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Hepatic deletion of serine palmitoyl transferase 2 impairs ceramide/sphingomyelin balance, bile acids homeostasis and leads to liver damage in mice

Justine Lallement, Ilyès Raho, Grégory Merlen, Dominique Raintean, Mikael Croyal, Melody Schiffano, Nadim Kassis, Isabelle Doignon, Maud Soty, Floriane Lachkar, Michel Krempf, Matthias Van Hul, Patrice D. Cani, Fabienne Foufelle, Chloé Amouyal, Hervé Le Stunff, Christophe Magnan, Thierry Tordjmann, Céline Cruciani-Guglielmacci

A B S T R A C T

Ceramides (Cer) have been shown as lipotoxic inducers, which disturb numerous cell-signaling pathways, leading to metabolic disorders such as type 2 diabetes. In this study, we aimed to determine the role of de novo hepatic ceramide synthesis in energy and liver homeostasis in mice. We generated mice lacking serine palmitoyltransferase 2 (Sptlc2), the rate limiting enzyme of ceramide de novo synthesis, in liver under albumin promoter. Liver function, glucose homeostasis, bile acid (BA) metabolism and hepatic sphingolipids content were assessed using metabolic tests and LC-MS. Despite lower expression of hepatic Sptlc2, we observed an increased concentration of hepatic Cer, associated with a 10-fold increase in neutral sphingomyelinase 2 (nSMase2) expression, and a decreased sphingomyelin content in the liver. Sptlc2-/- mice were protected against obesity induced by high fat diet and displayed a defect in lipid absorption. In addition, an important increase in tauro-muricholic acid was associated with a downregulation of the nuclear BA receptor FXR target genes. Sptlc2 deficiency also enhanced glucolipid tolerance and attenuated hepatic glucose production, while the latter effect was

Abbreviations: Akt/Pkb, Protein Kinase B; Alat, alanine amino-transferase; Asat, aspartate amino-transferase; BA, bile acids; Bax, Bcl-2-associated X protein; Bcl2, B-cell lymphoma 2; cAMP, cyclic adenosine monophosphate; CDCA, chenodeoxycholic acid; Cer, ceramides; CerS, ceramide synthase; Chop, C/EBP homologous protein; Ct19, cytokeratin 19; CycloA, cyclophilin A; ECM, extracellular matrix; EDL, extensor digitorum longus; ER, endoplasmic reticulum; FFA, free-fatty acid; Fxr, farnesoid X receptor; G6p, glucose-6-phosphate; G6pase, glucose-6-phosphatase; GlyTT, glycerol tolerance test; Grp78, G-protein-coupled receptor 78; Gr-1, granulocyte receptor-1; HFD, high fat diet; ITT, insulin tolerance test; KD, knock-down; NASH, non-alcoholic steatohepatitis; nSMase2, neutral sphingomyelinase 2; OGTT, oral glucose tolerance test; Pca, pyruvate carboxylase; PTT, pyruvate tolerance test; Ria, regular chow; Sm, sphingomyelin; Smase, sphingomyelinase; Smad3, sphingomyelin phosphodiesterase 3; Sptlc2, serine palmitoyltransferase 2; T2D, type 2 diabetes; Tbp, TATA-binding protein; Tgfr5, Takeda G protein-coupled receptor; T-MCA, tauro-muricholic acid; TNF receptor 1; TNfr, tumor necrosis alpha; Tβ-MCA, tauro-beta-muricholic acid; WT, wild type.

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dampened in presence of nSMase2 inhibitor. Finally, Sptlc2 disruption promoted apoptosis, inflammation and progressive development of hepatic fibrosis, worsening with age. Our data suggest a compensatory mechanism to regulate hepatic ceramides content from sphingomyelin hydrolysis, with deleterious impact on liver homeostasis. In addition, our results show the involvement of hepatic sphingolipid modulation in BA metabolism and hepatic glucose production in an insulin-independent manner, which highlight the still under-researched role of ceramides in many metabolic functions.

1. Introduction

In the last few decades, number of studies have pointed out deleterious roles of ceramides (Cer) especially in the development of metabolic disorders such as non-alcoholic steatohepatitis (NASH) or type 2 diabetes (T2D) [1]. Thus, this specific class of lipids, which belongs to sphingolipids (SL) family, are proposed to be important mediators of free-fatty acid (FFA)-induced β cell dysfunction and apoptosis, and FFA-induced insulin resistance in insulin target tissue [2]. At the cellular level, increased concentrations of Cer promote insulin resistance by inhibiting insulin signaling pathway, and Cer can regulate autophagy, reactive oxygen species production, cell proliferation, inflammation and apoptosis [3].

In mammals, three main pathways have been described to produce Cer. First, the *de novo* synthesis pathway starts on the cytoplasmic face of the endoplasmic reticulum (ER) with the condensation of a coenzyme A-linked fatty acid (palmitoyl-CoA) and L-serine palmitoyltransferase (SPT) to form 3-ketosphinganine. Three different subunits of SPT called as SPTLC1, SPTLC2, and SPTLC3 have been described. SPTCL1 could act as a dimer with SPTCL2 and/or SPTCL3, which carry out the pyridoxal 5-phosphate binding motif, essential for the catalytic activity [4]. Then, Cer are transported to the Golgi apparatus to be metabolized into more complex sphingolipids such as glucosylceramides and sphingomyelin (SM). Second, the catabolic sphingomyelinase (SMase) pathway leads to the degradation of SM into Cer and takes place at different sub-cellular localizations (plasma membrane, mitochondria, lysosome and Golgi) [5]. The third pathway is called the “salvage pathway” from late endosomes/lysosomes, which generate Cer from the breakdown of complex sphingolipids [6]. In both salvage and *de novo* synthesis pathways, Cer are produced by ceramide synthase (CerS) through N-acylation of a sphingoid base. There are six different isoforms of CerS, which lead to the production of different Cer species, according to the chain length of the acyl-CoA added [7]. Among them, C18:0-Cer and C16:0-Cer have been pointed out as apoptosis and inflammatory inducers and play a key role in the development of insulin resistance [8–10].

The liver is a key metabolic organ, which governs body energy metabolism. In particular, the intrahepatic accumulation of lipids, especially Cer, strongly correlates with the risk of T2D [11]. In addition, it has been demonstrated that *in vivo* or *in vitro* inhibition of SPT, using myriocin, decreases Cer accumulation in plasma and liver and improves hepatic fibrosis, steatosis and insulin sensitivity [12,13]. Hepatocytes, the major parenchymal cells in the liver, carry out many metabolic functions, including the production of bile acids (BA). BA are amphipathic molecules, which allow dietary lipid absorption and also act as signaling molecules through their action on nuclear receptors such as the Farnesoid X receptor (FXR) or G protein-coupled BA receptors, such as Takeda G protein-coupled receptor 5 (TGR5) or SIP receptor 2 (SIPR2) [14,15]. FXR is activated by chenodeoxycholic acid (CDCA) but inhibited by tauro-beta-muricholic acid (Tβ-MCA), and regulates BA synthesis [16,17]. Numerous studies have demonstrated the role of FXR signaling in glucose homeostasis and especially in hepatic glucose production [18]. Thus, beyond their detergent properties, BA modification could trigger endocrine-mediated metabolic disorders. Interestingly, inhibition of intestinal FXR signaling prevents obesity induced by high fat diet and metabolic disease such as insulin resistance and fatty liver [19]. Recently, SmpD3 (sphingomyelin phosphodiesterase 3), a gene encoded for the neutral sphingomyelinase 2 (nSMase2) and Sptlc2 gene, both involved in Cer production, have been identified as FXR target genes [20,21]. These results suggest the existence of a Cer/FXR/BA signaling axis, which could regulate glucose and lipid metabolism.

We investigated the role of *de novo* Cer synthesis in the liver of mice fed with regular chow (RC) or high fat diet (HFD) on energy homeostasis. Using the cre-lox system, we developed a mouse model to decrease Cer *de novo* synthesis in liver by targeting the rate-limiting enzyme subunit, Sptlc2, in hepatocytes.

2. Materials and methods

2.1. Animals

All procedures were carried out in accordance with the ethical standards of French and European regulations (European Communities Council Directive, 86/609/EEC). Animal use and procedures were approved by the ethics committee of the University of Paris and by the French ministry of research under the reference #20160404149129. *Sptlc2<sup>Δ<sub>WTLiv</sub></sup>* mice (kindly given by Dr. Xian-Cheng Jiang, SUNY Downstate Health Sciences University, New York, USA) were crossed with AlbCre<sup>lox/lox</sup> mice (kindly given by Dr. Catherine Postic, Cochin Institute, Paris, France) to generate mice lacking Sptlc2 expression in liver (albumin Cre expresses in both hepatocytes and cholangiocytes), *Sptlc2<sup>Δ<sub>WTLiv</sub></sup></sup>, or littermate controls, *Sptlc2<sup>Δ<sub>WTLiv</sub></sup></sup>. Male *Sptlc2<sup>Δ<sub>WTLiv</sub></sup></sup> and *Sptlc2<sup>Δ<sub>WTLiv</sub></sup></sup>* were housed in a controlled environment with tap water and ad libitum regular food (AD4 diet, SAFE, Augy, France) before starting a high fat diet (HFD260 diet, SAFE, Augy, France) at 8 weeks of age. Mice were maintained on a 12-h light/12-h dark cycle and cages were enriched with tunnels. Number of mice and suffering were minimized in accordance with the 3Rs.

2.2. Metabolic phenotyping

All the protocols used for metabolic phenotyping (body mass composition and metabolic tests) are described in the supplementary methods.

2.3. GW4869 treatment

Transgenic mice (*Sptlc2Δ<sub>WTLiv</sub>* and littermate controls) were orally treated at 45 day-old with a selective inhibitor of nSMase 2, GW4869 (catalog n° D1692, SIGMA-ALDRICH, Saint-Louis, MO, USA) in corn oil + DMSO 1 % (vehicle) at 15 mg/kg/day. After the treatment, sphingomyelin measurement was performed on primary hepatocytes to confirm the increase in sphingomyelin content.

2.4. Energy content of feces by bomb calorirometer

Feces were collected every 24 h during one week, dried to constant weight (0.001 g) at 60 °C, and energy content of dried feces (kJ/g) was analyzed for each individual mouse using a bomb caloriometer (IKA C200, Staufen, Germany).

2.5. RNA and protein analysis

Western blot analysis, liver histology for immunohistochemistry or
staining, RNA extraction, cDNA synthesis and real-time PCR were performed using standard protocols already described previously [22,23]. Detailed procedure with a list of the primer sequences can be found in the supplementary methods.

2.6. Quantification of phosphorylated (Ser473) Akt and total Akt

Protein extraction was carried out from liver, adipose tissue and muscle following the same protocol as was used for western blot analysis. Quantitative determination of phospho-Akt (Ser473) and total Akt was assessed by a Meso Scale Discovery (MSD, Kenilworth, NJ, USA) multiplex electrochemiluminescence immunoassay system (Phospho (Ser473)/Total Akt Whole Cell Lysate Kit, catalog no. K15100D) according to manufacturer’s instructions.

2.7. Biochemical analyses

Liver triglycerides were measured using triglyceride determination kit from Sigma (catalog no. TR0100, SIGMA-ALDRICH, Saint-Louis, MO, USA) according to the manufacturer’s instructions. Liver glycogen content was measured using amyloglucosidase enzyme (catalog no. A7095, SIGMA-ALDRICH, Saint-Louis, MO, USA) assay according to Roehrig and Allred method [24]. Glucose was then determined using Glucose GOD-PAP kit (catalog no. 87,109, BIOLABO, Maizy, France). Plasma concentrations of alanine amino-transferase (ALT), aspartate aminotransferase (ASAT), direct bilirubin and alkaline phosphatase were determined using an automated Monarch device (CEFI, IFRO2, Paris, France) as described previously [25].

2.8. Isolation of primary mouse hepatocytes

Hepatocytes from 45 day-old Sptlc2ΔLiv and Sptlc2WtLiv mice were isolated as previously described [26]. Procedure for glucose production measurement from primary hepatocytes is described in supplementary methods.

2.9. G6pase activity assay and liver G6P content measurement

G6Pase activity was assayed in liver homogenates for 10 min at 30 °C at pH 7.3 in the presence of a saturating glucose-6-phosphate concentration of 20 mM as described [27]. Hepatic glucose-6-phosphatase (G6P) determinations were carried out as previously described [28].

2.10. Quantification of sphingolipids

LC-MS/MS technic was used for sphingolipids quantification. Detailed procedure can be found in the supplementary methods.

2.11. Bile acids measurements

BA measurements were performed on mouse gallbladder bile, plasma, liver and feces by high-performance liquid chromatography-tandem mass spectrometry as described in [29,30]. The hydrophobicity index takes into account the retention time of different BA on a C18 column with a gradient of methanol; the LCA has the highest retention time, the TUDCA-3S has the lowest.

2.12. Gut microbiota analysis

We analyzed gut microbiota in Sptlc2ΔLiv and Sptlc2WtLiv mice under HFD, by collecting the caecum content at sacrifice and performing 16S ribosomal RNA sequencing. Detailed procedure can be found in the supplementary methods.

2.13. Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was performed using Student’s t-test, Mann-Whitney U test or two-way ANOVA followed by two-by-two comparisons using Bonferroni’s post hoc test (Graphpad Prism 6 Software, La Jolla, CA, USA). Differences were considered significant at p < 0.05.

3. Results

3.1. Genetic deletion of Sptlc2 in liver increases selective ceramide content in the liver but decreases sphingomyelin content associated with an up-regulation of nSmase2/SmpD3

Sptlc2Δlox/lox mice were crossed with heterozygous mice, which expressed the proteinCre driven by the albumin promoter (Fig.S1A). Sptlc2 hepatic mRNA level was reduced to 60 % of wild type (WT) level both in Sptlc2ΔLiv mice fed with RC or HFD (Fig.S1B), consistent with the fact that hepatocytes (main albumin producers) represent about 70 % of all liver cells [31]. Enzymatic SPT2 activity was accordingly reduced to 60 % of controls (Fig. S1C). Surprisingly, in our genetic model, Sptlc2 deficiency does not induce Cer reduction in liver of RC mice (Fig. 1A) and even elicits hepatic Cer accumulation in HFD fed mice (Fig. 1B). Moreover, Sptlc2 deletion modifies hepatic Cer species distribution. Thus, we showed C16:0-Cer, C18:0-Cer and C24:1-Cer accumulation and even elicits hepatic Cer accumulation in HFD fed mice (Fig. 1B). Furthermore, Sptlc2 deletion modifies hepatic Cer species distribution. Thus, we showed C16:0-Cer, C18:0-Cer and C24:1-Cer accumulation and even elicits hepatic Cer accumulation in HFD fed mice (Fig. 1B). C16:0-Cer and C18:0-Cer synthesis were up regulated in Sptlc2ΔLiv mice and their littermate controls confirmed the increase in C16:0-Cer and the decrease in C22:0-Cer in the liver of mutant mice (Fig. 1C-D). Quantification of Cer in isolated hepatocytes from Sptlc2ΔLiv mice and their littermate controls confirmed the increase in C16:0-Cer and the decrease in C22:0-Cer (Fig. S1C). Interestingly, Ces5 and Ces6, which catalyze C16:0-Cer and C18:0-Cer synthesis were up regulated in Sptlc2ΔLiv mice liver upon RC and HFD (Fig. 1E-F). Please refer to Fig. S1D for ceramide synthesis pathways scheme.

We measured a reduction in SM content in Sptlc2ΔLiv mice liver, under both diets (Fig. 1A-B). These results are also confirmed in primary hepatocytes from RC mice (Fig.S1E). These data suggest that the SMase pathway is over-activated to produce Cer through the hydrolysis of sphingomyelin. In line, we measured a dramatic increase in neutral sphingomyelinase 2 (nSmase2) mRNA level, which is specifically expressed at plasma membrane, in the liver of KD mice, independently of the diet (Fig. 1G-H). Moreover, up-regulation of nSmase2 protein levels was also detected by western blot in the liver of RC Sptlc2ΔLiv mice (Fig. 1I). The analysis of SM species distribution reveals that the modification of hepatic SM profile is similar to the one observed for Cer species (i.e increase of C16:0:SM and decrease of C22:0:SM) (Fig. 1J-K, Fig. S1F) suggesting a remodeling of Cer species profile due to Ces5 and Ces6 up-regulation. Of note, Cer and SM concentration in the plasma were found to be increased in mice lacking Sptlc2 as compared with WT. Then, hepatic Cer and especially hepatic SM, substrate of nSmase2, could be provided by plasma sphingolipids (Fig. S2). Cer and SM contents have been also measured in epididymal adipose tissue, and showed a non-significant trend towards increased SM in the adipose tissue of Sptlc2ΔLiv mice (data not shown).

Overall, these data suggest a compensatory mechanism through the SMase pathway to maintain an elevated Cer content in the liver despite the inhibition of de novo synthesis pathway.

3.2. Sptlc2 deficiency in liver impairs bile acid homeostasis leading to a defect in lipid absorption and resistance against obesity induced by high fat diet

We measured BA composition and concentration in the gallbladder bile, liver, ileum and plasma and showed that Sptlc2ΔLiv compared to WT mice exhibited substantially elevated BA concentrations in these compartments (Fig. 2A, Fig. S3A), consistent with the reduction of bile flow (i.e. cholestasis) described in prior studies [32]. Moreover, we showed in this study that BA composition was strongly modified between the two
groups of mice, independently of the diet (Fig. 2B, Fig. S3B). A dramatic increase in β-MCA, the dominant form of muricholic acid and in its preponderant conjugated form, tauro-muricholic acid (T-MCA) was observed in mutant mice (Fig. 2B-C, Fig. S3B—C) while secondary BA, most hydrophobic, were almost absent. Except in the plasma, cholic acid (CA) concentration was also reduced in enterohepatic tissues in Sptlc2ΔLiv mice (Fig. 2B, Fig. S3B). In line with these data, the hydrophobicity index of the BA pool was decreased in liver, gallbladder bile, ileum and plasma from Sptlc2ΔLiv as compared with WT mice (Fig. 2D, Fig. S3D). We measured serum C4 levels, which reflect BA synthesis, and showed higher levels in Sptlc2ΔLiv mice whatever the diet (Fig. S3E). Elevated plasma BA were associated with severe jaundice in mutant mice, along with moderate cytolyis and strong elevation of alkaline phosphatase and bilirubin (Fig. 2E and Table 1), consistent with cholestasis. These changes in BA homeostasis were accompanied with altered gut microbiota profile, consistent with the fact that BA and gut microbiota are in close interrelationship as microbiota converts primary BA into secondary BA. At the phylum level, we evidenced increased Bacteroides and decreased Proteobacteria in Sptlc2ΔLiv mice (Fig. S6A), while at the family level, Sptlc2ΔLiv mice microbiome showed the presence of bacteria almost absent in the controls such as Sutterellaceae and Erysipelotrichaceae (Fig. S6B).

Interestingly in this cholestatic context, mutant mice exhibited disrupted localization of cytotherakerin 19 (Ck19) immunostaining (a marker of cholangiocytes) (Fig. 2F), suggesting that cholangiocyte injury occurred in these mice and may contribute to bile flow impairment, i.e. to cholestasis. Furthermore, we measured main BA transporters and BA synthesis enzymes mRNAs. Interestingly, Cyp8b1 (CA synthesis) and Cyp27a1 (alternative pathway) mRNAs were both reduced in Sptlc2ΔLiv mice liver (Fig. 2G). Together with a strong decrease in mRNA Ntcp level (hepatocyte basolateral uptake BA transporter) (Fig. 2H), these variations could be part of an adaptive response to BA accumulation in hepatocytes as already well described [33]. In addition, consistent with the dramatic increase in T-MCA, a BA reported as an FXR antagonist [17], a significantly decreased expression in the Fxr target genes Shp and Fgft15 was observed in ileum and liver from Sptlc2ΔLiv as compared with WT mice (Fig. 2I-J). Thus, Sptlc2 deficiency in liver leads to profound changes in BA synthesis and transport together with a decreased FXR target genes expression in both ileum and liver.

As modifications of BA composition, synthesis and transport could impair lipid absorption [34], we fed Sptlc2ΔLiv mice and their littersmate controls with HFD for 8 weeks in an attempt to induce obesity. Interestingly we measured an increased energetic density of KD mice feces (Fig. 3A) associated with impaired triglyceride absorption, as revealed by reduced plasma triglycerides in Sptlc2ΔLiv as compared with WT mice up to 6 h after an oral olive oil charge (Fig. 3B). In line with these data, Sptlc2 mutant mice upon HFD (starting from week 4) gained significantly less body weight than control animals, despite similar food intake (Fig. 3C-D). Of note, RC fed Sptlc2ΔLiv and WT mice exhibited similar body weight gain (Fig. 3E). However, mass composition analysis revealed a decreased fat mass in KD compared to WT mice upon both RC and HFD (Fig. 3F). Altogether, these results suggest that Sptlc2ΔLiv mice exhibited a defect in lipid absorption leading to body weight gain resistance upon HFD.

3.3. Genetic deletion of Sptlc2 in liver impairs hepatic glucose production and storage in an insulin-independent manner

The liver is at the core of glucose metabolism: it produces glucose from the breakdown of glycogen, or through gluconeogenesis from lactate, pyruvate, glycerol and amino acids [35]. Interestingly, decreased level of hepatic Sptlc2 prevents glucose intolerance in mice challenged with HFD (Fig. 4A-C). This could be due, in part, to the lack of weight gain upon HFD in Sptlc2 KD mice (see above). However, de novo glucose production from pyruvate (Fig. 4D-F) and glycerol (Fig. 4G-I) were decreased in KD mice either fed with HFD or RC. This observed in vivo defect in hepatic glucose production is supported by glucose production measurements isolated on primary hepatocytes (Fig. S4A–B). Two and three hours after hepatocyte stimulation induced by medium without glucose containing pyruvate and glycerol, we detected a tendency of a reduced glucose production in isolated hepatocytes from Sptlc2ΔLiv mice. Indeed, hepatic Sptlc2 deficiency decreases glucose-6-phosphatase (G6pase) and pyruvate carboxylase (Pc) expression levels (Fig. 4J). Furthermore, G6Pase activity and glucose-6-phosphate (G6P) content were decreased in mutant mice liver (Fig. 4K-L).

Another series of mice were orally treated with a Nsmase2 inhibitor, GW4869 (4M-P). Sptlc2ΔLiv treated with GW4869 (15/mg/kg/day) displayed no significant difference for AUCs of PTT and GlyTT compared with WT, treated or not (Fig. 4 O–P), suggesting a normalization of their hepatic glucose production. We checked ex vivo, on isolated hepatocytes, the effect of GW4869 treatment on SM hydrolysis and thus, SM concentration, and measured as expected an overall increase in SM concentration (P = 0.024, n = 6, not shown) consistent with decreased sphingomyelinase activity.

In order to evaluate the role of insulin on glucose homeostasis in our transgenic model, we measured insulin secretion during the course of glucose tolerance test and insulin sensitivity. Hepatic deletion of Sptlc2 did not impair insulin secretion or systemic insulin sensitivity (Fig. 5A-C). Moreover, as expected based on C16:0-Cer and C18:0-Cer accumulation in the liver, activation by phosphorylation on serine 473 of Akt/Pkb (protein kinase B), is reduced in the liver of mutant mice (Fig. 5D-F). Thus, systemic insulin sensitivity is likely compensated by an enhanced Akt/Pkb phosphorylation in the muscle (Extensor Digitorum Longus, EDL). In addition, reduced liver glycogen content was also observed in Sptlc2ΔLiv mice at fed state or at 5 h fasted state, as compared with WT mice (Fig. 5G) and we also measured in mutant mice a lower glycemia after 6 h fast (Fig. S4 C–D). Glycogen production in the liver is also controlled by insulin, thus these results are in line with hepatic insulin resistance observed in Sptlc2ΔLiv mice. Altogether, these results demonstrated that hepatic Sptlc2 deficiency impairs hepatic glucose production in an insulin-independent manner.

3.4. Hepatic Sptlc2 deficiency leads to liver injury and proliferation, inflammation and fibrosis

Based on our data, we next asked whether modulating Cer levels could lead to liver damage. Sirius red and H&E liver section staining revealed that Sptlc2ΔLiv mice exhibited necrosis, inflammatory infiltration and fibrosis, increasing progressively with age (Fig. 6A). At 20 days, bile infarcts (i.e. necrotic cell clusters characteristic of BA overload), were observed in livers from Sptlc2ΔLiv mice but not in WT, without any significant fibrosis. At 45 days and even more at 120 days, patent fibrosis was observed, with both perisinusoidal and portal-portal accumulation.
Fig. 2. Genetic deletion of Sptlc2 in hepatocytes impairs BA composition and metabolism. (A-B) Total bile acids content and BA composition in liver, gallbladder bile, ileum and plasma of HFD fed mutant and control mice. (C) Tauro-muricholic acid concentration in liver and ileum. (D) Hydrophobicity index of bile acids pool contained in liver, gallbladder bile, ileum and plasma of HFD fed mice. (E) Photograph of representative plasma samples from Sptlc2WT/Liv or control mice upon RC or HFD. (F) Representative immunofluorescence image showing Ck19 in liver sections of RC fed 3-month-old mice. (G) Relative expression of main enzymes involved in bile acids synthesis in liver (G) and bile acids transporters in the liver (Bsep, Ntcp) or terminal ileum (Astb, Ostα/β) (H) of HFD fed mice, data are expressed in fold change relative to control. (I-J) Relative expression of Fxr and its target gene in the liver (Shp) or terminal ileum (Fgf15) of HFD fed mice, data are expressed in fold change relative to control. Analysis was performed in 4-month-old mice and HFD mice were fed with HFD for 2 months starting at 2 month-old; \( n = 4 \)–10 mice per groups; error bars represent SEM; \( *p < 0.05, **p < 0.01, ***p < 0.001 \) via Mann-Whitney U test, Student’s \( t\)-test when appropriate or two-way ANOVA followed by two-by-two comparisons using Bonferroni's post hoc test.
of extracellular matrix (ECM). Interestingly, HFD did not appear to exacerbate ECM accumulation in liver, demonstrating strong impact of Sptlc2 disruption on the setting of liver fibrosis. These results were associated with an increased liver weight without triglyceride accumulation, as compared to the littermate controls (Fig. 6.B-C).

As inflammation, which could be triggered by unbalanced Cer proportions, is known to be associated with liver disorders [36,37], we investigated the role of hepatic Cer metabolism in the development of liver inflammation, as compared to the littermate controls (Fig. 6.B-C).

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Sptlc2 WTm</th>
<th>Sptlc2 ΔLiv</th>
<th>p-value (Mann-Whitney U test)</th>
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<tr>
<td>Albumin (g/L)</td>
<td>28.3 ± 0.18</td>
<td>27.3 ± 0.24</td>
<td>0.243</td>
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<tr>
<td>ALAT (U/L)</td>
<td>47.6 ± 8.165</td>
<td>167.9 ± 11.99</td>
<td>0.00002 ***</td>
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<tr>
<td>ASAT (U/L)</td>
<td>140.7 ± 26.59</td>
<td>325.5 ± 49.09</td>
<td>0.0001 ***</td>
</tr>
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<td>Direct Bilirubin (μmol/L)</td>
<td>0.6 ± 0.1017</td>
<td>15.5 ± 3.929</td>
<td>0.0003 ***</td>
</tr>
<tr>
<td>Alkaline Phosphatase (U/L)</td>
<td>42.1 ± 3.491</td>
<td>469.1 ± 22.7</td>
<td>0.0003 ***</td>
</tr>
</tbody>
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Analysis were performed in 4 months-old mice and HFD mice were fed with HFD for 2 months starting at 2 months-old; values are means ± SEM; n = 6 mice per group; **p < 0.001 via Student’s t-test.

We also show that the modulation of sphingolipid metabolism in liver leads to a strong alteration of liver homeostasis, in particular we and even increases plasma and liver Cer content in HFD fed mice. However, we show a significant decrease of the C22:0-Cer specie in liver, and both C22:0-Cer and C24:0-Cer diminution is evidenced in primary hepatocytes. These Cer species are the most abundant in liver, partially because of the high expression level of CerS2 involved in C22:0-Cer and C24:0-Cer formation from dihydro sphingosine [38]. Thus, in our model, Sptlc2 deficiency reduces Cer production from de novo synthesis for the benefit of another production pathway. Indeed, nSmase2 mRNA and protein levels are up-regulated in Sptlc2ΔLiv mice, suggesting over-activation of the sphingomyelinase pathway at plasma membrane. Our data suggest for the first time to our knowledge a compensatory mechanism to maintain (and even to increase) Cer production though the dramatic up-regulation of nSmase2 expression.

A direct regulatory interaction between SPTLC2 and nSmase2 is unlikely due to different intracellular localization, however the decreased utilization of serine and palmitoyl-CoA (SPTLC2 substrates) could lead to a modulation of nSmase2 activity. Indeed, previous studies showed that nSmase2 was activated by fatty acids and phosphatidylserine [39-41]. In addition, the development of pro-inflammatory environment due to the lack of ceramide de novo synthesis could also induce nSmase2 up-regulation [42,43]. Interestingly, nSmase2 expression has been correlated with inflammation in different organs [43-45] and in particular the activation of TNF-R by a ligand as TNF-alpha has been shown to induce nSmase2 translocation to the plasma membrane and ceramides synthesis [46-48]. These is consistent with the up-regulation of both TNF-R and TNF-alpha expression in Sptlc2ΔLiv mice. Although the fine mechanisms leading to an overproduction of Cer remain unclear, these data suggest that Cer are essential components for the cell and that an attempt to alter their de novo synthesis would trigger a cellular response to avoid a drop in Cer content, at the expense of SM concentration.

Interestingly, acute reduction of Sptlc2 expression or activity using adenovirus targeting Sptlc2 in liver, or pharmacological inhibitors such as myricin, does not induce Cer accumulation [12,41], in contrast to chronic Sptlc2 deletion using genetic approach which led to Cer and especially C16:0-Cer increase in liver and adipose tissue [32,49]. In the liver, authors have shown that specific deletion of Sptlc2 induces SM reduction and Cer accumulation in plasma membrane of hepatocytes, supporting the hypothesis of a compensatory mechanism through over-activation of SM hydrolysis at plasma membrane [32]. Interestingly, CerS2 null mice display changes in the composition of liver Cer species, which are similar to these highlighted in our transgenic mouse model. In addition, the authors have also demonstrated an up-regulation of CerS5 and nSmase2 gene expression. These results corroborate the existence of a compensatory mechanism between the different pathways of Cer synthesis in liver [50].

We also observe the up-regulation of CerS5 and CerS6, which allows C16:0-Cer and C18:0-Cer formation from dihydro sphingosine. As neutral SMase 2 is known to preferentially use very long acyl chain SM (reduced in the liver and primary hepatocytes in our model), C16:0-Cer and C18:0-Cer accumulation could result from CerS5 and CerS6 up-regulation and their substrates would likely be provided by SM hydrolysis and ceramide action [51]. Moreover, C16:0-Cer accumulation in liver is consistent with the observed C16:0:SM increase in plasma and suggests C16:0:SM supply from one or several other tissues. This hypothesis suggests the existence of a crosstalk between the different tissues to normalize Cer production. Of note, besides SM transport to the liver by classical lipoproteins, SM species could be transported by extracellular vesicles [52]. Then, we demonstrated in mutant mice a dramatic increase in hepatic C16:0-Cer and C18:0-Cer which are well described as apoptotic inducers and inflammation triggers [53]. Taken altogether, our data suggest two distinct pools of Cer (in plasma membrane and endoplasmic reticulum) with likely different cellular functions.

We also show that the modulation of sphingolipid metabolism in liver leads to a strong alteration of liver homeostasis, in particular we...
evidenced an impairment of BA pool composition associated with a defect in intestinal lipid absorption. Our findings show that primary BA, especially T-MCA, are increased in \textit{Sptlc2}\textsuperscript{\textsubscript{ΔLiv}} mice and that secondary BA are almost absent. Bile acids and gut microbiota are in close interrelationship as microbiota converts primary BA into secondary BA, through first the action of Bile salt hydrolases, which allow BA deconjugation. Then, the action of other specific enzymes will end up the conversion of primary BA in secondary BA [54, 55]. In addition, BA composition could influence gut microbiome and then BA synthesis, transport or removal impairment could directly induce dysbiosis [56, 57].

We accordingly showed strong alteration of \textit{Sptlc2}\textsuperscript{\textsubscript{ΔLiv}} mice gut microbiota in accordance with the changes in BA homeostasis. Secondary BA, which are more hydrophobic than primary BA, are essential for intestinal lipid absorption [58]. Accordingly, we measured lower hydrophobic index in BA pool of \textit{Sptlc2}\textsuperscript{\textsubscript{ΔLiv}} mice leading to lower triglycerides absorption and increased energy density in feces. Taken together, these data are consistent with a full resistance to high-fat induced obesity in \textit{Sptlc2}\textsuperscript{\textsubscript{ΔLiv}} mice due to altered BA-dependent lipid absorption in the intestine. Of interest, food intake is similar in \textit{Sptlc2}\textsuperscript{\textsubscript{ΔLiv}} compared to the controls, supporting post-ingestive events rather than nervous perturbation of food behavior.

\textit{Sptlc2}\textsuperscript{\textsubscript{ΔLiv}} mice also displayed jaundice as reported [32]. Prior study has also reported alterations in BA transporters localisation and

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**Fig. 3.** Genetic deletion of \textit{Sptlc2} in hepatocytes prevents HFD-induced obesity.

(A) Energy density of feces collected every 24h of HFD fed mice. (B) Plasma triglycerides measurement after oral olive oil charge in HFD fed mice. (C–D) Body weight change in \textit{Sptlc2}\textsuperscript{\textsubscript{ΔLiv}} and their littermate controls upon HFD or RC. (E) Time course of food intake in HFD fed mice. (F) Body fat and lean composition, in grams. Analysis were performed in 4 months-old mice and HFD mice were fed with HFD for 2 months starting at 2 month-old; \(n=4–10\) mice per groups; error bars represents SEM; *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\) via Mann-Whitney U test, Student\'s t-test when appropriate or two-way ANOVA followed by two-by-two comparisons using Bonferroni\'s post hoc test.
Fig. 4. Genetic deletion of Sptlc2 in hepatocytes impairs hepatic glucose production and storage.
OGTT (A-B), PTT (D-E) and GlyTT (G-H) at 1 g/kg performed in RC or HFD fed mice (overnight fasting). (C) Respective areas under the curve for the OGTT (C), PTT (F) and GlyTT (I) in RC or HFD mice. (J) Relative expression of main enzymes involved in gluconeogenesis in liver of HFD fed mice, data are expressed in fold change relative to control. (K-L) G6Pase specific activity and G6P content in liver of HFD fed mice. PTT (M) and GlyTT (N) with respective areas under the curve (O–P) at 1 g/kg performed in RC fed mice orally treated with GW4869 (15 mg/kg/day for 14 days). n = 3–10 mice per group; error bars represents SEM; *p < 0.05, **p < 0.01, ***p < 0.001 via Mann-Whitney U test, Student’s t when appropriate or two-way ANOVA followed by two-by-two comparisons using Bonferroni’s post hoc test.
hepatocyte polarity in liver-specific Sptlc2 KO mice [32]. Although precise mechanisms of cholestasis development in this model remain to be fully deciphered, our data suggest that, in the lack of SPTLC2 in hepatocytes and cholangiocytes, intrahepatic bile duct cells were disorganized, a feature that likely contributed to bile flow impairment. How cholangiocytes would be injured in these mice remains to be elucidated, but we speculate that the accumulation of pro-inflammatory and pro-apoptotic Cer observed in Sptlc2 ΔLiv mice might contribute to biliary epithelial damage. Consistently, Cer have been shown to up-regulate hepatic miRNA expression of lipid metabolism-related genes, inflammation-related (as evidenced in our model) and fibrogenesis-related genes [59].

The nuclear BA receptor FXR is a transcription factor involved in the regulation of both BA metabolism and glucose homeostasis. There are in vitro and in vivo evidences that modulation of FXR pathway regulates gluconeogenesis in rodent [60,61]. Consistent with the dramatic increase in T-MCA, a reported FXR antagonist [17], in our mutant mice, we show that genetic deletion of hepatic Sptlc2 inhibits expression of FXR signaling pathway. Recently, it has been shown that nSmase2 is a target gene of FXR [20,21]. However, Fxr and nSmase2 vary in opposite ways in our model, suggesting that sSmase2 upregulation is not linked to FXR but rather to other factors (intracellular substrates, pro-inflammatory environment).

In addition, Sptlc2 ΔLiv mice have a defect in hepatic glucose production from pyruvate and glycerol, which probably explains, associated with better insulin sensitivity in the muscle, the better glucose

Fig. 5. Impact of hepatic deletion of Sptlc2 on insulin sensitivity. ITT at 0.25 UI/kg (A) or 0.5 UI/kg (B) and inverse areas under the curve performed in RC or HFD fed mice. (C) Plasma insulin secretion during OGTT test of RC or HFD fed mice. (D–F) Phosphorylated Akt (Ser473)/Akt ratio measured in cells lysates from liver, adipose tissue or muscle (extensor digitorum longus) of HFD fed mice stimulated with insulin (10 UI/kg) 30 min before tissue collection. (G) Glycogen content in liver of HFD fed or 5-h-fasting mice. Analysis were performed in 45 day-old mice (for primary hepatocytes collection) or 4 month-old mice and HFD mice were fed with HFD for 2 months starting at 2 month-old . Data are expressed relative to insulin response. Analysis were performed in 4 month-old mice and HFD mice were fed with HFD for 2 months starting at 2 month-old; n = 3-10 mice per groups; error bars represents SEM; *p < 0.05, **p < 0.01, ***p < 0.001 via Mann-Whitney U test, Student's t when appropriate or two-way ANOVA followed by two-by-two comparisons using Bonferroni’s post hoc test.
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A. Sptlc2\textsuperscript{WT/Lv} HF Sptlc2\textsuperscript{Ltv/Ltv} HF

20 day-old mice RC

45 day-old mice RC

120 day-old mice RC

B. Liver weight (g per body weight (g))

\begin{itemize}
  \item Sptlc2\textsuperscript{WT/Lv} HF
  \item Sptlc2\textsuperscript{Ltv/Ltv} HF
\end{itemize}

C. Triglyceride concentrations (mg/g liver) or weight (g liver)

\begin{itemize}
  \item Sptlc2\textsuperscript{WT/Lv} HF
  \item Sptlc2\textsuperscript{Ltv/Ltv} HF
\end{itemize}

D. GR1

\begin{itemize}
  \item Sptlc2\textsuperscript{WT/Lv} RC
  \item Sptlc2\textsuperscript{Ltv/Ltv} RC
\end{itemize}

E. Relative expression of cytokines

\begin{itemize}
  \item TNF\textsubscript{a}
  \item Th17
  \item IFN\gamma
\end{itemize}

F. Cleaved Casp3 (17 kDa)

CYCLO a (18 kDa)

Sptlc2\textsuperscript{WT/Lv} HF Sptlc2\textsuperscript{Ltv/Ltv} HF

\begin{itemize}
  \item Sptlc2\textsuperscript{WT/Lv} HF
  \item Sptlc2\textsuperscript{Ltv/Ltv} HF
\end{itemize}

G. Relative expression of pro-apoptotic genes

\begin{itemize}
  \item Bad
  \item Bim
  \item Bax
  \item Bal2
\end{itemize}

H. Ki67

\begin{itemize}
  \item Sptlc2\textsuperscript{WT/Lv} RC
  \item Sptlc2\textsuperscript{Ltv/Ltv} RC
\end{itemize}

I. Relative expression of immune response genes

\begin{itemize}
  \item Gpr78
  \item Chop
\end{itemize}

(caption on next page)
tolerance under HF. Interestingly, Sptlc2\textsuperscript{ΔLiv} mice treated with a selective nSmae2 inhibitor show a partial normalization of their glucose production in response to pyruvate or glycerol, suggesting that increased SM hydrolysis by nSmae2/Smpr3 upregulation plays a causal role in the hepatic glucose production defect. Of note, 6-hour-fasting glycemia is reduced in Sptlc2\textsuperscript{ΔLiv} mice. These traits are unlikely linked to a liver-specific increase in insulin sensitivity, while Cer, and especially C16:0-Cer and C18:0-Cer, well-known inducers of insulin resistance, accumulate in liver [7]. Consistently, glycogen content is reduced, and phosphorylation of Akt/ protein kinase B (Pkb), a major mediator of insulin anabolic effects is reduced in the liver of mutant mice, while systemic insulin sensitivity seems to be compensated by an enhanced Akt/Pkb phosphorylation in the muscle. Altogether, these results demonstrated that hepatic Sptlc2 deficiency impaired hepatic glucose production in an insulin independent way.

We also evidenced a previously undescribed age-related effect of SPTLC2 deficiency that favours hepatic fibrogenesis. Although precise mechanisms remain to be investigated, the decreased amount of membrane SM in the lack of hepatic SPTLC2, associated with a reduction of membrane lipids rafts, may favour beta catenin translocation to the nucleus and contribute to the onset of fibrogenesis [32,62]. Moreover, several studies showed that Cer themselves are involved in hepatic fibrogenesis [63]. Interestingly, we demonstrated that the hepatic glucose production defect was already present in 20-day-old Sptlc2\textsuperscript{ΔLiv} mice before the neat onset of fibrosis and altogether our results suggest that hepatic defect in glucose production is independent of fibrosis development over time.

In conclusion, our findings suggest a compensatory mechanism for Cer production in liver, and highlight a link between sphingolipid production, BA metabolism, and glucose production in an insulin-independent way. The BA nuclear receptor FXR, which is involved in both BA homeostasis and energy metabolism, is likely to be at the core of a sphingolipids/BA/neoglucogenesis axis.

CRediT authorship contribution statement


Christophe Magnan: Writing - Review & Editing, Supervision. Thierry Tordjmann: Writing - Review & Editing, Supervision. Céline Cruciani-Guglielmacci: Conceptualization, Methodology, Visualization, Investigation, Data curation, Writing- Original draft preparation, Writing - Review & Editing, Supervision.

Declaration of competing interest

P.D.C. is an inventor on patent applications dealing with the use of specific bacteria and components in the treatment of different diseases. P.D.C. was co-founder of The Akkermansia Company SA and Enterosys. The other authors declare no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbapap.2023.159333.

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