determined. (D) TG levels in serum and in lipoprotein subfractions were measured in mice fed a control diet. (E) Serum samples from NAFLD patients were obtained after a 12 h overnight fast. Serum samples from NAFLD-1 and NAFLD-2 patients were pooled and the TG content in serum lipoprotein subfractions was quantified. (F) Liver samples from patients with NAFLD (n = 17) (6 from NAFLD-1 and 11 from NAFLD-2) were obtained by liver biopsy GNMT immunohistochemistry. Magnification 40×. Quantitative assessments of IHC staining were performed using FRIDA image analysis software, expressed in % of positive staining per area. A negative control (NC) was performed by omission of primary antibody. Values are mean ± SEM of 5–6 animals per group and of the previously indicated patients. Statistical differences between GNMT-KO and WT mice or NAFLD-1 and NAFLD-2 patients are denoted by ⁄ p < 0.05; ⁄⁄ p < 0.01; ⁄⁄⁄ p < 0.001 (Student’s t test); and differences between GNMT-KO and GNMT-KO fed a MDD are denoted by # p < 0.05; ## p < 0.01 (Student’s t test).
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(Fig. 1F). When compared with normal livers (NL), GNMT protein expression was not changed in livers from NAFLD-1 subjects, while in livers from NAFLD-2 subjects the decrease was evident (Supplementary Fig. 2A). The hepatic PE content was decreased in NAFLD-2 patients, making the PC to PE ratio higher (Supplementary Fig. 2B), which suggests increased PEMT flux and VLDL secretion. Thus, in NAFLD-2 patients decreased TG levels in serum could be due to increased VLDL clearance as a consequence of increased hepatic SAMe levels.

A high fat diet (HFD) induces VLDL clearance and TG storage in the livers of GNMT-KO mice

We observed that excess SAMe in the liver increased VLDL-TG secretion as a consequence of increased PEMT flux, increased MTP activity, and increased turnover of TG lipid stores. High levels of hepatic SAMe also transformed VLDL features and increased VLDL clearance from blood (Fig. 2). We wondered whether challenging the mice with a HFD for six weeks could affect differently WT and GNMT-KO mice, exacerbating VLDL secretion and clearance in GNMT-KO mice. The metabolic characterization of mice fed a HFD showed that in GNMT-KO mice the food intake was slightly decreased as compared to their WT counterparts (Supplementary Fig. 3A). The body weight and the % of WAT did not change (Supplementary Fig. 3B and C), while the % of liver weight was increased (Supplementary Fig. 3D) as when fed a control diet (CD) [19]. GNMT-KO mice fed a HFD did not exhibit insulin resistance (Supplementary Fig. 3E). The HFD induced VLDL-TG secretion was observed only in GNMT-KO mice if compared with animals that were fed the control diet (CD) (Fig. 3A and Fig. 1A). The HFD did not alter VLDL-PE or VLDL-PC secretion nor VLDL size since the observed changes, when

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**Fig. 2. Proposed model of disrupted VLDL assembly, secretion and blood stream metabolism in GNMT-KO mice.** Excess levels of SAMe induce the secretion of less but enlarged, PE-poor and TG-enriched VLDL particles. ApoB availability is decreased in the liver due to lower mRNA synthesis and stability. There is an increased hepatic TG secretion, mainly due to a stimulated PEMT flux, which will increase TG storage in lipid droplets. The rise in mobilization of TGs from cytoplasmic lipid droplet stores will enhance the availability of TGs to be channelled towards VLDL secretion. MTP activity and DGAT activity will promote this process. VLDL particles will be rapidly metabolized from the blood stream, due to their specific VLDL features, leading to increased lipid supply in tissues such as liver and heart. Abbreviations: Apo, apolipoprotein; DGAT, diacylglycerol O-acyltransferase; ER, endoplasmic reticulum; MTP, microsomal triglyceride transfer protein; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine N-methyltransferase; PLIN2, perilipin 2; SAMe, S-adenosylmethionine; TG, triglyceride; TGL, TG lipase; VLDL, very-low-density lipoprotein.

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compared to their controls (Fig. 3A), were of the same magnitude as when fed a CD (Fig. 1A). Even though the HFD increased VLDL-TG secretion, serum TG levels were still half those in WT mice, indicating higher VLDL clearance in GNMT-KO mice fed the HFD compared to the CD (Fig. 3B). The low TG levels in serum were also due to a decrease in VLDL and some LDL subfractions (Fig. 3B).

In a condition in which VLDL clearance is increased, VLDL lipid supply to tissues will also be increased. After feeding the HFD the liver TG levels were 2.75 fold increased as compared to their WTs (Fig. 3C and D), whereas when fed a CD it was not increase (data not shown).

The HFD did not modify mRNA levels of proteins involved in lipid synthesis and NADPH production in GNMT-KO mice if compared to their controls (Supplementary Fig. 3F). Serum ketone bodies and fatty acids (Supplementary Fig. 3G) were not changed, and the hepatic CD36 protein content was not increased (Supplementary Fig. 3H).

**Fig. 3.** HFD induces VLDL clearance and liver TG storage in GNMT-KO mice. Wild type mice fed a high fat diet (WT HFD) and GNMT-KO mice fed a HFD (KO HFD) were fasted for 2 h. (A) Before the 1 g/kg poloxamer (P-407) injection and 6 h later, VLDL particles were isolated from the serum and characterized for triglyceride (TG), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) content and VLDL size. (B) TG levels in serum and in lipoprotein subfractions were measured. (C) Liver triglyceride (TG), diglyceride (DG), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) levels from WT and KO mice fed the HFD were quantified. (D) Representative liver haematoxylin and eosin staining. (E) Percentage of heart weight. Heart TG levels were quantified after lipid extraction. Values are mean ± SEM of 4–5 animals per group. Statistical differences between KO and WT mice are denoted by *p <0.05; **p <0.01; ***p <0.001 (Student’s t test).
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Specific VLDL features in GNMT-KO mice are linked with increased VLDL clearance

To define if the increased VLDL clearance depended on specific features of GNMT-KO VLDL particles, human VLDL particles were labelled and administered intravenously to GNMT-KO and WT mice. We found that the kinetic of clearance of labelled human VLDL was similar in WT and GNMT-KO mice (Fig. 4A), and that the % of injected dose per g of tissue after 30 min of injection was not altered in GNMT-KO mice (Fig. 4A), verifying that the increased clearance depended only on specific features of GNMT-KO VLDL particles.

To go further in the characterization of VLDL features from GNMT-KO mice we analysed VLDL-ApoB and -ApoE secretion. In GNMT-KO mice the secretion of VLDL-ApoB100 was decreased, while no changes were observed in those VLDL particles bearing ApoB48 as compared to their WT controls (Fig. 4B). Each VLDL particle contains a single copy of ApoB but several copies of ApoE. The results showed that in GNMT-KO mice, VLDL-ApoE secretion was increased compared to WT mice (Supplementary Fig. 4A), an increase even higher when fed the HFD (Supplementary Fig. 4A). This made each secreted VLDL particle to be ApoE-enriched (Fig. 4C). We observed that the altered VLDL-ApoE (Supplementary Fig. 4A) and VLDL-ApoB100 secretion (Fig. 4B) responded to changes in the hepatic content of these proteins and restored after re-establishing hepatic SAMe levels (Fig. 4D and E).

It has been suggested that lysosomal enzymes may degrade part of ApoE [27], so we investigated the effect of NH4Cl/leupeptin inhibitors. The treatment did not induce changes in GNMT-KO or WT hepatocytes ApoE levels (Supplementary Fig. 4B). In addition, we observed that in GNMT-KO mice there is an accumulation of ubiquitinated proteins (Supplementary Fig. 4C) and since part of ApoE is degraded through proteasomes in HepG2 cells [28], we wondered whether the increased ApoE content could be due to decreased proteasomal degradation. Inhibition of proteasome function using MG132 did not increase ApoE levels in WT hepatocytes (Supplementary Fig. 4C) nor VLDL-ApoE secretion (data not shown) and decreased ApoE content in GNMT-KO mice hepatocytes (Supplementary Fig. 4C) and secretion in VLDL (data not shown).

Increased ApoE levels in the liver were not linked with changes in ApoE mRNA (Supplementary Fig. 4D). However, the decreased hepatic ApoB100 content, driven by the excess hepatic SAMe, as shown after feeding the MDD (Fig. 4E), was coupled to lower levels of ApoB mRNA (Fig. 4F), in which binding to the mRNA stabilizing HuR protein was reduced (Fig. 4F). Knowing that global DNA methylation is increased in GNMT-KO mice [17], we wondered whether the ApoB promoter could also be more methylated than in WT mice. We analysed six CpG sites within the liver ApoB promoter; of which three were significantly hypermethylated in KO compared to WT mice (Fig. 4F). After restoring hepatic SAMe levels, ApoB mRNA levels re-established as did binding to HuR and methylation levels in those three CpG sites (Fig. 4F).

Factors that regulate cytosolic lipid storage, such as PLIN2 also influence VLDL secretion [29]. We have previously shown that HFD induces hepatic TG storage and increases VLDL-ApoE secretion in GNMT-KO mice, whereas deletion of PLIN2 in GNMT-KO mice results in the reduction of hepatic TG levels [19]. We wanted to know whether absence of PLIN2 in GNMT-KO mice could disturb VLDL assembly, VLDL-ApoE secretion and VLDL clearance. PLIN2 deletion in GNMT-KO mice (GNMT-PLIN2-KO) leads to increased VLDL-TG secretion, decreased VLDL-PE secretion and increased size of VLDL already observed in GNMT-KO mice when compared to their controls [Supplementary Fig. 5A]. Surprisingly, the absence of PLIN2 in GNMT-KO mice led to an increase in VLDL-PC secretion not found in GNMT-KO mice (Supplementary Fig. 5A), supporting a described role of PLIN2 binding PC [30,31]. In PLIN2-KO mice VLDL-TG secretion was also increased while no other changes were found (Supplementary Fig. 6A and B). Turnover of TG stores for secretion into VLDL and serum TG levels were decreased in the double KO mice if compared to their controls (Supplementary Fig. 5B and C). VLDL-ApoE and -ApoB secretion changed in the same way as in GNMT-KO mice, leading also to the secretion of ApoE-enriched VLDL particles (Supplementary Fig. 5D). Thus, the storage of TGs in lipid droplets in GNMT-KO mice does not have any effect on VLDL-ApoE secretion. Instead, the increased ApoE availability is directly linked with excess SAMe levels.

Discussion

VLDL assembly depends on the hepatic availability of lipids, certain proteins and multiple enzymatic reactions [32]. Specific features of VLDL, among other factors, define the rate of VLDL clearance from the blood stream [33]. VLDL particles export lipids from the liver to tissues and when lipid supply is excessive, metabolic homeostasis in peripheral tissues may disrupt [34]. Excess SAMe levels in GNMT-KO mice induce PC synthesis, through the PEMT pathway [19]. The PEMT pathway is required to ensure VLDL secretion [20], and the conversion of PE into PC seems to be involved in the formation of lipid droplets [35], in which mobilization of lipids represents a regulated step in VLDL secretion [15,16]. The increased PEMT flux in GNMT-KO mice is responsible for the rise in VLDL-TG secretion, which is linked with the increased mobilization of hepatic TG stores, and higher MTP and DGAT activity. Inhibition of DGAT1 reduces liver fibrosis in mice with NASH [36] while inhibition of DGAT2 results in decreased hepatosteatosis but higher fibrosis and liver damage [37]. In 8-month-old GNMT-KO mice, liver fibrosis is more prominent [17] and DGAT activity is increased to a higher degree than in 3-month-old KO mice, while DGAT2 mRNA is decreased 40% as compared to their WTs. No changes have been found in DGAT2 or DGAT1 mRNA in 3-month-old KO mice (data not shown). All these suggest a role of DGAT1 and DGAT2 in the progression of NAFLD in GNMT-KO mice.

The increased PEMT flux also causes a reduction in hepatic PE [19], reproduced in decreased VLDL-PE secretion. Surprisingly, even though PC synthesis was increased in GNMT-KO mice no changes were found in VLDL-PC secretion; however, deletion of PLIN2 in GNMT-KO mice, in which PLIN2 expression is increased [19], results in the rise of VLDL-PC secretion, suggesting a role of PLIN2 in the retention and catabolism of PC. PLIN2 binds lipids such as PC with high affinity, which supports this hypothesis [31]. As expected, these changes were not observed in PLIN2-KO mice, in which PC synthesis was not increased.

High levels of SAMe in GNMT-KO mice led to the secretion of enlarged, PE-poor and TG- and ApoE-enriched VLDL particles. These features facilitate VLDL clearance from the blood. We have also observed that in a group of patients with NAFLD, serum TG levels were decreased and the VLDL size increased when hepatic GNMT protein levels were lower. Moylan et al (2014) [25] have...
recently found that patients with severe NAFLD exhibit a significant downregulation of \textit{MAT1A} and \textit{GNMT} gene expression. We do not know whether MAT protein expression, involved in SAMe synthesis, may be decreased in this group of NAFLD patients but the enlarged VLDL size, decreased hepatic PE levels and increased PC/PE ratio suggest that the balance between GNMT
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and MAT activity results in increased hepatic SAMe levels, which enhance the PEMT flux. Unfortunately, the SAMe content could not be measured in liver biopsies from patients with NAFLD used in the present study. All this supports the hypothesis that administration of SAMe, to improve liver disease and lipoprotein metabolism, will have beneficial effects only in that group of patients in which the hepatic SAMe content is decreased. When the SAMe content is increased, probably a methionine deficient diet could be the chosen treatment, according to the results obtained previously [19] and in this work.

In the absence of ApoE, VLDL catabolism from the blood is heavily impaired [38], resulting in increased levels of TGs and a decreased uptake of TG-rich lipoproteins as has been observed in ApoE-deficient mice [39]. Since overexpression of ApoE markedly reduces ApoB-containing lipoproteins in plasma [13], we propose that high hepatic SAMe levels lead to VLDL-ApoE enrichment. This will explain the increased VLDL clearance in GNMT-KO mice, a feature more marked in animals fed a HFD, in which clearance is higher and the heart and liver TG storage is increased, which could be taken as a consequence of excessive lipid supply to these tissues. The fact that no changes in serum fatty acids or ketone bodies, together with the lack of an increase in CD36 protein levels or in genes related with lipogenesis in GNMT-KO animals fed the HFD, support this proposal. CD36 is upregulated in livers of NASH patients [40] and CD36 mRNA increases in animals fed a HFD after partial hepatectomy [41]. However, in obese fa/fa Zucker rats, where CD36 mRNA levels are increased, the protein content in hepatocytes did not change [42]. mTOR selectively regulates the expression of CD36 at a translational level [43,44]. We have found that phosphorylation of S6 and 4E-BP1, which are mTOR targets, (data not shown) are decreased in GNMT-KO mice fed the HFD, while CD36 mRNA is increased (data not shown), suggesting that downregulation of the mTOR pathway in this model could suppress, at least in part, CD36 translation.

ApoE secretion in VLDL was increased as a consequence of the higher liver ApoE content, which is also modulated by hepatic SAMe levels. In HepG2 ApoE is partially degraded through the proteasome [28] and partially through lysosomal enzymes [27]; however, our results show that in hepatocytes from GNMT-KO mice and their WT controls other mechanisms are involved in regulating ApoE availability. Interestingly, ApoE can escape degradation and can be re-secreted when following LDLR-mediated internalization by liver cells [45]. This is an attractive hypothesis since VLDL clearance is increased in GNMT-KO mice; therefore, ApoE could be recycled by the cell several times until final degradation occurs, as has been suggested before [45].

Factors that regulate cytosolic lipid storage, such as PLIN2 or CIDEB also influence VLDL secretion [29,46]. We observed that the HFD induced hepatic TG storage and increased VLDL-ApoE secretion in GNMT-KO mice whereas the absence of PLIN2 in GNMT-KO mice resulted in the reduction of hepatic TG levels, so we thought that cytosolic lipid storage could influence ApoE secretion into VLDL particles. However, while deletion of PLIN2 in GNMT-KO mice resulted in a decreased turnover of TGs for secretion in VLDL, ApoE secretion was still increased through VLDL clearance from the blood, discarding this hypothesis.

Excess SAMe levels in the livers of GNMT-KO mice were associated with decreased ApoB secretion. We found that the liver content of ApoB100 was decreased as a consequence of decreased ApoB mRNA. We also found that 3 CpG islands were hypermethylated within the ApoB promoter and that ApoB mRNA binding to the mRNA-stabilizing HuR protein was decreased in GNMT-KO mice, suggesting that ApoB mRNA synthesis and stability were decreased in GNMT-KO mice.

In conclusion, all these findings show that in NAFLD increased SAMe disrupts VLDL features and enhances VLDL clearance from the blood stream, which may lead to an improvement in the serum lipid profile of NAFLD patients. However, the increased lipoprotein lipid supply to tissues could also impair intracellular lipid homeostasis and be a cause of lipid storage in muscle, heart or adipose tissue, being involved in the extrahepatic complications of NAFLD.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors’ contributions

MM-U, MV-R, AG-O, CG-M, MLM-C, JMM, and PA study concept and design; MM-U, DM, LF-A; OF, DFR, VG-de-J, JM-G, IA, XB, and CG-M acquisition of data; ZL, CW, SCL, RHF technical and material support; MM-U, MV-R, MLM-C, JMM, and PA analysis and interpretation of data; MM-U and PA preparing the figures; MM-U and PA preparing the manuscript; MM-U, DM, LF-A; OF, DFR, VG-de-J, IM-G, IA, XB, and CG-M acquisition of data; ZL, CW, SCL, RHF technical and material support; MM-U, MV-R, MLM-C, JMM, and PA critical revision of the manuscript for important intellectual content; PA drafting of the manuscript and study supervision.

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Supplementary data

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