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Vitamin E alleviates non-alcoholic fatty liver disease in phosphatidylethanolamine *N*-methyltransferase deficient mice

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ABSTRACT

Phosphatidylethanolamine *N*-methyltransferase (PEMT) converts phosphatidylethanolamine (PE) to phosphatidylcholine (PC), mainly in the liver. *Pemt*$^{-/-}$ mice are protected from high-fat diet (HFD)-induced obesity and insulin resistance, but develop severe non-alcoholic fatty liver disease (NAFLD) when fed a HFD, mostly due to impaired VLDL secretion. Oxidative stress is thought to be an essential factor in the progression from simple steatosis to steatohepatitis. Vitamin E is an antioxidant that has been clinically used to improve NAFLD pathology. Our aim was to determine whether supplementation of the diet with vitamin E could attenuate HFD-induced hepatic steatosis and its progression to NASH in *Pemt*$^{-/-}$ mice. Treatment with vitamin E (0.5 g/kg) for 3 weeks improved VLDL-TG secretion and normalized cholesterol metabolism, but failed to reduce hepatic TG content. Moreover, vitamin E treatment was able to reduce hepatic oxidative stress, inflammation and fibrosis. We also observed abnormal ceramide metabolism in *Pemt*$^{-/-}$ mice fed a HFD, with elevation of ceramides and other sphingolipids and higher expression of mRNAs for acid ceramidase (*Asah1*) and ceramide kinase (*Cerk*). Interestingly, vitamin E supplementation restored *Asah1* and *Cerk* mRNA and sphingolipid levels. Together this study shows that vitamin E treatment efficiently prevented the progression from simple steatosis to steatohepatitis in mice lacking PEMT.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is increasingly recognized as the liver component of metabolic syndrome. According to recent data, it is estimated that approximately 25% of the adult population in developed countries suffers from the disease [1]. NAFLD is defined as the presence of > 5% fat in the liver without any other liver disease etiologies and/or use of medication, and it includes a histological range from simple steatosis to nonalcoholic steatohepatitis (NASH), which can further progress to cirrhosis and liver cancer [2]. NAFLD is linked to a low molar ratio of hepatic phosphatidylcholine (PC) to phosphatidylethanolamine (PE) [3–5]. PC and PE are the two main phospholipids in plasma membranes of all mammalian cells. In mice, around 70% of hepatic PC is produced via the CDP-choline pathway and the other 30% via phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway, by which PE is converted to PC via three sequential methylation reactions. PC is the major phospholipid in plasma lipoproteins. PC species derived from both synthesis pathways are crucial for the assembly and secretion of very low-density lipoprotein (VLDL) particles, as deletion of either pathway results in a strong reduction of VLDL secretion [6–9]. Mice lacking PEMT are protected from high-fat diet (HFD)-induced obesity and insulin resistance, but develop severe NASH when fed a HFD, mostly due to impaired VLDL secretion [8,10,11].

Currently, there is no definitive treatment for NALFD, mainly because the precise mechanism underlying the disease and especially its progression to more severe stages is still poorly understood. In *Pemt*$^{-/-}$ mice, a well-known model for NASH with hepatic fibrosis, it has not been established what the “second hit” is that progresses simple

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steatosis into hepatic inflammation and fibrosis. One of the lipid classes that have been associated with NASH development is sphingolipids and it is unknown whether sphingolipid metabolism is affected by PEMT-deficiency. Sphingolipids are a major class of membrane lipids that play a fundamental role in membrane architecture and in the regulation of key physiologic processes. Among the sphingolipid family, ceramides have been linked to insulin resistance, oxidative stress, and inflammatory processes [12–18], which suggest that ceramides may play a critical role in development of fatty liver disease [19–22]. Ceramides have been demonstrated to act on the mitochondrial electron transport chain leading to hydrogen peroxide and reactive oxygen species (ROS) generation, thereby inducing oxidative stress and promoting inflammation in different systems [15,23–25]. It is thought that oxidative stress plays a key role contributing to hepatocellular injury. Recent reports have focused on the biological activities of vitamin E in hepatic steatosis development [26–28]. Vitamin E (α-tocopherol) is a lipid-soluble antioxidant that defends cells against radical-induced damage [29]. Vitamin E is absorbed in the intestine, transported in plasma mainly with apolipoprotein B-containing chylomicrons, and transferred to parenchymal cells in liver. Subsequently, vitamin E is secreted from liver, mainly in association with VLDL, to be distributed to other tissues [28]. We have previously demonstrated that livers from HFD-fed *Pemt*$^{-/-}$ mice exhibit increased oxidative stress [9]; however, its importance in the development of NASH had not been established. Thus, the aims of this study were to investigate whether dietary supplementation of vitamin E can attenuate the development of HFD-induced NASH in *Pemt*$^{-/-}$ mice, and whether aberrant sphingolipid metabolism is involved in the disease progression in this model.

2. Materials and methods

2.1. Animals and diets

All experimental procedures were approved by the University of Alberta's Institutional Animal Care Committee in accordance with guidelines of Canadian Council on Animal Care. Male *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice (backcrossed into C57B1/6 for seven generations; five animals per group), were fed a semi-synthetic HFD (catalog no.F3282, Bio-Serv, Flemington, NJ; 60% kcal fat) or HFD supplemented with vitamin E (0.5 g/kg) for 3 weeks. This concentration of vitamin E results in an approximate dose of 133 IU/kg/day, which is equivalent to the recommended dose for human vitamin E supplements. Animals were fasted for 12 h before collection of different tissues. Blood was collected from the heart to an EDTA-containing tube, mixed, separated by centrifugation, and the plasma was stored at −80 °C. The liver was rinsed with ice-cold saline, weighed, immediately frozen in liquid nitrogen, and stored at −80 °C until further analyses. Formalin-fixed paraffin-embedded livers were sectioned for histological analysis using hematoxylin-eosin staining. Fibrillar collagen was visualized by Picro-Sirius red staining (5-μm sections, formalin-fixed livers) and confocal microscopy, as described elsewhere [30].

2.2. In vivo metabolic tests

VLDL-TG secretion rates were measured in vivo. After a 12 h fast, baseline blood samples were collected after which mice received an intra-peritoneal injection of Poloxamer 407 (1 g/kg). Poloxamer 407 inhibits lipoprotein lipase (LPL)-mediated plasma triglyceride (TG) hydrolysis and clearance [31]. Additional blood samples were collected by tail bleeding at 1, 2, 3, and 4 h after the injection of Poloxamer 407, and TG and cholesterol concentrations were measured.

2.3. Analytical procedures

Hepatic and plasma TG were measured by a commercially available kit from Roche Diagnostics. Plasma cholesterol was measured using a commercially available kit from Wako Pure Chemical Industries according to manufacturer's instructions. Hepatic PC and PE were isolated by thin-layer chromatography and quantified using a phosphorous assay. Hepatic α-tocopherol levels were quantified by HPLC [32]. Thiobarbituric acid-reactive substances (TBARS) were measured in liver homogenates as a marker for oxidative stress, using a commercially available kit from R&D Systems (catalog no. KGE013) according to manufacturer's instructions. Oxidized (GSSG) and reduced (GSH) glutathione was measured in liver homogenates using a commercially available kit from Abcam (catalog no. ab138881). Sphingolipid measurements were performed by mass spectrometry as previously described [33].

2.4. Real-time quantitative PCR

Total liver RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was treated with DNase I (Invitrogen) to degrade genomic DNA, then reverse-transcribed using oligo(dT)12–18 primers and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time quantitative PCR was performed using a Step One Plus qPCR system. Data analyses were performed using the Step One software v2.3 (Applied Biosystems) and mRNA levels were normalized to cyclophilin mRNA.

2.5. Western blot analysis

For immunoblotting, livers were homogenized in buffer (100 mM Tris-HCl, 150 mM sodium chloride, 1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF, pH 7.4) containing a protease inhibitor cocktail. Proteins were electrophoresed on 8–12.5% polyacrylamide gels containing 0.1% SDS and transferred to a polyvinylidene difluoride membrane for immunoblotting with antibodies against APOA1 (1:1000; catalog no. K23001R, Biodesign), ApoB (1:5000; catalog no. AB742, Chemicon), BiP (1:1000; catalog no. 3183, Cell Signaling), CHOP (1:1000; catalog no. 2895, Cell Signaling), PDI (1:4000; catalog no. SPA-890, Assay Designs), PLIN2 (1:1000; catalog no. ab108323, Abcam), SR-B1 (1:1000; catalog no. ab52629, Abcam) and GAPDH (1:1000; catalog no. ab8245, Abcam). Secondary antibodies conjugated to horseradish peroxidase were used for detection of immunoreactive proteins by enhanced chemiluminescence system (GE Healthcare) according to the manufacturer's instructions. Protein levels were quantified using GeneTools software (Syngene) and normalized to GAPDH protein expression.

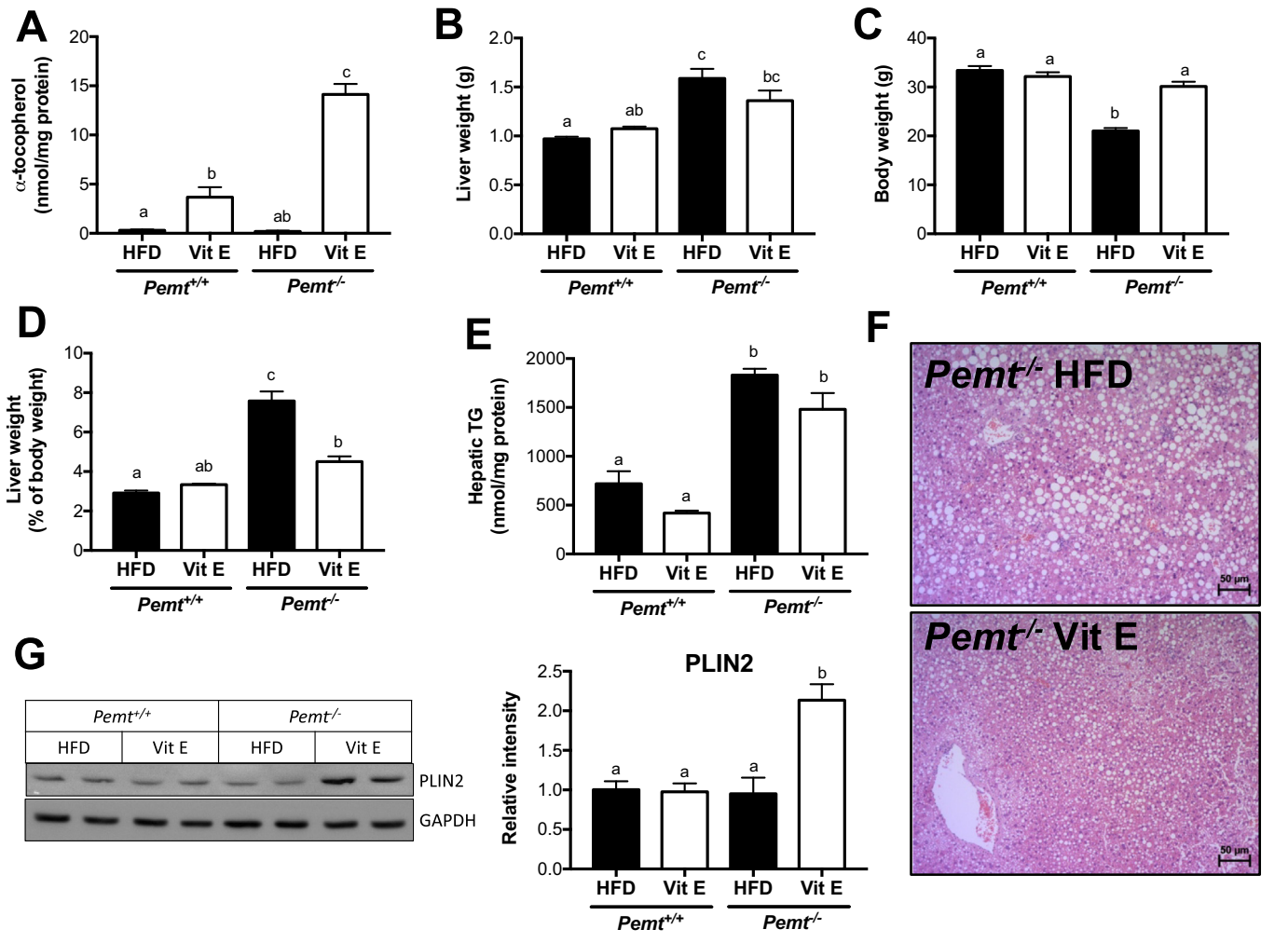
2.6. Statistical analysis

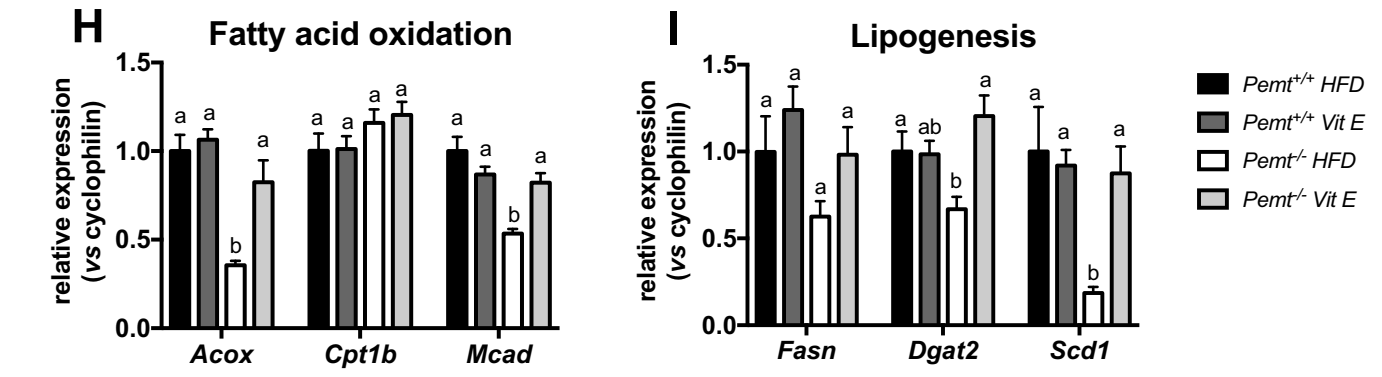
GraphPad Prism (version 6.01) was used to perform all the analyses. Results are expressed as the mean ± SEM. Comparisons between experimental groups were performed using two-way analysis of variance (ANOVA) with Tukey's post-hoc test for multiple comparisons. *P*-values of < 0.05 ($P < 0.05$) were considered statistically significant.

3. Results

3.1. Vitamin E supplementation reduced liver weight and improved hepatic lipid secretion in *Pemt*$^{-/-}$ mice

Recent reports have indicated that vitamin E supplementation can attenuate hepatic steatosis [26–28]. Therefore, we fed *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice a HFD or a HFD supplemented with 0.5 g/kg vitamin E for 3 weeks to determine whether it could prevent fatty liver development in *Pemt*$^{-/-}$ mice. Three weeks of HFD-feeding causes *Pemt*$^{-/-}$ mice to develop severe NASH [34] and is thus sufficient to investigate the effect of vitamin E on NASH development in these mice. In order to verify that the vitamin E supplemented to the diet was sufficiently





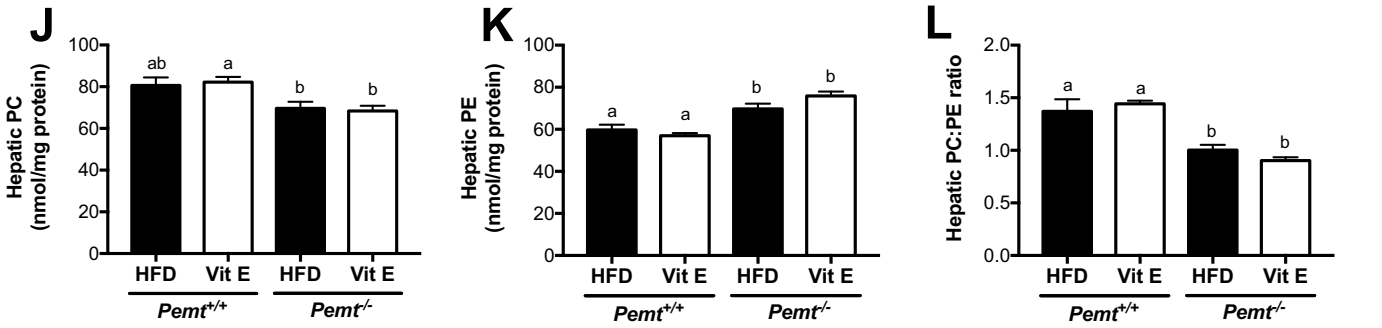


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Fig. 1. Vitamin E supplementation reduced liver weight but did not alter hepatic lipid profile in *Pemt*$^{-/-}$ mice. *A:* Vitamin E levels in liver after 3-week treatment. *B-D:* Liver weight (*B*), body weight (*C*) and liver weight relative to body weight (*D*) after 3-week treatment on a HFD with or without vitamin E. *E:* Hepatic TG in livers from *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed a HFD or a HFD supplemented with vitamin E. *F:* Representative pictures of hematoxylin-eosin staining of liver samples from untreated and vitamin E supplemented *Pemt*$^{-/-}$ mice. *G*: Immunoblot and quantification of PLIN2 in liver samples from *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed HFD ± vitamin E. *H* and *I*: mRNA levels of genes encoding proteins involved in fatty acid oxidation (*H*) and de novo lipogenesis (*I*) relative to cyclophilin expression and normalized to the corresponding control group. *J-L:* PC, PE, and PC:PE ratio in livers from *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed a HFD or a HFD supplemented with vitamin E. All values are the means ± SEM ($n = 5$ per group for all panels except for G: $n = 4$ per group). Values that do not share a letter are significantly different ($P < 0.05$).

absorbed, we measured vitamin E concentrations in liver. As seen in Fig. 1A, vitamin E concentrations were strongly increased in livers of supplemented animals. It is noteworthy that it was significantly higher in *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice. Vitamin E is a hydrophobic molecule that is stored in lipid droplets, and it is secreted from the liver as a component of VLDL particles. The higher vitamin E concentrations in supplemented *Pemt*$^{-/-}$ mice are thus likely due to impaired VLDL secretion and subsequent higher hepatic lipid content in these mice.

After 3 weeks on the diet, livers from *Pemt*$^{-/-}$ mice were larger than those from *Pemt*$^{+/+}$ mice (Fig. 1B). Even though the liver weights did not reduce with vitamin E supplementation, body weights were normalized to *Pemt*$^{+/+}$ values, resulting in a decrease in relative liver weight upon vitamin E (Fig. 1C and D). Hepatic TG levels were 2.5-fold higher in *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice, and vitamin E treatment seemed to result in a slight reduction in TG levels; however, this observation did not reach statistical significance (Fig. 1E). Interestingly, hematoxylin-eosin staining showed large lipid droplets in liver samples of *Pemt*$^{-/-}$ mice, whereas the lipid droplets appeared smaller after treatment with vitamin E (Fig. 1F). This implies that the same amount of neutral lipids is stored in more lipid droplets, which was supported by an increase in PLIN2 expression in *Pemt*$^{-/-}$ mice upon vitamin E supplementation (1G). In NAFLD subjects, hepatic fatty acid oxidation is often reduced. In accordance, we observed a reduction in mRNA levels of some (*Acox* and *Mcad*), but not all (*Cpt1b*) genes involved in fatty acid oxidation in HFD-fed *Pemt*$^{-/-}$ mice (Fig. 1H). Vitamin E supplementation completely restored the expression level of *Acox* and *Mcad* to *Pemt*$^{+/+}$ control levels. This supports the idea that smaller lipid droplets allow the lipids to be more accessible to fatty acid oxidation. In addition, mRNA levels of *Dgat2* and *Scd1*, but not of *Fasn*, were reduced in HFD-fed *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice, and were completely restored upon vitamin E treatment (Fig. 1I). Even though the increased number of lipid droplets would require more PC for the surface of the droplets, hepatic PC levels in *Pemt*$^{-/-}$ mice were not increased upon treatment with vitamin E (Fig. 1J). Hepatic PE levels were increased in *Pemt*$^{-/-}$ animals (Fig. 1K), but unaffected by vitamin E. Thus, the lower PC:PE ratio in *Pemt*$^{-/-}$ mice was not restored by vitamin E supplementation (Fig. 1L).

Although hepatic TG concentrations were not reduced by vitamin E supplementation, vitamin E was able to increase VLDL-TG secretion in *Pemt*$^{-/-}$ animals compared to those without supplementation, as demonstrated by plasma TG quantification before and after the inhibition of lipoprotein lipase (LPL) by Poloxamer 407 (Fig. 2A and B). However, both VLDL-TG secretion rates and fasting plasma TG levels were still lower in vitamin E-supplemented *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice. Plasma ApoB-100 and ApoB-48 levels were not different between HFD-fed *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice, indicating poorly lipidated VLDL particles, rather than fewer VLDL particles, in *Pemt*$^{-/-}$ mice (Fig. 2C). Vitamin E did not affect ApoB-100 or ApoB-48 in either genotype. No changes in ApoB levels combined with an increase in VLDL-TG production suggests that vitamin E supplementation improved the lipidation of VLDL particles in *Pemt*$^{-/-}$ mice.

Interestingly, plasma HDL was also reduced in HFD-fed *Pemt*$^{-/-}$ mice, as indicated by strongly reduced plasma total cholesterol (Fig. 2D) and ApoA1 levels (Fig. 2E). Vitamin E supplementation normalized HDL levels in *Pemt*$^{-/-}$ mice. To determine whether the low plasma concentrations of HDL-cholesterol were due to increased hepatic uptake, we measured hepatic SR-B1. Surprisingly, there was a reduction of hepatic SR-B1 levels in *Pemt*$^{-/-}$ mice, suggesting that low HDL-cholesterol levels were likely due to reduced production of HDL particles rather than increased uptake of HDL-cholesterol into the liver. The reduction in SR-B1 levels in *Pemt*$^{-/-}$ mice was completely restored when the mice received diet supplemented with vitamin E (Fig. 2F).

3.2. Vitamin E treatment prevented hepatic oxidative stress, inflammation and fibrosis in *Pemt*$^{-/-}$ mice

Since vitamin E is an antioxidant and oxidative stress is an important trigger for the development of NASH [35,36], we determined levels of oxidative stress in all groups of animals. Hepatic lipid peroxidation, an indicator of oxidative stress in liver [37,38], was 4-fold higher in *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice, as shown by higher amounts of TBARS. Vitamin E supplementation completely normalized TBARS values to levels observed in *Pemt*$^{+/+}$ mice (Fig. 3A). Similarly, the ratio GSSG/GSH was increased in HFD-fed *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice, and this increase was completely prevented by vitamin E supplementation (Fig. 3B). Moreover, mRNA levels of genes related to oxidative stress NADPH oxidase 2 (*Nox2),* heme oxygenase 1 (*Hmox1)*, and mitochondrial uncoupling protein 2 (*Ucp2)* were 2.5 to 4-fold increased in *Pemt*$^{-/-}$ mice and were reduced by vitamin E (Fig. 3C-E). Next, we evaluated whether the oxidative stress in *Pemt*$^{-/-}$ mice would lead to endoplasmic reticulum (ER) stress in these mice. Protein levels of CCAAT-enhancer-binding protein homologous protein (CHOP), Binding immunoglobulin protein (BiP) and protein disulfide-isomerase (PDI) were all elevated in livers from HFD-fed *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice (Fig. 3F). Dietary supplementation with vitamin E prevented the induction of these proteins as the protein levels in vitamin E-treated *Pemt*$^{-/-}$ mice were indistinguishable from the *Pemt*$^{+/+}$ controls. Together, this suggests that oxidative stress resulted in ER stress in livers of *Pemt*$^{-/-}$ mice, and that treatment with vitamin E prevented oxidative stress, thereby alleviating ER stress.

Next, we evaluated the effect of vitamin E supplementation on hepatic inflammation and fibrosis, two important hallmarks of NASH. We measured mRNA levels of genes involved in inflammation (*Tnfα,* an inflammatory cytokine, and *Cd68,* a general macrophage marker). Both were > 5-fold increased in *Pemt*$^{-/-}$ mice fed the HFD, indicating increased number of macrophages in the liver (Fig. 4 A and B). Vitamin E supplementation prevented the increase in *Tnfα* and *Cd68* expression. Since both oxidative stress and inflammatory cytokines can activate hepatic stellate cells, we investigated if vitamin E supplementation could prevent the development of hepatic fibrosis in *Pemt*$^{-/-}$ mice. mRNA levels of alpha-1 type I collagen (*Col1a1)*, responsible for collagen synthesis, as well as those of tissue inhibitor of metalloproteinases 1 (*Timp1*) were strongly increased in HFD-fed *Pemt*$^{-/-}$ mice and this increase was partially prevented by vitamin E supplementation (Fig. 4C and D). Moreover, Picro-Sirius Red staining confirmed that the amount of fibrillar collagen was lower when *Pemt*$^{-/-}$ mice were fed the vitamin E-supplemented diet compared to the HFD-fed *Pemt*$^{-/-}$ mice (Fig. 4E). Together these data indicated a protective role of vitamin E in the development of NASH.

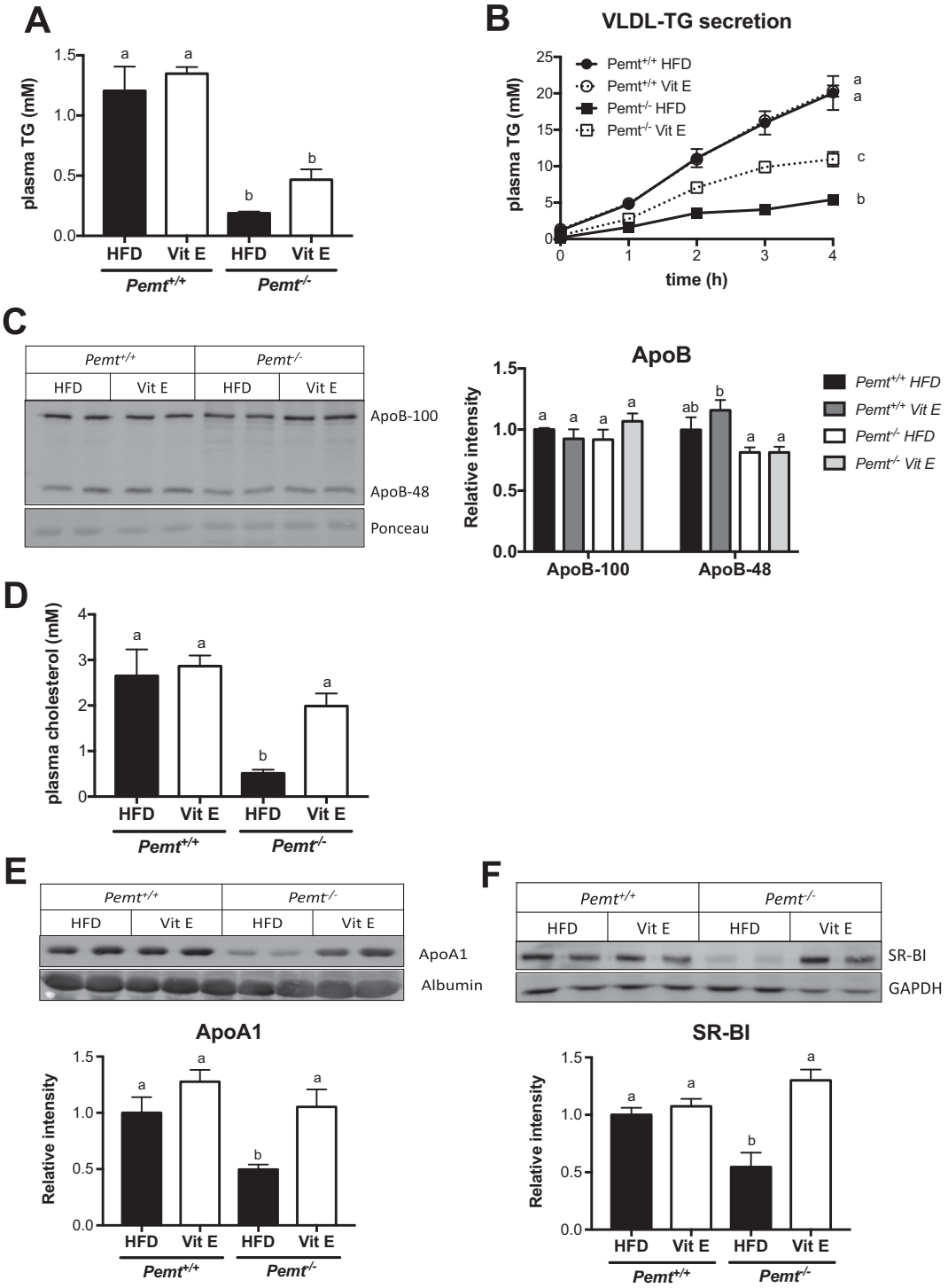

Fig. 2. Vitamin E supplementation improved hepatic lipid secretion in *Pemt*$^{-/-}$ mice. *A:* Plasma TG concentrations in fasting conditions in *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed the HFD ± vitamin E. *B:* TG secretion curves after Poloxamer 407 injection. *C:* Immunoblot of ApoB-100 and ApoB-48 in plasma in fasting conditions and its quantification. *D:* Plasma total cholesterol concentrations in fasting conditions in *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed the HFD ± vitamin E. *E, F:* Immunoblot and quantification of APOA1 in plasma samples and SR-B1 in liver samples, respectively. All values are mean ± SEM ($n = 5$ per group for A, B and D; $n = 4$ per group for C, E and F). Values that do not share a letter are significantly different ($P < 0.05$).

3.3. Ceramide metabolism is normalized in vitamin E-treated Pemt$^{-/-}$ *mice*

Due to the increasingly recognized role of ceramides and their derivatives in the development of NAFLD [19–22], we investigated ceramide metabolism in HFD-fed *Pemt*$^{-/-}$ mice that developed NASH, and determined whether vitamin E could influence the master enzymes of the different routes for ceramide synthesis and/or breakdown (Fig. 5A). We observed no changes in the mRNA levels of serine palmitoyltransferase 1 (*Spt1*), ceramide synthase 2 and 6 (*CerS2* and *CerS6*), but a slight reduction in acidic sphingomyelinase (*Asmase*) in *Pemt*$^{-/-}$ mice, which was not reversed with vitamin E treatment (Fig. 5B-E). However, acidic ceramidase (*Asah1*) and ceramide kinase (*Cerk*) mRNA levels were 1.5- and 2.5-fold higher in *Pemt*$^{-/-}$ mice, and vitamin E

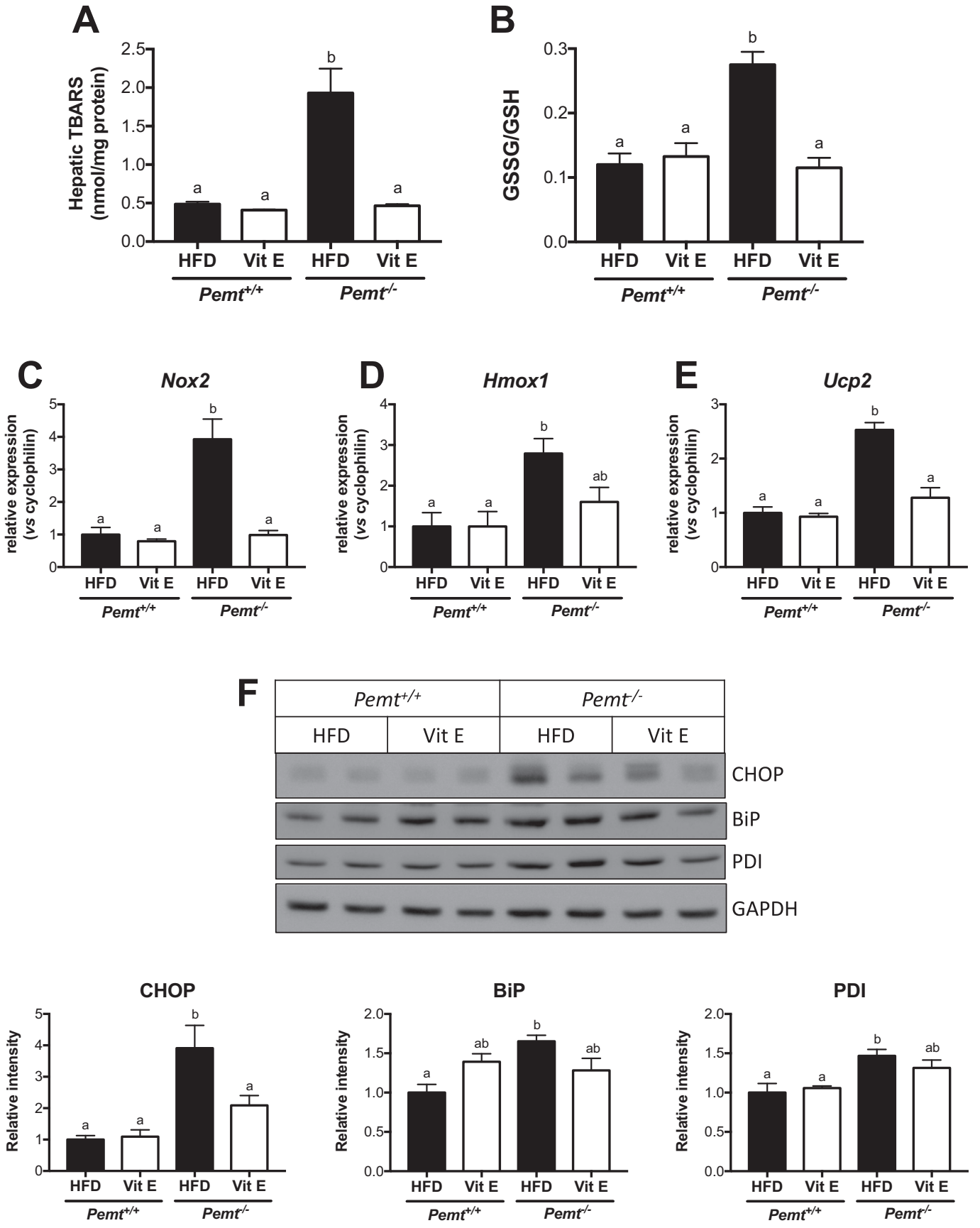

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Fig. 3. Vitamin E supplementation prevented hepatic oxidative stress and ER stress in *Pemt*$^{-/-}$ mice. *A:* Hepatic concentration of thiobarbituric acid-reactive substances (TBARS), a marker for lipid peroxidation. B: Hepatic ratio of GSSG/GSH as a marker for oxidative stress. *C-E:* mRNA levels for oxidative stress related genes NADPH oxidase 2 (*Nox2*), heme oxygenase 1(*Hmox1*) and mitochondrial uncoupling protein 2 (*Ucp2*) relative to cyclophilin expression and normalized to the corresponding control group. *F:* Immunoblot of proteins involved in ER stress CCAAT-enhancer-binding protein homologous protein (CHOP), binding immunoglobulin protein (BiP), and protein disulfide-isomerase (PDI). *D:* Quantification of *C* relative to the amount of the loading control, GAPDH. Values are means ± SEM ($n = 5$ per group for A-E, and $n = 4$ per group for F). Values that do not share a letter are significantly different ($P < 0.05$).

supplementation normalized those levels to *Pemt*$^{+/+}$ levels (Fig. 5F-G).

Mass-spectrometry analyses revealed that ceramide levels were indeed elevated in livers from *Pemt*$^{-/-}$ mice, and they were reduced upon vitamin E supplementation in *Pemt*$^{-/-}$ mice (Fig. 6A). Similarly, sphingomyelin, sphinganine, and sphingosine were also increased in *Pemt*$^{-/-}$ mice, and vitamin E supplementation normalized all of them to control levels (Fig. 6B-D). Interestingly, the concentration of 1-deoxy-ceramides, which were one of the most correlated biomarkers for

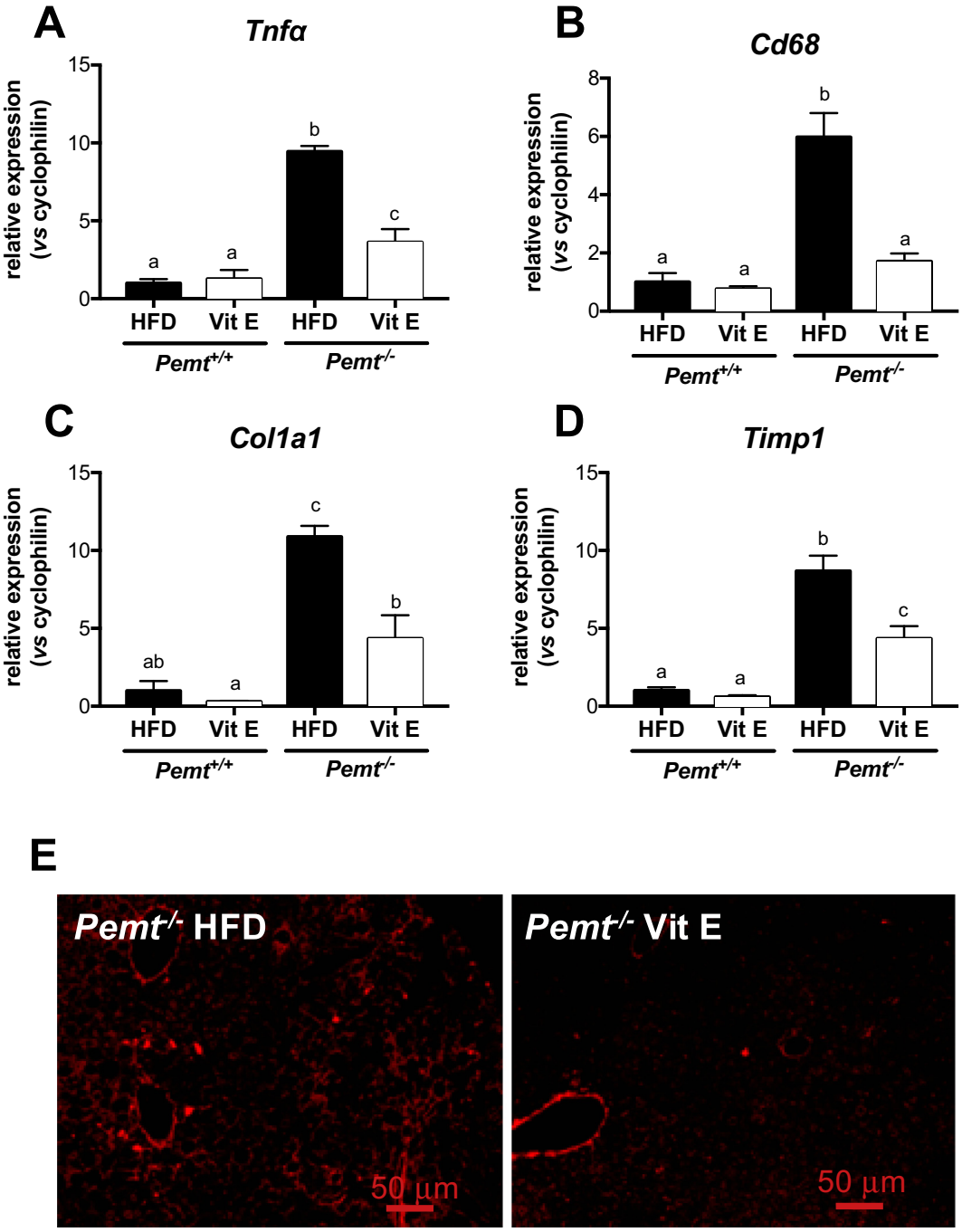

Fig. 4. Vitamin E supplementation prevented hepatic inflammation and fibrosis in *Pemt*$^{-/-}$ mice. *A* and *B:* mRNA levels for inflammation related genes (*Tnfα* and *Cd68*) relative to cyclophilin expression and normalized to the corresponding control group. *C and D:* mRNA levels of markers for fibrosis (*Col1a1* and *Timp1*) relative to cyclophilin expression and normalized to the corresponding control group. All values are means ± SEM ($n = 5$ per group). *E:* Histological assessment of hepatic fibrosis in untreated and vitamin E-supplemented *Pemt*$^{-/-}$ mice using Picro-Sirius Red. Values that do not share a letter are significantly different ($P < 0.05$).

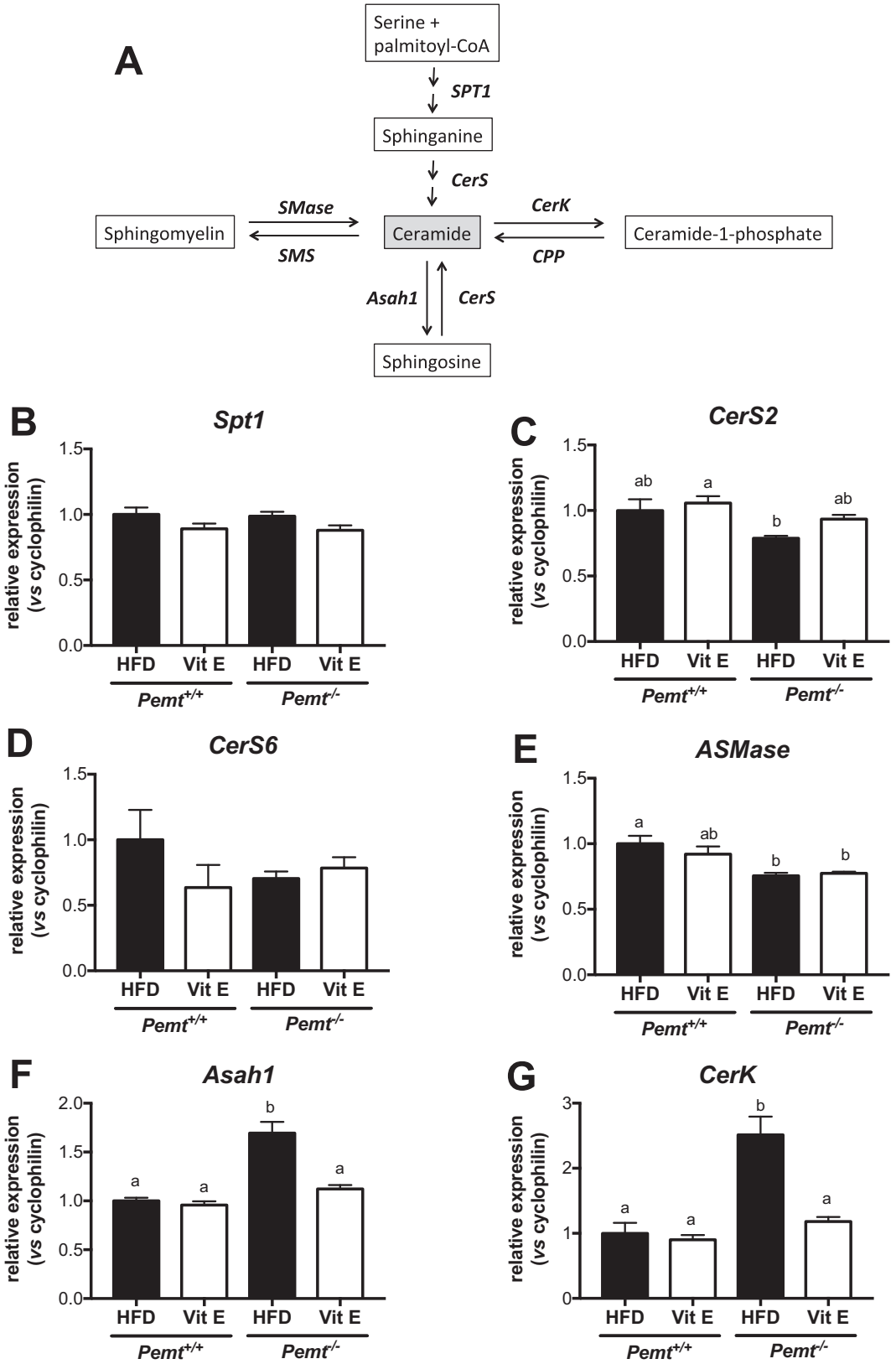

Fig. 5. Ceramide metabolism is normalized in *Pemt*$^{-/-}$ mice treated with vitamin E. *A:* Schematic representation of ceramide biosynthesis pathways. mRNA levels after 3-week treatment on HFD with or without vitamin E supplementation relative to cyclophilin expression of: *B:* de novo ceramide synthesis pathway enzyme SPT1 (serine palmitoyltransferase subunit 1), *C* and *D: salvage* and de novo synthesis pathway enzymes CERS2, CERS6 (ceramide synthase 2 and 6), *E:* SMase synthesis pathway enzyme ASMase (acid sphingomyelinase), *F:* ASAH1 (acidic ceramidase), and *G:* Ceramide 1-phosphate converting enzyme CERK (ceramide kinase). All values are means ± SEM ($n = 5$ per group). Values that do not share a letter are significantly different ($P < 0.05$).

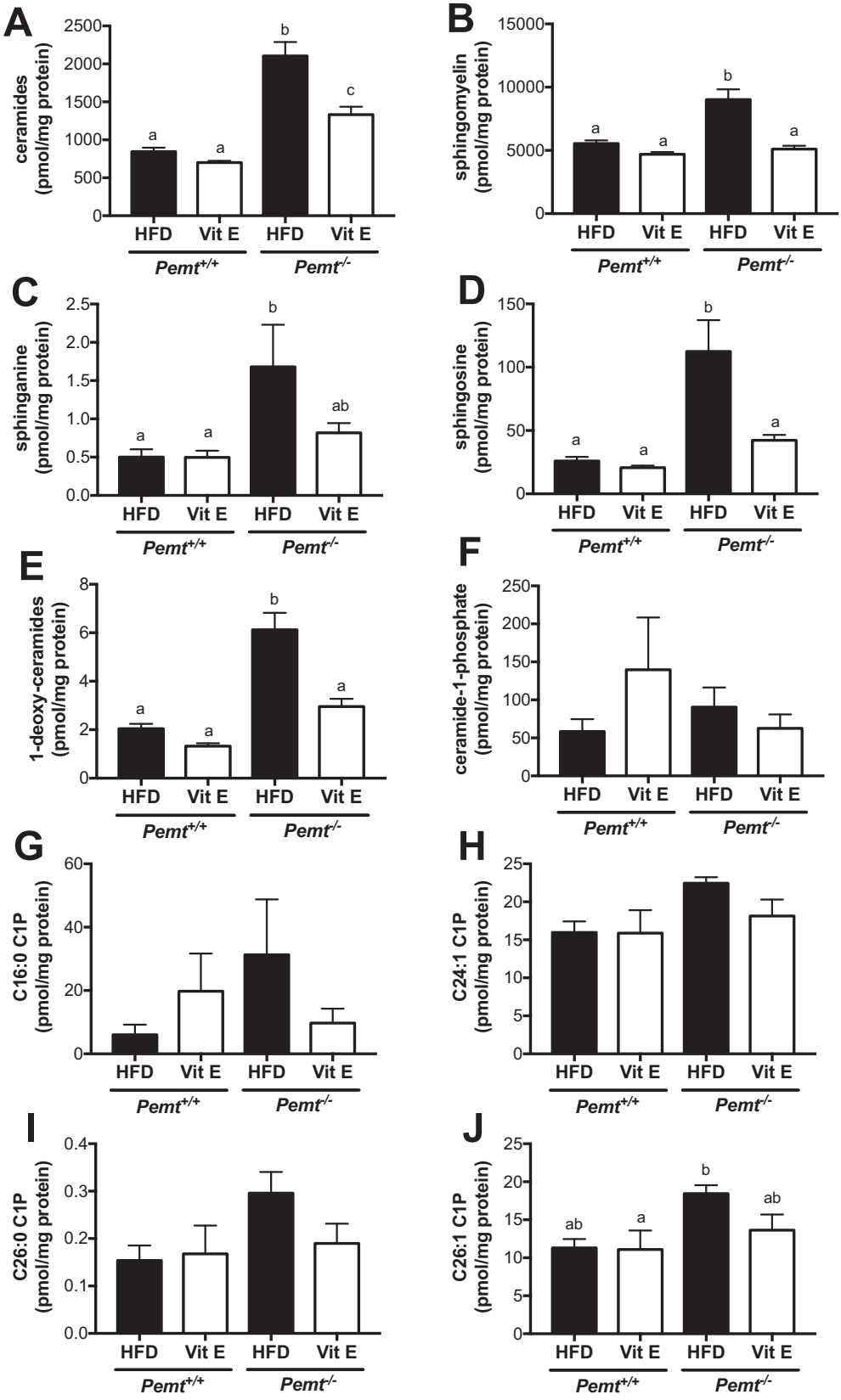

Fig. 6. Ceramide metabolism is normalized in $Pemt^{-/-}$ mice treated with vitamin E. Quantification by mass-spectrometry of *A*: total ceramides; *B*: total sphingomyelin; *C*: total sphinganine; *D*: total sphingosine; *E*: total 1-deoxyceramides; *F*: total ceramide 1-phosphate; *G-I*: ceramide 1-phosphate species (C16:0, C24:1, C26:0, C26:1), in liver samples from $Pemt^{+/+}$ and $Pemt^{-/-}$ mice fed a HFD or a HFD supplemented with vitamin E. All values are means ± SEM ($n = 5$ per group). Values that do not share a letter are significantly different ($P < 0.05$).

the progression of NASH [22], was also strongly increased in *Pemt*$^{-/-}$ mice compared to *Pemt*$^{+/+}$ mice and treatment with vitamin E reduced these levels by > 50% (Fig. 6E). In contrast, mass-spectrometry analysis did not show any significant differences in total ceramide 1-phosphate (C1P) levels or in the individual species of C1P (C16:0, C24:1, C26:1, and C26:0; Fig. 6F-J).

4. Discussion

Although the exact mechanism responsible for NAFLD development and progression is still poorly understood, oxidative stress could be the "second hit" triggering the transition from steatosis to steatohepatitis, and promoting hepatic damage, inflammation and fibrosis [39,40]. We investigated whether treatment with vitamin E could prevent the development of NASH in a mouse model of reduced PC synthesis. In summary, we observed improved VLDL-TG secretion from the liver, and normalization of cholesterol metabolism, but no reduction in hepatic TG upon vitamin E supplementation in HFD-fed *Pemt*$^{-/-}$ mice. Nevertheless, vitamin E supplementation efficiently prevented hepatic oxidative stress, inflammation and fibrosis, Moreover, aberrant ceramide metabolism in *Pemt*$^{-/-}$ mice was restored with vitamin E supplementation. Thus, vitamin E supplementation prevented the progression from simple steatosis to steatohepatitis in mice lacking PEMT.

4.1. Experimental and clinical use of vitamin E to treat NAFLD

Previous studies in animals with fatty liver disease have demonstrated a beneficial effect of vitamin E on liver health. In mice fed a methionine- and choline-deficient diet, vitamin E treatment ameliorated steatosis, oxidative stress, hepatic apoptosis, inflammation and fibrosis [41,42]. In obese (*ob/ob*) mice, treatment with α- or γ-tocopherol prevented lipopolysaccharide-induced NASH, but not steatosis [43], which is similar to what we observed in *Pemt*$^{-/-}$ mice. Furthermore, the progression of NAFLD caused by partial hepatectomy was also attenuated by vitamin E treatment [26]. In contrast to the studies with experimental animals, the benefits of vitamin E in humans have been less clearly delineated. Two large randomized clinical trials have been conducted to evaluate the efficacy of vitamin E to ameliorate NASH. The Pioglitazone versus Vitamin E versus Placebo for the treatment of nondiabetic Patients with Non-alcoholic Steatohepatitis (PIVENS) study investigated the effect of vitamin E (800 IU/day) in non-diabetic, non-cirrhotic adults with NASH [44]. Vitamin E compared to placebo significantly improved NASH, as it reduced steatosis, inflammation, hepatocellular ballooning, but not fibrosis. This outcome in humans is somewhat different than our data in *Pemt*$^{-/-}$ mice, where vitamin E did reduce fibrosis, but not steatosis. The Treatment of NAFLD in Children (TONIC) study investigated vitamin E in children with NASH [45]. Resolution of NASH was significantly higher in vitamin E versus placebo treatment group, due to reduced hepatocellular ballooning. However, vitamin E did not reduce steatosis, inflammation or fibrosis in this study. Although vitamin E seems effective in improving NASH in non-diabetic subjects, there have not been clinical trials carried out in diabetic subjects. Considering that up to 75% of people with NASH also suffers from type 2 diabetes, this is an area that needs to be addressed. We believe that our study provides new insights in the mechanism by which vitamin E ameliorates NASH, which might result in better designed interventions for NASH in humans.

4.2. Oxidative stress and NAFLD

Oxidative stress occurs when there is an imbalance between reactive oxygen species production and antioxidant defenses. In the liver, increased oxidative stress and chronic inflammation are considered key features for the progression from simple hepatic steatosis to steatohepatitis or NASH. Several studies show a connection between the severity of NASH and the degree of oxidative stress [35,36,46,47]. During hepatic steatosis, there is an increased influx of fatty acids derived from the circulation and/or from *de novo* lipogenesis, often in combination with reduced fatty acid oxidation. In NAFLD patients, mitochondrial respiratory chain complexes are often decreased, resulting in an increased production of reactive oxygen species (ROS) [48,49]. In addition, NASH patients exhibit insufficient antioxidant defenses, such as GSH, superoxide dismutase and catalase, resulting in oxidative stress in the liver [36,50]. Increased ROS can cause cellular damage and detrimental responses in several cell types of the liver. In Kupffer cells, oxidative stress can induce the production of cytokines, including TNFα, TGF-β, FAS-ligand and IL-8 [51]. ROS exposure can also activate hepatic stellate cells and induce proliferation and collagen synthesis [52]. Thus, oxidative stress can cause apoptosis, inflammation and fibrosis in the liver and thereby progress simple steatosis into NASH. The main triggers of these cellular responses are products of ROS-induced lipid peroxidation. Due to its hydrophobic nature, vitamin E is especially effective in stopping the chain reaction of lipid peroxidation. We demonstrated that HFD-fed *Pemt*$^{-/-}$ mice indeed exhibit increased lipid peroxidation, which was completely prevented by dietary vitamin E supplementation. By preventing this type of oxidative stress, vitamin E likely prevented the activation of both Kupffer cells and stellate cells, thereby preventing the development of inflammation and fibrosis in livers of *Pemt*$^{-/-}$ mice.

4.3. Sphingolipid metabolism in NAFLD

In the past decades ceramides have emerged as intracellular signaling molecules involved in the regulation of differentiation, proliferation, and apoptosis. Recently, ceramides have also been linked to fatty liver disease development, as they can act as key lipid mediators of insulin resistance, oxidative stress, and inflammation in different organs [12,18–22]. In response to inflammatory signals, such as TNFα or IL-1β, ceramide formation and release from membrane sphingomyelin is increased [25,53]. Elevated ceramide levels can promote ROS production in the mitochondria by interfering with the electron transport chain [15,23–25]. This increase in oxidative stress can then again lead to progression from NAFLD to NASH. Thus, ceramides seem to be part of a perpetual cycle in which ROS-induced cytokine production causes ceramide levels to increase, which in turn exacerbates oxidative stress and inflammation. Hence, we sought to determine whether ceramide metabolism was altered in HFD-induced NAFLD in *Pemt* $^{-/-}$ mice, and whether vitamin E could influence ceramide metabolism or ceramide-mediated events. We observed marked accumulation of ceramides in the livers of *Pemt*$^{-/-}$ mice, but no increases in the mRNA levels of the enzymes involved in ceramide synthesis (*Spt1*, *Cers2*, *Cers6* or *Asmase*). Nevertheless, inflammatory cytokines may have induced the activity of these enzymes, rather than transcription, resulting in elevated ceramide formation. Besides, given that liver is a key site for ceramide synthesis, that up to 80% of plasma ceramides are associated with VLDL/LDL particles [54,55], and that VLDL secretion from liver is impaired in HFD-induced NAFLD in *Pemt*$^{-/-}$ mice, the majority of hepatic ceramides is likely accumulating due to impaired secretion from the liver. Interestingly, *Cerk*, the enzyme that catalyzes the production of anti-apoptotic ceramide 1-phosphate from ceramide, and *Asah1*, an enzyme that degrades ceramides to sphingosine, which has opposing effects to ceramides in some cell types [56,57], were both overexpressed in *Pemt*$^{-/-}$ animals fed a HFD. The induction of *Cerk* and *Asah1* expression could serve to reduce ceramide levels within the cell in an attempt to limit the detrimental accumulation of ceramides in the liver. VLDL-TG secretion was partially restored in *Pemt*$^{-/-}$ mice upon vitamin E supplementation, which could be linked to the prevention of ER stress in these mice. As a result of the increase in VLDL production, ceramides would also be secreted from the liver at a higher rate. Together with the possibly reduced cytokine-mediated release of ceramides from the plasma membrane, this prevented the accumulation of ceramides and other sphingolipids in hepatocytes and normalized *Cerk* and *Asah1*

expression, since their induction would no longer be needed.

Interestingly, hepatic TG concentrations in *Pemt*$^{-/-}$ mice were not lowered upon vitamin E supplementation. Even though VLDL-TG secretion in *Pemt*$^{-/-}$ mice was increased by vitamin E supplementation, it was still strongly impaired compared to *Pemt*$^{+/+}$ mice. In addition, *de novo* lipogenesis seemed to be increased and fatty acid oxidation decreased in *Pemt*$^{-/-}$ livers after treatment with vitamin E. The increased adiposity in *Pemt*$^{-/-}$ mice could also supply more fatty acids to the liver for storage in lipid droplets. These processes could counteract the increase in VLDL-TG secretion, leaving hepatic TG levels unaltered. The lipid droplets in vitamin E-supplemented *Pemt*$^{-/-}$ livers were remarkably smaller but more abundant, which was supported by an increase in PLIN2 protein. Smaller lipid droplets would render the lipids more metabolically accessible, i.e. used more readily for oxidation, and thereby also contribute to improved liver health.

4.4. Conclusion

Altogether, our data support the therapeutic potential of vitamin E in the treatment of NASH. Through the prevention of oxidative stress, dietary vitamin E supplementation reduced hepatic inflammation and fibrosis, and it restored aberrant ceramide metabolism in this mouse model of impaired PC synthesis. Dietary vitamin E supplementation could thus be a viable therapeutic option in the continuously growing population of patients with hepatic steatosis to prevent the progression into the more detrimental stages of NAFLD.

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Author contributions

D.E.V., R.L.J. and J.N.V. designed the experiments. N.P., R.D.C., S.L., S.E.K., A.H.M. and J.N.V. performed the experiments. N.P. wrote the first draft of the paper. R.D.C., A.H.M., A.G-M., D.E.V., R.L.J. and J.N.V. read and revised the manuscript. N.P., D.E.V., R.L.J. and J.N.V. are the guarantors of this research and had full access to all the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis.

Disclosures

The authors confirm that there are no conflicts of interest relevant to this article.

Transparency document

The Transparency document associated with this article can be found, in online version.

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