

Changes in bile acid composition are correlated with reduced intestinal cholesterol uptake in intestine-specific WASH-deficient mice

Andries Heida^a, Theo van Dijk^b, Marieke Smit^a, Martijn Koehorst^b, Mirjam Koster^a, Niels Kloosterhuis^a, Rick Havinga^a, Vincent W. Bloks^a, Justina C. Wolters^a, Alain de Bruin^{a,c}, Jan Albert Kuivenhoven^a, Jan Freark de Boer^{a,b}, Folkert Kuipers^{a,d}, Bart van de Sluis^{a,*}

^a Department of Pediatrics, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands

^b Department of Laboratory Medicine, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands

^c Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, the Netherlands

^d European Research Institute for the Biology of Ageing (ERIBA), University of Groningen, University Medical Center Groningen, Groningen, the Netherlands

ARTICLE INFO

Keywords:

Endosome
Ezetimibe
Bile acids
Cholesterol
Intestine

ABSTRACT

The Wiskott-Aldrich syndrome protein and SCAR homolog (WASH) complex is a pentameric protein complex localized at endosomes, where it facilitates the transport of numerous receptors from endosomes toward the plasma membrane. Recent studies have shown that the WASH complex plays an essential role in cholesterol and glucose homeostasis in humans and mice. To investigate the physiological importance of intestinal WASH, we ablated the WASH component WASHC1 specifically in murine enterocytes. Male and female intestine-specific WASHC1-deficient mice (*Washc1*^{IKO}) were challenged with either a standard chow diet or a high-cholesterol (1.25 %) diet (HCD). *Washc1*^{IKO} mice fed a standard diet did not present any apparent phenotype, but when fed an HCD, their hepatic cholesterol levels were ~ 50 % lower compared to those observed in control mice. The intestinal cholesterol absorption was almost 2-fold decreased in *Washc1*^{IKO} mice, which translated into increased fecal neutral sterol loss. The intestinal expression of cholesterologenic genes, such as *Hmgcs1*, *Hmgcr*, and *Ldlr*, was significantly higher in *Washc1*^{IKO} mice than in control mice and correlated with increased whole-body de novo cholesterol synthesis, likely to compensate for impaired intestinal cholesterol absorption. Unexpectedly, the ratio of biliary 12 α -/non-12 α -hydroxylated bile acids (BAs) was decreased in *Washc1*^{IKO} mice and reversing this reduced ratio by feeding the mice with the HCD supplemented with 0.5 % (w/w) sodium cholate normalized the improvement of hepatic cholesterol levels in *Washc1*^{IKO} mice. Our data indicate that the intestinal WASH complex plays an important role in intestinal cholesterol absorption, likely by modulating biliary BA composition.

1. Introduction

Intestinal cholesterol absorption is a critical process in the maintenance of systemic cholesterol homeostasis [1] and is highly dependent on the Niemann-Pick C1-Like 1 (NPC1L1) protein [2,3]. Blocking the function of this protein with ezetimibe in humans decreases intestinal cholesterol absorption and subsequently lowers plasma cholesterol by approximately 17 % [4,5]. Ezetimibe treatment is often combined with cholesterol-lowering statins, and together they strongly reduce plasma cholesterol levels and cardiovascular events in humans [6]. The importance of NPC1L1 in intestinal cholesterol absorption is further supported in experimental mouse models. *Npc1l1* knockout mice display

a substantially impaired cholesterol uptake from the intestine [2,7,8]. This reduced intestinal cholesterol absorption protects NPC1L1-deficient mice against diet-induced accumulation of cholesterol in plasma and liver [3,7–9].

NPC1L1 is a transmembrane protein localized to the apical plasma membrane and perinuclear endocytic recycling compartment (ERC) of enterocytes [10]. Its subcellular localization highly depends on the cholesterol concentration within the cell. Depletion of cholesterol promotes transport of NPC1L1 from the ERC to the plasma membrane [5,11], whereas restoring cellular cholesterol triggers trafficking of NPC1L1 from the plasma membrane to the ERC. Although the endocytic itinerary of NPC1L1 has been well described [5,12,13] the mechanism

* Corresponding author.

E-mail address: A.J.A.van.de.sluis@umcg.nl (B. van de Sluis).

<https://doi.org/10.1016/j.bbalip.2023.159445>

Received 20 October 2023; Received in revised form 4 December 2023; Accepted 6 December 2023

Available online 11 December 2023

1388-1981/© 2023 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

by which NPC1L1 is transported from the ERC to the apical plasma membrane remains poorly understood.

Wiskott-Aldrich syndrome protein and SCAR homolog (WASH) complex is a pentameric protein complex comprised of WASHC1 - WASHC5 (previous known as WASH, FAM21, CCDC53, SWIP, and strumpellin, respectively) [14,15]. The WASH complex is targeted to endosomes in a retromer-dependent or independent manner [15,16], where it promotes the generation of branched F-actin through activation of the Arp2/3 complex [17,18]. These branched actin networks form domains on the endosomal surface from where receptors, such as integrins, lipoprotein receptors and glucose transporter, are transported to their respective destination in the cell [18,19]. Mutations in genes encoding for the WASH components have been linked to neurological and intellectual disability disorders [20–23]. Mutation in *WASHC4* causes non-syndromic autosomal recessive intellectual disability [20], and a homozygous hypomorphic *WASHC5* allele is reported to cause Ritscher-Schnitzel/3C syndrome [23], while dominant-negative or gain-of-function mutations in *WASHC5* cause Hereditary spastic paraplegia type SPG8 [21,22]. Tissue-specific knockout mouse models have highlighted the importance of the WASH complex also in various metabolic processes, including lipid and glucose metabolism [24–26]. Hepatic WASHC1 deficiency results in hypercholesterolemia in mice due to reduced hepatic cholesterol uptake caused by impaired endosomal recycling of lipoprotein receptors, such as low-density lipoprotein receptor (LDLR) and LDLR-related protein 1 (LRP1) [24,25]. Ritscher-Schnitzel/3C syndrome patients also have a relatively high plasma levels of low-density lipoprotein (LDL) cholesterol, implicating that the action of WASH in hepatic cholesterol uptake is conserved between mice and humans [24].

Ablation of *Washc1* in pancreatic β -cells illustrated the physiological relevance of the WASH complex in recycling the glucose transporter GLUT2 to maintain glucose homeostasis [26]. A recent study linked the WASH complex also to NPC1L1-mediated intestinal cholesterol uptake [27]. In this study, it was found that LIMA1 facilitates the transport of NPC1L1 along microfilaments from the endocytic recycling compartment (ERC) to the plasma membrane [27]. Here, they also showed that intestinal LIMA1 can physically interact with the WASH component WASHC2, but whether LIMA1 acts in concert with WASH to assist the endosomal transport of NPC1L1 in enterocytes remains to be defined.

To gain more insights in the physiological role of the WASH complex in the intestine, we generated intestine-specific WASHC1-deficient mice (*Washc1*^{IKO} mice). Our findings suggest that the WASH complex plays a significant role in intestinal cholesterol absorption, primarily by influencing biliary BA composition, rather than through facilitating NPC1L1-mediated cholesterol uptake.

2. Material & methods

2.1. Mice

Mice carrying *Washc1* floxed allele (*Washc1*^{f/f}) [28] were intercrossed with mice expressing Cre recombinase driven by the villin promoter (Villin-Cre, Jackson#021504) to ablate *Washc1* exclusively in the intestine. These mice were maintained on a C57BL/6 J genetic background. They were housed individually in climate-controlled rooms with a 12-h light/12-h dark cycle and were fed ad libitum with a standard laboratory diet (RM1, Special Diet Services, UK), a 1.25 % cholesterol-rich diet (research diets, D12108C) or 1.25 % cholesterol-rich diet supplemented with 0.5 % sodium cholate (research diets, D12109C). Littermates without the Villin-Cre allele were used as wild-type controls. Mice were fasted for 4 h before sacrifice, intestine, and liver tissue were collected for histological analysis in paraffin, and tissues were snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Blood was obtained by cardiac puncture, and plasma was collected after centrifugation at 1000 $\times g$ for 10 min at 4°C . All animal experiments were approved by The Central Authority for

Scientific Procedures on Animals (CCD), as well as by the Animal Welfare Body (IvD) of the University of Groningen (Groningen, the Netherlands). All experiments were performed according to institutional, national, and European animal regulations.

2.2. Bioinformatical gene expression analysis

WASH components (*Washc1–5*), *Npc1l1*, and *Lima1* gene expression along the intestinal tract were analyzed and visualized using GENEVESTIGATOR® (<https://genevestigator.com/>). Affymetrix Microarray data (MOE430_2) of control C57BL/6 mice were extracted from the Genevestigator database (NEBION AG, Zurich, Switzerland) and used to generate a heatmap. The heatmap (Fig. 1A) shows the average log2 expression of the selected genes in the gastrointestinal tract.

2.3. Gene expression analysis

Snap-frozen tissue was homogenized in QIAzol Lysis Reagent (Qia-gen), and total RNA was isolated according to the manufacturer's instructions. cDNA synthesis was performed according to the manufacturer's instructions (Invitrogen, #28025013). mRNA expression levels were determined using quantitative real-time PCR (qRT-PCR) analysis with SYBR Green Master Mix (Roche) and qRT-PCR primers (Supplemental Table 1). Analysis was performed in QuanStudio™ Real-Time PCR software using the $\Delta\Delta\text{-Ct}$ method. Gene expression was normalized to *Ppia* and expressed as fold-change compared to control mice.

2.4. Histology

Histological analysis of intestinal samples was performed on sections fixed in 4 % paraformaldehyde. Sections were embedded in paraffin and sliced at 4 μm for hematoxylin-eosin (H&E). Histological images were taken using the Leica DM3000 microscope with a mounted Leica DFC420 camera. Intestinal integrity was judged unbiased by a board-certified veterinary pathologist of the Dutch Molecular Pathology Center (UU, Utrecht, the Netherlands).

2.5. Cholesterol and triglyceride analysis in plasma and liver

Hepatic lipids were extracted according to Bligh & Dyer [29]. Lipid extracts were dissolved in 2 % TX100 and cholesterol, and triglyceride concentrations were measured using colorimetric assays (11,489,232, Roche) and Trig/GB kit (1,187,771, Roche), respectively.

2.6. Fast-performance liquid chromatography (FPLC)

Pooled plasma samples were fractionated by FPLC, as previously described [30]. In short, plasma lipoproteins are separated by size on a column, and a UV detector measures cholesterol concentrations via an enzymatic colorimetric reaction determination post-column.

2.7. Cholesterol flux measurements

A previously described dual stable isotope tracer method was used to measure the cholesterol fluxes [31]. On day 0, mice received intravenous cholesterol-D₅ and oral gavage of cholesterol-D₇ to determine cholesterol absorption. Intravenous injections were 0.3 mg cholesterol-D₅ (Medical Isotopes Inc., Pelham, NH) dissolved in 150 μL 20 % intralipid (Fresenius Kabi, Den Bosch, the Netherlands). Oral gavages contained 0.6 mg cholesterol-D₇ (Cambridge Isotope Laboratories, Inc., Andover, MA) dissolved in 200 μL medium-chain triglyceride oil. Bloodspots were collected at 0, 3, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, and 240 h after administration of labeled cholesterol. On day 10, the bile, blood, and livers of the mice were collected for biochemical, molecular, and histological characterization. Bodyweight was

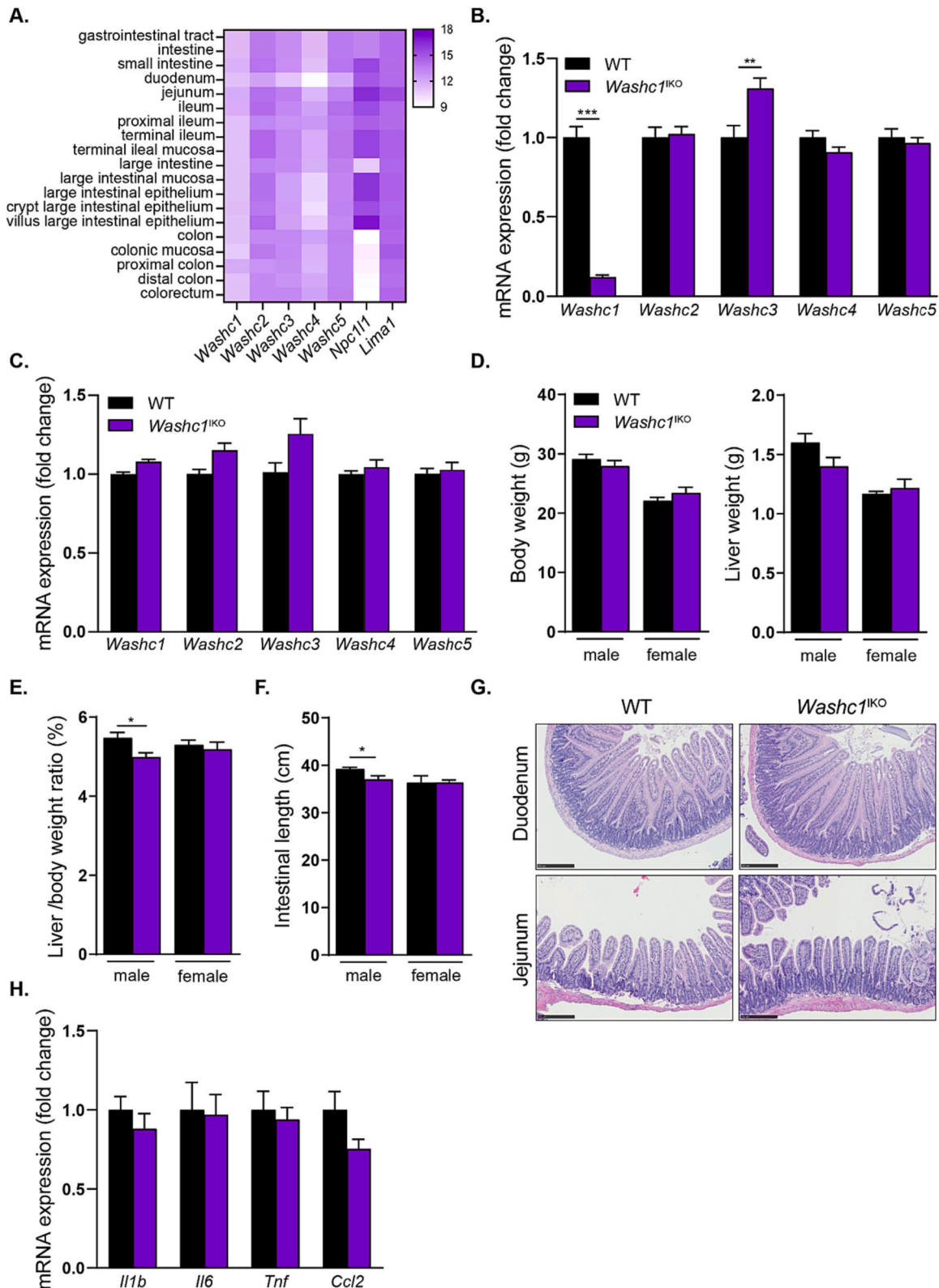


Fig. 1. Intestine-specific deletion of *Washc1* does not result in an overt phenotype. (A) Heat map of mRNA expression of *Washc1–5*, *Npc1l1*, and *Lima1* in various sections of the murine gastrointestinal tract, generated by GENEVESTIGATOR®. (B) Relative mRNA expression of *Washc1–5* in proximal intestines and in (C) livers of *Washc1*^{IKO} mice compared to wildtype (WT) mice, as determined by qRT-PCR (δ n = 9). (D) Body weight, liver weight, (E) liver-to-body weight ratio, and (F) intestinal length of WT and *Washc1*^{IKO} mice fed a standard diet (δ n = 9, η n = 6). (G) Representative H&E staining of small intestinal (duodenum and jejunum) sections from WT and *Washc1*^{IKO} mice; scale bars represent 250 μ m. (H) Relative mRNA expression of inflammatory markers in the duodenum of *Washc1*^{IKO} mice compared to WT mice as determined by qRT-PCR (δ n = 9). Data are presented as mean \pm SEM, * P < 0.05, ** P < 0.01, *** P < 0.001, as determined by Student's t -test.

determined daily, and food intake data and feces were collected for 72 h before sacrifice. Bile was collected for 30 min while the anesthetized mice were placed in a humidified incubator (37 °C) to maintain body temperature. Three days before sacrifice the mice received drinking water containing 2 % [1-¹³C] acetate to determine whole-body cholesterol synthesis by mass isotopomer distribution analysis (MIDA). Bloodspots were collected at 0, 24, 32, 48, 56, and 72 h after administration of labeled cholesterol 2 % [1-¹³C] acetate water.

2.8. Proteomics

Enterocytic protein levels were determined by mass spectrometry. Enterocytes were collected via intestinal scrapes of the intestine. In detail, the small intestine was removed and divided into three equal sections. Individual sections were flushed using PBS and cut longitudinally. Leftover feces were removed with additional PBS flushing. The intestinal mucosa was scraped off and collected using a glass cover, and snap-frozen in liquid nitrogen. Samples were stored at -80 °C until further processing. Samples were homogenized in RIPA buffer (10 mL 25 mM KPI buffer, pH 7.4, 39 mL 0.5 M EDTA, pH 8.0) supplemented with protease and phosphatase inhibitors (Roche). Bradford assay (Bio-Rad) was used to determine protein concentrations.

Subsequently, targeted proteomics, with isotopically labeled standards, was employed to quantify a subset of proteins, similar to previous studies [32]. In brief, in-gel digestion was performed on an equivalent of 37.5 µg total protein in the intestinal mucosa lysates. Samples were reduced with 10 mmol/L dithiothreitol, alkylated with 55 mmol/L iodoacetamide, and trypsinized (1:100 g/g sequencing grade modified trypsin V5111; Promega). This was followed by solid-phase extraction (SPE C18-Aq 50 mg/1 mL, Gracepure, Thermo Fisher Scientific) for sample clean-up.

For NPC1L1 in intestinal mucosa samples, three peptides (YDSLGLGPK, SLEDEINR, and NSQDFTEALR) were selected, using previously described criteria [33]; however, assays were developed based on the endogenous peptides without the addition of isotopically-labeled standards and yielded only relative changes. The relative changes in the endogenous levels of pertinent proteins, including isotope-labeled standards, were examined to assess the risk of run-to-run variation, which is not corrected in the absence of isotopically-labeled standards.

2.9. Statistics

All data are presented as the mean ± SEM. Statistical analyses were performed using GraphPad version 8.4.0 (GraphPad Software), and an unpaired 2-tailed Student's *t*-test was used to compare differences between any two groups. *P*-values <0.05 were considered statistically significant: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

3. Results

3.1. Depletion of intestinal *Washc1* does not result in an overt phenotype

The WASH complex is ubiquitously expressed [14], to confirm its expression in the intestine we examined the expression patterns of all *Wash* components (*Washc1*–5) in various sections of the gastrointestinal tract using the bioinformatics tool GENEVESTIGATOR®. We compared this pattern with the gene expression pattern of *Npc1l1* and *Lima1* (Fig. 1A). Similar to *Lima1*, all WASH components were expressed along the entire length of the intestinal tract, whereas *Npc1l1* was expressed mainly in the small intestine. To explore the functions of the WASH complex in intestinal physiology, we generated intestine-specific *Washc1* knockout (*Washc1*^{IKO}) mice. These mice were generated by intercrossing mice carrying *Washc1* floxed allele (*Washc1*^{fl/y}) [28] with mice expressing Cre recombinase specifically in the intestinal epithelium (Villin-Cre). Intestinal ablation of *Washc1* was confirmed by qRT-PCR analysis (Fig. 1B). Loss of intestinal WASHC1 did not affect the

mRNA levels of other *Wash* components (*Washc2*–*Washc5*) (Fig. 1B), nor the mRNA expression of the WASH components in the livers of these mice (Fig. 1C). Although no effects on body and liver weights were observed in male and female mice upon intestinal *Washc1* ablation (Fig. 1D), a slight decrease in the liver-to-body weight ratio was seen in male *Washc1*^{IKO} mice compared to control mice (Fig. 1E). In addition, the length of the small intestine of male *Washc1*^{IKO} mice was reduced compared to control mice, a phenotype that was not seen in female mice (Fig. 1F). Hematoxylin-eosin (H&E) staining of the duodenum and jejunum indicated neither gross abnormalities nor intestinal inflammation in male *Washc1*^{IKO} (Fig. 1G). The absence of intestinal inflammation was confirmed by gene expression analysis of several inflammatory markers (Fig. 1H).

Plasma and hepatic cholesterol and triglyceride levels also did not differ between intestinal WASHC1-deficient mice and control mice fed a standard chow diet (Fig. 2A, B). Altogether these data demonstrate that loss of intestinal WASHC1 affects neither intestinal morphology nor causes any other overt phenotype in mice.

3.2. Intestinal WASHC1-deficient mice fed a cholesterol-rich diet display decreased hepatic cholesterol accumulation compared to control mice

Next, we challenged *Washc1*^{IKO} and control mice with a high-cholesterol diet (HCD, 1.25 % cholesterol) to assess the role of intestinal WASHC1 in diet-induced plasma and hepatic cholesterol accumulation. The body and liver weights of *Washc1*^{IKO} and control mice fed the HCD did not differ (Fig. 3A). Although plasma cholesterol levels between male *Washc1*^{IKO} and control mice were similar, plasma cholesterol levels in female *Washc1*^{IKO} mice were slightly elevated compared to female control mice (Fig. 3B). This increase was due to elevated LDL as well as HDL cholesterol levels, as determined by FPLC analysis (Fig. 3C). Plasma triglyceride contents in male and female mice were not affected by intestinal *Washc1* ablation (Fig. 3D). However, hepatic cholesterol contents in *Washc1*^{IKO} mice were approximately 50 % lower than those of control mice fed the HCD, this reduction in hepatic cholesterol concentration was seen in both genders (Fig. 3E). Hepatic triglyceride contents were similar between *Washc1*^{IKO} and control mice (Fig. 3E). H&E staining of liver sections did not reveal any histological differences between the groups (Fig. 3F). Next, we studied the expression of the sterol regulatory element-binding protein 2 (SREBP2) target genes, *HMG-CoA reductase* (*Hmgcr*), *HMG-CoA synthase* (*Hmgcs1*), and *low-density lipoprotein receptor* (*Ldlr*) in duodenum and liver of male mice fed the HCD. The mRNA levels of *Hmgcr*, *Hmgcs1*, and *Ldlr* in the duodenum of *Washc1*^{IKO} mice were higher than those in control mice (Fig. 3G), indicating that intestinal WASHC1 deficiency causes a decrease in dietary cholesterol uptake, resulting in higher expression of genes involved in cholesterol synthesis and uptake to compensate for the reduced intestinal cholesterol absorption. A similar trend in the mRNA expression pattern of *Hmgcs1*, *Hmgcr*, and *Ldlr* was observed in the livers of *Washc1*^{IKO} mice compared to controls (Fig. 3H).

3.3. Intestinal cholesterol absorption is reduced upon intestinal ablation of WASHC1

To quantify the effect of WASHC1 deficiency on intestinal cholesterol absorption, *Washc1*^{IKO} and control mice were administered cholesterol-D₅ and cholesterol-D₇ by intravenous injection and oral gavage, respectively, and the enrichment of these tracers in plasma was monitored for 7 days to calculate fractional cholesterol absorption [31,34]. Fractional cholesterol absorption in *Washc1*^{IKO} mice was significantly reduced compared to control mice (Fig. 4A, B). In line, fecal excretion of neutral sterols (NS) in *Washc1*^{IKO} mice was increased (Fig. 4C). It is well-known that reduced intestinal cholesterol absorption is compensated by elevated cholesterol synthesis [27,35]. To this end, we calculated de novo cholesterol synthesis using MIDA following the addition of ¹³C-acetate to the drinking water for three days [31], and found that

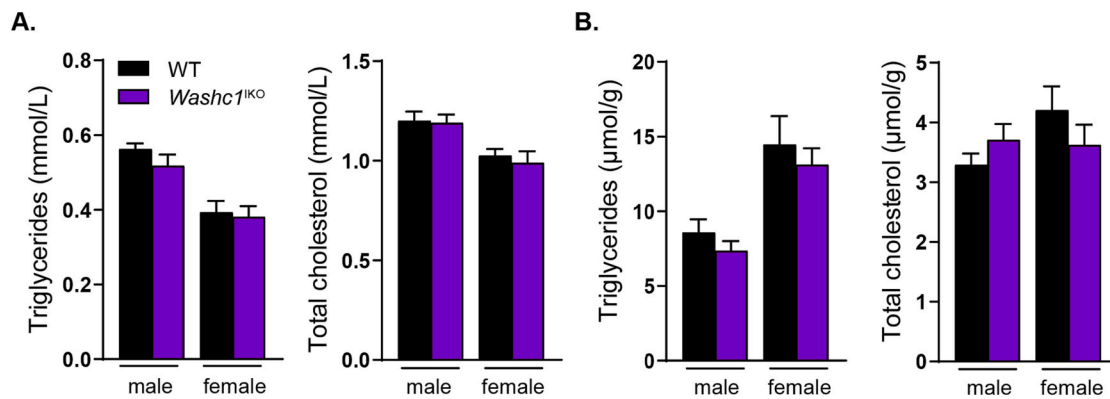


Fig. 2. Intestinal WASHC1 deficiency does not affect plasma or hepatic lipid levels. (A) Plasma and (B) hepatic triglyceride and cholesterol concentrations ($\mu\text{mol/g}$ wet liver weight) of WT and *Washc1*^{KO} mice fed a standard diet (δ $n = 9$, φ $n = 6$). Data are presented as mean \pm SEM.

Washc1^{KO} mice had significantly increased cholesterol synthesis rates compared to control mice (Fig. 4F). Using targeted proteomics, we found that enterocytic loss of WASHC1 resulted in a 2-fold reduction of NPC1L1 protein levels compared to control enterocytes (Fig. 4D), and a similar reduction in *Npc1l1* mRNA levels, but this reduction failed to reach significance (Fig. 4E). Altogether, these results demonstrate that the WASH complex is required for efficient intestinal cholesterol absorption by controlling the levels of NPC1L1 in enterocytes.

3.4. Changes in bile acid composition is causally related to reduced intestinal cholesterol absorption in *Washc1*^{KO} mice

Bile acids (BAs) are synthesized from cholesterol in the liver and secreted into the intestine, where they play an essential role in micellar solubilization and subsequent cholesterol absorption [36,37]. To assess whether changes in BA composition contribute to the reduced intestinal cholesterol absorption in *Washc1*^{KO} mice, we measured biliary BA secretion and composition. Bile flow and biliary BA secretion did not differ between control and *Washc1*^{KO} mice (Fig. 5A, B). We also observed no differences in biliary cholesterol and phospholipid secretion (Figs. 5C, D). Although total biliary BA secretion was unaffected in mice lacking intestinal WASHC1, the BA composition was altered in *Washc1*^{KO} mice, mainly due to a reduction in TDCA and a tendency for a decrease in TCA (Fig. 5E). Consequently, the ratio of 12 α /non-12 α -hydroxylated BAs present in bile was significantly decreased in *Washc1*^{KO} mice compared to control mice (Fig. 5F). Hepatic mRNA levels encoding the BA synthesis enzymes CYP7A1, CYP27A1, and CYP8B1, the latter responsible for 12 α -hydroxylation in the BA synthesis cascade, were not altered (Fig. 5G). We found that the ratio of 12 α /non-12 α -hydroxylated BAs strongly and positively correlated with fractional cholesterol absorption (Fig. 5H).

To further investigate the contribution of the BA composition to the reduced intestinal cholesterol absorption in *Washc1*^{KO} mice we fed control and *Washc1*^{KO} mice the HCD (1.25 % cholesterol) supplemented with 0.5 % (w/w) sodium cholate for one week. Feeding mice a diet supplemented with sodium cholate increases the presence of 12 α -hydroxylated BAs in bile, thereby enhancing intestinal cholesterol absorption [35,38]. Body and liver weights were not different between control and *Washc1*^{KO} mice fed the cholate-supplemented diet (Fig. 6A). Although hepatic cholesterol accumulation is diminished in HCD-fed *Washc1*^{KO} mice when compared to control mice (Fig. 3E), adding sodium cholate to the HCD restored lower hepatic cholesterol levels in *Washc1*^{KO} mice to levels that were also seen in control mice (Fig. 6B). Given that hepatic cholesterol content serves as an indicator of intestinal cholesterol absorption, these findings suggest that cholesterol absorption between *Washc1*^{KO} mice and control mice fed the cholate-supplemented HCD is similar. Plasma cholesterol levels were significantly reduced by ± 10 % in male *Washc1*^{KO} mice compared to control

male mice, whereas no difference was seen in female mice (Fig. 6C). Altogether, these results indicate that changes in BA composition likely explain the reduced intestinal cholesterol absorption in *Washc1*^{KO} mice.

4. Discussion

The WASH complex is involved in the control of various metabolic processes, including lipid and glucose metabolism [24–26]. A previous study linked this endosomal sorting complex to intestinal NPC1L1 through its interaction with LIMA1, a component involved in NPC1L1-mediated cholesterol uptake [27]. In this study, we found that the WASH complex is essential for intestinal cholesterol absorption, likely by influencing the composition of biliary BA, rather than regulating the function of NPC1L1 in enterocytes.

Prior mouse studies have shown that the WASH complex is essential in several metabolic pathways by facilitating the endosomal transport of various transmembrane proteins, including GLUT2 and the lipoprotein receptors LDLR and LRP1 [24–26]. Here, we assessed the role of WASH in intestinal physiology by deleting the WASH component WASHC1 specifically in enterocytes. Under chow-fed conditions, intestinal ablation of WASHC1 does not result in an apparent phenotype in mice. However, upon HCD feeding, intestinal WASH deficiency causes a marked reduction in hepatic cholesterol levels without significant changes in plasma cholesterol or triglyceride levels. A possible explanation for the decrease in hepatic cholesterol levels could be ascribed to the reduced intestinal cholesterol absorption in *Washc1*^{KO} mice compared to the control mice (Fig. 4). This aligns with previous mouse studies that show the importance of intestinal cholesterol uptake in contributing to hepatic cholesterol content: in fact, hepatic cholesterol appears to serve as a proxy of intestinal cholesterol absorption in mice [2,5,7,27,39]. Building on a previous study that connected the WASH complex with NPC1L1 through an interaction with LIMA1 [27] and its known function in endosomal transport of transmembrane proteins [19,40], we speculated that the loss of WASH might impede the retrieval of NPC1L1 from lysosomal degradation, thereby hampering its transport from the endosome to the cell surface. This disruption could subsequently lead to impaired cholesterol uptake from the intestinal lumen. Although our hypothesis is supported by the reduced NPC1L1 protein levels in WASHC1-deficient enterocytes (Fig. 4), we also showed that a decrease in 12 α /non-12 α -hydroxylated BA ratio correlates with the reduced intestinal cholesterol absorption in *Washc1*^{KO} mice (Fig. 5). There is a substantial body of evidence highlighting the crucial role of 12 α -hydroxylated BAs, i.e. cholic acid (CA) and its bacterial metabolite deoxycholic acid, in intestinal cholesterol absorption [37,41,42]. BAs form mixed micelles in the bile and upper intestine to facilitate lipid absorption at the apical brush border membrane and disruption in BA composition strongly affects intestinal cholesterol absorption [37]. For example, ablating sterol 12 α -hydroxylase (CYP8B1), a BA 12 α -

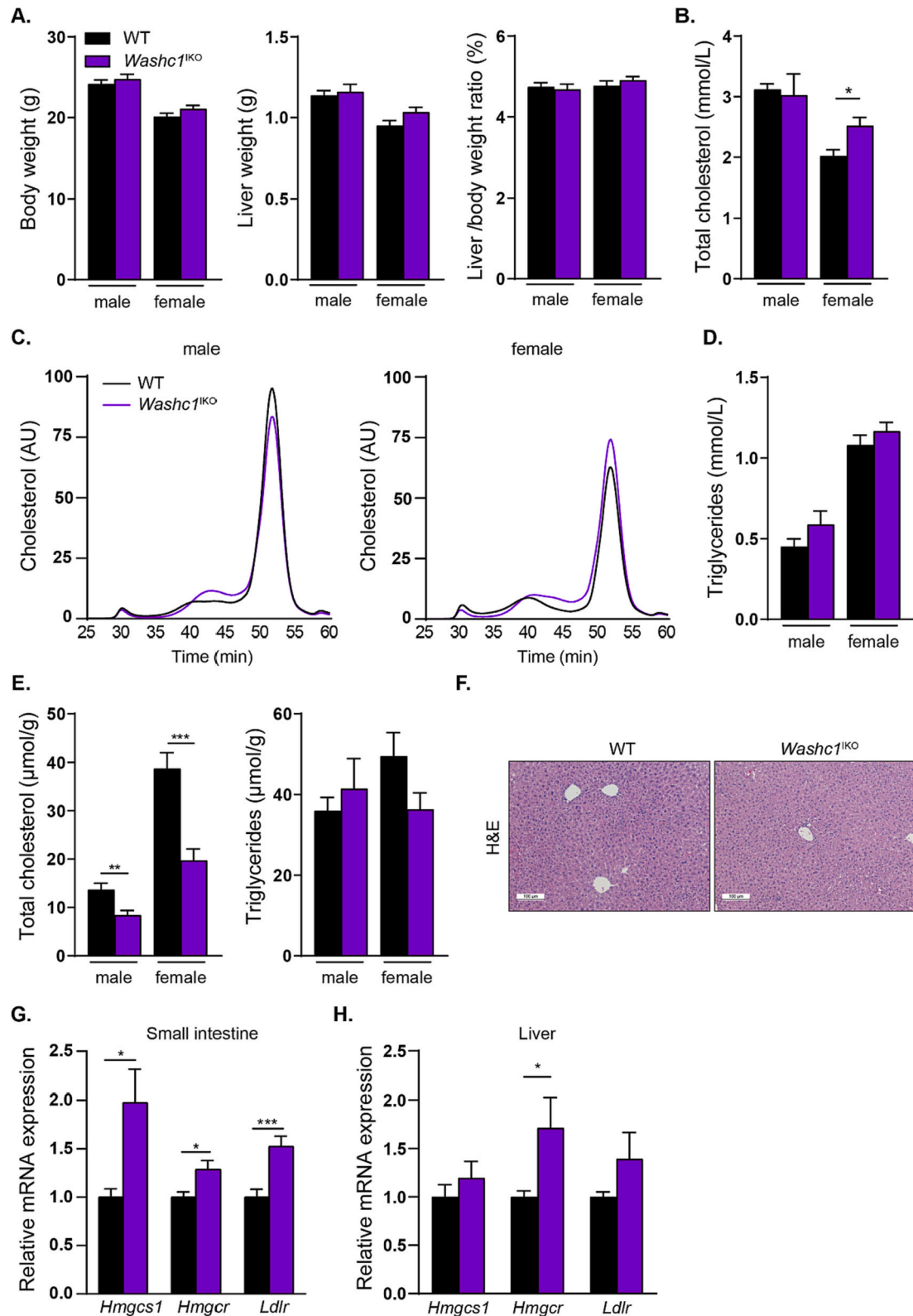


Fig. 3. Intestinal loss of WASHC1 decreases hepatic cholesterol accumulation upon HCD feeding. (A) Body weight, liver weight, and liver-to-body weight ratio of WT and *Washc1*^{IKO} mice fed a HCD ($n = 10$); (B) Plasma cholesterol concentrations; and (C) total plasma cholesterol levels of FPLC fractionated plasma pools of WT and *Washc1*^{IKO} mice ($n = 10$), with total cholesterol levels expressed in arbitrary units (AU). (D) Plasma triglyceride concentrations of WT and *Washc1*^{IKO} ($n = 10$). (E) Hepatic cholesterol and triglyceride concentrations (μmol/g wet liver weight) of WT and *Washc1*^{IKO} mice fed a HCD diet ($n = 10$). (F) Representative H&E-stained sections of livers of HCD-fed WT and *Washc1*^{IKO} mice; scale bars represent 100 μm. Relative mRNA expression of SREBP2-target genes in duodenum (G) and in liver (H) of *Washc1*^{IKO} mice, compared to wild-type controls, as determined by qRT-PCR ($\delta n = 10$). Data are presented as mean \pm SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as determined by Student's t -test.

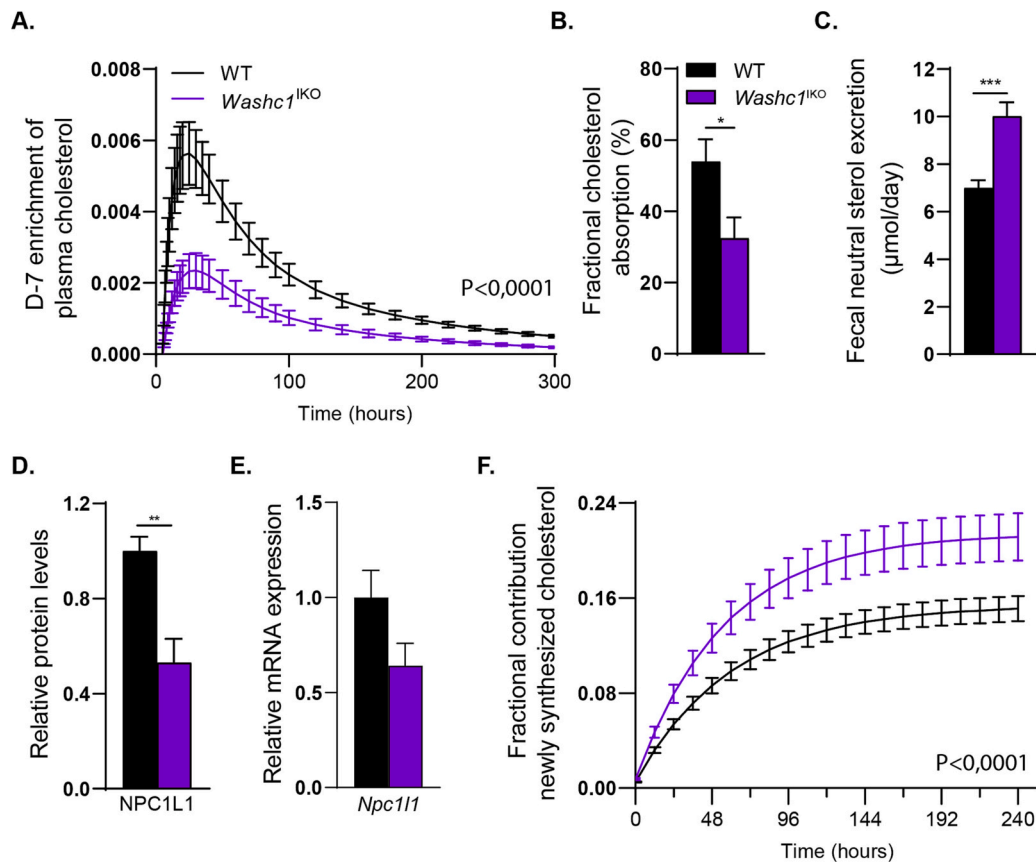


Fig. 4. Intestinal cholesterol absorption is impaired in *Washc1*^{IKO} mice. (A) The fractional enrichment of orally administered cholesterol-D₇, and (B) fractional cholesterol absorption in WT and *Washc1*^{IKO} mice (*n* = 7–11) fed a standard diet. (C) Fecal neutral sterol excretion in WT and *Washc1*^{IKO} mice (*n* = 7–11) fed a standard diet. (D) Relative NPC1L1 protein levels in duodenal enterocytes from *Washc1*^{IKO} mice as compared to WT enterocytes, determined by targeted proteomics. (E) Relative mRNA expression of intestinal *Npc1l1* in *Washc1*^{IKO} mice, compared to WT mice, as determined by qRT-PCR (*δ n* = 9). (F) Fraction of de novo synthesized cholesterol in the blood in WT and *Washc1*^{IKO} mice (*n* = 7–11) fed a standard diet following administration of [1-¹³C] acetate via the drinking water. Data are presented as mean ± SEM, * *P* < 0.05, ***P* < 0.01, and ****P* < 0.001, as determined by Student's *t*-test.

hydroxylase that determines the production of CA, in mice strongly decreases the levels of CA accompanied by reduced intestinal cholesterol absorption and lower hepatic cholesterol levels [35,43]. Similar as in *Washc1*^{IKO} mice (Fig. 4), this decrease in absorption of cholesterol and hepatic cholesterol levels is compensated by increased cholesterol synthesis, and all these phenotypes can be normalized by feeding the mice a diet supplemented with sodium cholate [35,38,43] (Fig. 6). In contrast to *Washc1*^{IKO} and CYP8B1-deficient mice, when *Npc1l1* knockout mice are fed a cholesterol-enriched diet supplemented with sodium cholate, the decreased hepatic cholesterol levels cannot be restored. Instead, both plasma and hepatic cholesterol levels are further reduced when compared to control mice [8,27,44]. Moreover, heterozygous *Npc1l1* knockout (*Npc1l1*^{+/-}) mice do not show a decrease in intestinal cholesterol absorption [2]. This observation suggests that intestinal NPC1L1 expression at 50 % of normal levels, which is also observed in *Washc1*^{IKO} mice, is sufficient for maintaining cholesterol absorption akin to that in wild-type mice. In addition, when *Npc1l1*^{+/-} mice are fed a cholate-supplemented diet, intestinal cholesterol absorption is slightly reduced [2]. Based on the reduced *Npc1l1* mRNA levels in enterocytes of *Washc1*^{IKO} mice (Fig. 4E), we cannot rule out that the reduced NPC1L1 levels are caused by lower *Npc1l1* expression instead of increased lysosomal degradation due to impaired retrieval away from the lysosomal degradation pathway. Intestinal ablation of WASHC1 did not affect the mRNA expression of the WASH components in the liver (Fig. 1C) to compensate for the intestinal loss of WASH. These data also ruled out the possibility that Cre recombinase is expressed at low levels in hepatocytes as shown in previous models [45]. Based on all these data together, we,

therefore, conclude that the reduced intestinal cholesterol absorption in *Washc1*^{IKO} mice is mainly caused by changes in the composition of BAs and is not caused by changes in the expression of the WASH complex in the liver. However, at this moment, we cannot rule out the possibility of other pathways, including NPC1L1-mediated cholesterol uptake, also contributing to the reduced intestinal cholesterol absorption in *Washc1*^{IKO} mice.

The mechanism by which the intestinal loss of WASHC1 affects the BA composition remains unclear. Although no differences in mRNA levels encoding enzymes responsible for BA production, i.e. CYP7A1 (catalyzing the first committed step in the formation of all BAs) and CYP8B1 (catalyzing 12 α -hydroxylation), were observed, we cannot exclude the potential contribution of their zonal expression along the acinus combined with different BA synthesis from different cholesterol precursor pools [46,47]. It has been shown that cholesterol carried in differently-sized vehicles is metabolized into different BA species [46]. Based on the current understanding that *Cyp7a1* is predominantly expressed in pericentral hepatocytes while *Cyp8b1* expression peaks in hepatocytes slightly toward periportal areas of the acinus, it might be that loss of intestinal WASHC1 affects the size of chylomicrons, which subsequently provide their cholesterol to a pool in hepatocytes that is less converted into 12 α -hydroxylated BAs [46,47].

Another explanation for the changes in the BA pool in *Washc1*^{IKO} mice that merits exploration is the role of gut microbiota. It is widely recognized that specific bacterial species in the gut can modulate the BA composition in the intestinal lumen [48]. A prior study showed that the abundance of *Erysipelotrichaceae* in cytochrome P450, family 2,

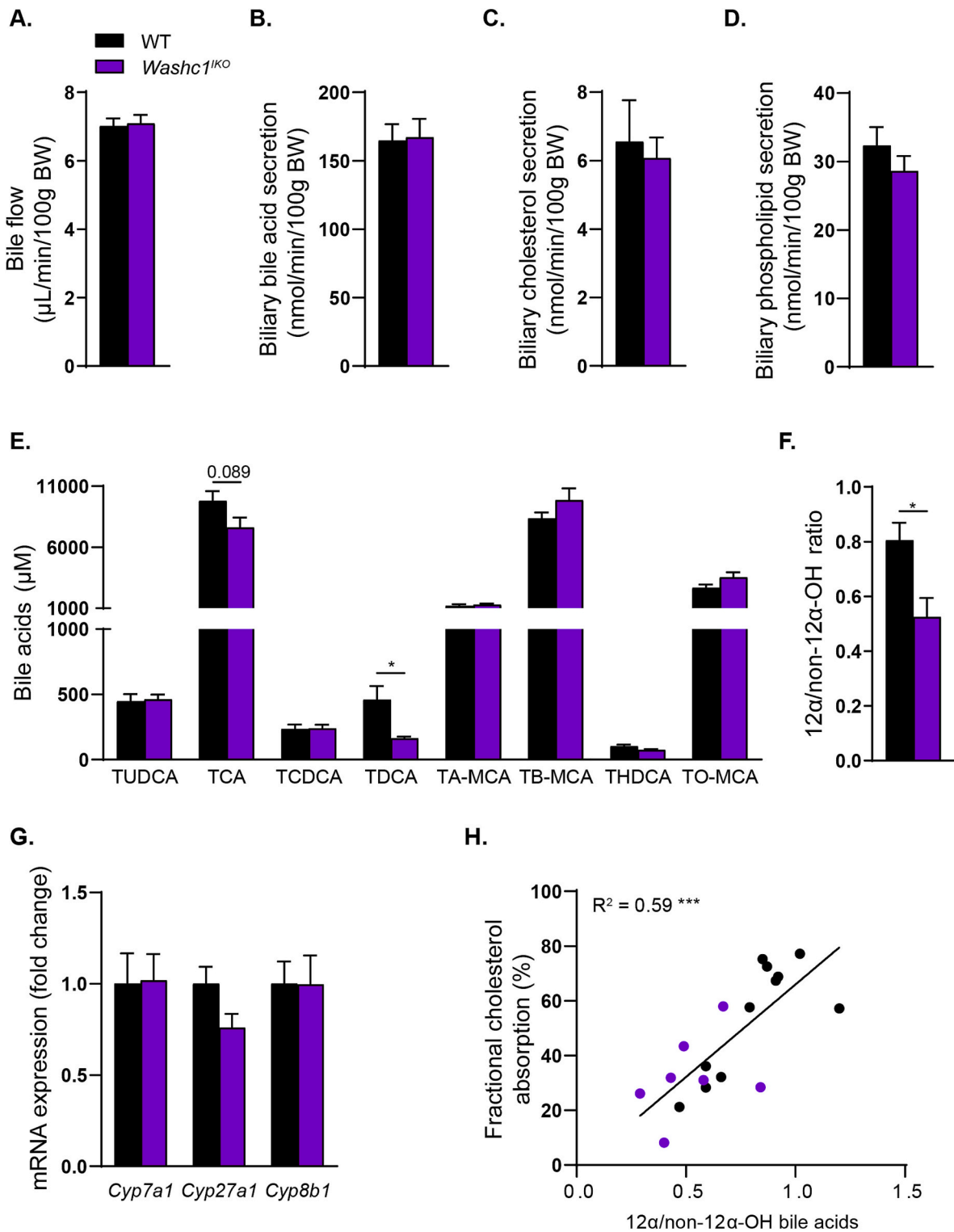


Fig. 5. Cholesterol absorption is positively correlated with 12α -/non- 12α hydroxylated BA ratio. (A) Bile flow and biliary (B) bile acid, (C) cholesterol (D), and phospholipid secretion rates in WT and *Washc1*^{IKO} mice ($n = 7$ – 11) fed a standard diet. (E) Biliary bile acid composition of WT and *Washc1*^{IKO} mice ($n = 7$ – 11) fed a standard diet. (F) $12\alpha/\text{non-}12\alpha$ -hydroxylated bile acid ratios in WT and *Washc1*^{IKO} mice ($n = 7$ – 11) fed a standard diet. (G) Relative mRNA expression encoding enzymes involved in bile acid synthesis in livers of male *Washc1*^{IKO} mice compared to WT mice, as determined by qRT-PCR ($n = 9$) fed a standard diet. (H) Correlation between $12\alpha/\text{non-}12\alpha$ -hydroxylated bile acid ratios and fractional cholesterol absorption ($n = 7$ – 11) using a simple linear regression model, *** $P < 0.001$. Data are presented as mean \pm SEM, * $P < 0.05$ as determined by Student's t -test.

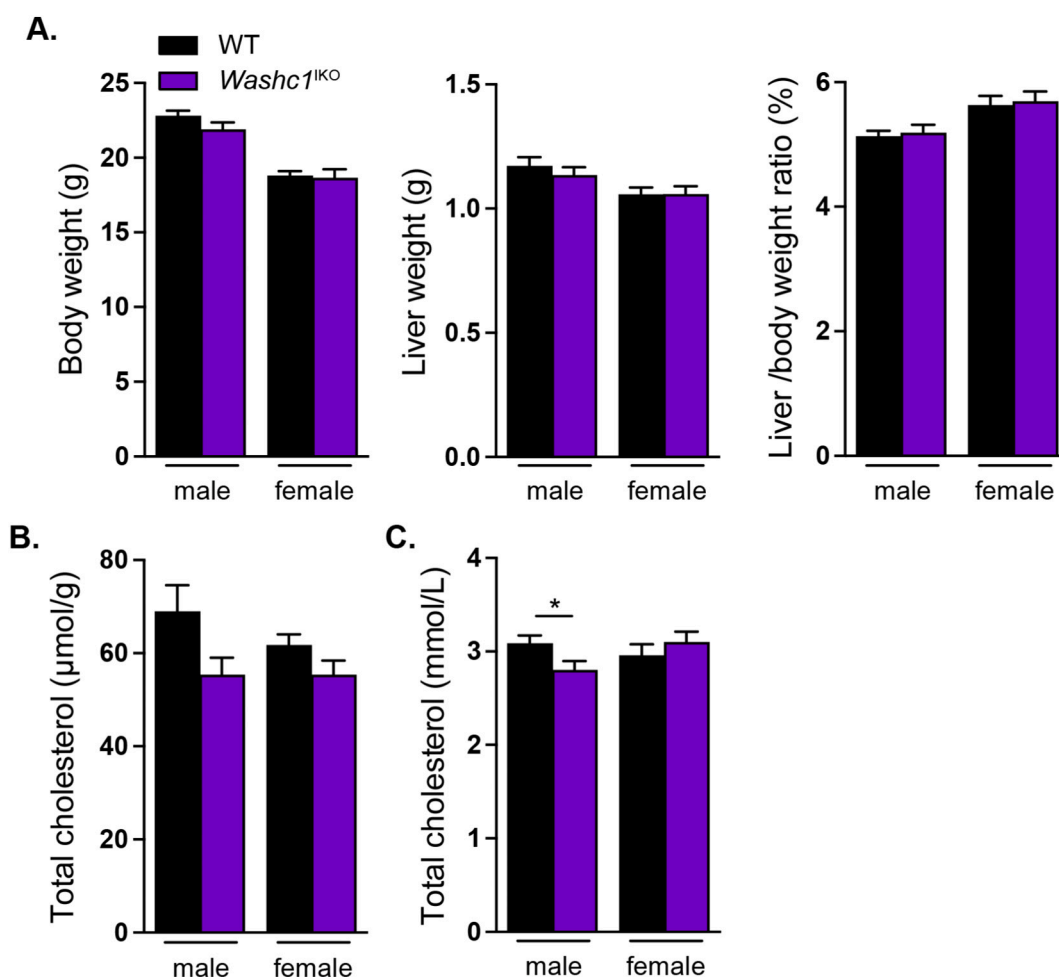


Fig. 6. Increasing 12 α -hydroxylated BA reverses the reduced hepatic cholesterol levels in *Washc1*^{KO} mice fed the HCD. (A) Body weight, liver weight, and liver-to-body weight ratio of WT and *Washc1*^{KO} mice fed a HCD supplemented with 0.5 % (w/w) sodium cholate ($n = 9-10$). (B) Hepatic ($\mu\text{mol/g}$ wet liver weight) and (C) plasma cholesterol concentrations of WT and *Washc1*^{KO} mice fed a HCD diet supplemented with 0.5 % (w/w) sodium cholate ($n = 9-10$). Data are presented as mean \pm SEM, * $P < 0.05$, as determined by Student's t -test.

subfamily C, polypeptide 70 (*Cyp2c70*)-deficient mice is negatively correlated with both biliary ratio of 12 α /non-12 α -hydroxylated BAs and the total hepatic cholesterol content [42]. However, whether the gut microbiota has a causal role in altering BA composition and subsequently reducing intestinal cholesterol uptake or is merely a consequence of changes in the composition of BAs itself remains to be determined.

5. Conclusion

This study provides the first evidence that WASH, a protein complex that participates in the endosomal recycling of plasma membrane receptors, plays an essential role in efficient intestinal cholesterol absorption in mice, likely by modulating the composition of BAs. It supports the well-accepted concept that the BA composition influences intestinal cholesterol uptake, but the mechanism by which intestinal WASH regulates the BA composition in mice remains to be elucidated.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbalip.2023.159445>.

CCRediT authorship contribution statement

Andries Heida: Conceptualization, Data curation, Formal analysis, Visualization, Writing – original draft. **Theo van Dijk:** Data curation, Formal analysis. **Marieke Smit:** Formal analysis, Methodology. **Martijn**

Koehorst: Formal analysis, Methodology. **Mirjam Koster:** Formal analysis, Methodology. **Niels Kloosterhuis:** Data curation, Formal analysis, Methodology. **Rick Havinga:** Methodology. **Vincent W. Bloks:** Data curation, Formal analysis, Writing – review & editing. **Justina C. Wolters:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Alain de Bruin:** Supervision. **Jan Albert Kuivenhoven:** Funding acquisition, Writing – review & editing. **Jan Freark de Boer:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Folkert Kuipers:** Conceptualization, Resources, Writing – review & editing. **Bart van de Sluis:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This study was financially supported by the University Medical Center Groningen (UMCG), partially financed by the European Union (MSCA-ITN-2020, 953489, Acronym EndoConnect coordinated by B.v.d.S.), and The Netherlands Cardiovascular Research Initiative: 'the Dutch Heart Foundation, Dutch Federation of University Medical Centers, The Netherlands Organization for Health Research and Development and the Royal Netherlands Academy of Sciences' (CVON2017-2020; Acronym Genius2) to J.A.K. and B.v.d.S.; ZonMW OC grant (2471032) to J.A.K. and B.v.d.S.; NWO ENW grant (OCENW.M.22.034) to B.v.d.S., J.A.K. is an established investigator of the Netherlands Heart Foundation (2015T068); the Graduate School for Drug Exploration (GUIDE). JfFdB is supported by the Nutrition and Health initiative of the University of Groningen. FK is supported by the Netherlands Heart Foundation (IN CONTROL, CVON2018-27) and the Noaber Foundation (Lunteren, the Netherlands). The authors would like to thank A. Gerding, R.C. Verzijl, U. Tharehalli Mathada, and D.Y. Vos of the Department of Pediatrics, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands, for their assistance with experiments and data analysis.

References

- [1] P. Xie, H. Zhu, L. Jia, Y. Ma, W. Tang, Y. Wang, B. Xue, H. Shi, L. Yu, Genetic demonstration of intestinal NPC1L1 as a major determinant of hepatic cholesterol and blood atherogenic lipoprotein levels, *Atherosclerosis* 237 (2014) 609–617, <https://doi.org/10.1016/j.atherosclerosis.2014.09.036>.
- [2] S.W. Altmann, H.R. Davis, L.-J. Zhu, X. Yao, L.M. Hoos, G. Tetzloff, S.P.N. Iyer, M. Maguire, A. Golovko, M. Zeng, L. Wang, N. Murgolo, M.P. Graziano, Niemann-pick C1 like 1 protein is critical for intestinal cholesterol absorption, *Science* 303 (2004) 1201–1204, <https://doi.org/10.1126/science.1093131>.
- [3] H.R. Davis, L. Zhu, L.M. Hoos, G. Tetzloff, M. Maguire, J. Liu, X. Yao, S.P.N. Iyer, M.-H. Lam, E.G. Lund, P.A. Detmers, M.P. Graziano, S.W. Altmann, Niemann-pick C1 like 1 (NPC1L1) is the intestinal Phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis*, *J. Biol. Chem.* 279 (2004) 33586–33592, <https://doi.org/10.1074/jbc.m405817200>.
- [4] R.H. Knopp, H. Gitter, T. Truitt, H. Bays, C.V. Manion, L.J. Lipka, A.P. LeBeaut, R. Suresh, B. Yang, E.P. Veltri, E.S. Group, Effects of ezetimibe, a new cholesterol absorption inhibitor, on plasma lipids in patients with primary hypercholesterolemia, *Eur. Heart J.* 24 (2003) 729–741, [https://doi.org/10.1016/s0195-668x\(02\)00807-2](https://doi.org/10.1016/s0195-668x(02)00807-2).
- [5] L. Ge, J. Wang, W. Qi, H.-H. Miao, J. Cao, Y.-X. Qu, B.-L. Li, B.-L. Song, The cholesterol absorption inhibitor ezetimibe acts by blocking the sterol-induced internalization of NPC1L1, *Cell Metab.* 7 (2008) 508–519, <https://doi.org/10.1016/j.cmet.2008.04.001>.
- [6] C.P. Cannon, M.A. Blazing, R.P. Giugliano, A. McCagg, J.A. White, P. Theroux, H. Darius, B.S. Lewis, T.O. Ophuis, J.W. Jukema, G.M.D. Ferrari, W. Ruzyllo, P. D. Lucca, K. Im, E.A. Bohula, C. Reist, S.D. Wiviott, A.M. Tershakovec, T. A. Musliner, E. Braunwald, R.M. Califf, I.-I. Investigators, Ezetimibe added to statin therapy after acute coronary syndromes, *N. Engl. J. Med.* 372 (2015) 2387–2397, <https://doi.org/10.1056/nejmoa1410489>.
- [7] L. Jia, Y. Ma, S. Rong, J.L. Betters, P. Xie, S. Chung, N. Wang, W. Tang, L. Yu, Niemann-pick C1-like 1 deletion in mice prevents high-fat diet-induced fatty liver by reducing lipogenesis[S], *J. Lipid Res.* 51 (2010) 3135–3144, <https://doi.org/10.1194/jlr.m006353>.
- [8] L. Jia, Y. Ma, G. Liu, L. Yu, Dietary cholesterol reverses resistance to diet-induced weight gain in mice lacking Niemann-pick C1-like 1[S], *J. Lipid Res.* 51 (2010) 3024–3033, <https://doi.org/10.1194/jlr.m008599>.
- [9] E.D. Labonté, L.M. Camarota, J.C. Rojas, R.J. Jandacek, D.E. Gilham, J.P. Davies, Y.A. Ioannou, P. Tso, D.Y. Hui, P.N. Howles, Reduced absorption of saturated fatty acids and resistance to diet-induced obesity and diabetes by ezetimibe-treated and Npc1l1^{-/-} mice, *Am. J. Physiol.-Gastrointest. Liver Physiol.* 295 (2008) G776–G783, <https://doi.org/10.1152/ajpgi.90275.2008>.
- [10] L. Yu, S. Bharadwaj, J.M. Brown, Y. Ma, W. Du, M.A. Davis, P. Michael, P. Liu, M. C. Willingham, L.L. Rudel, Cholesterol-regulated translocation of NPC1L1 to the cell surface facilitates free cholesterol uptake*, *J. Biol. Chem.* 281 (2006) 6616–6624, <https://doi.org/10.1074/jbc.m51123200>.
- [11] B.A.P. Phan, T.D. Dayspring, P.P. Toth, Ezetimibe therapy: mechanism of action and clinical update, *Vasc. Heal. Risk Manag.* 8 (2012) 415–427, <https://doi.org/10.2147/vhrm.s33664>.
- [12] J.P. Davies, B. Levy, Y.A. Ioannou, Evidence for a Niemann-Pick C (NPC) gene family: identification and characterization of NPC1L1, *Genomics* 65 (2000) 137–145, <https://doi.org/10.1006/geno.2000.6151>.
- [13] J.L. Betters, L. Yu, NPC1L1 and cholesterol transport, *FEBS Lett.* 584 (2010) 2740–2747, <https://doi.org/10.1016/j.febslet.2010.03.030>.
- [14] E.V. Linardopoulou, S.S. Parghi, C. Friedman, G.E. Osborn, S.M. Parkhurst, B. J. Trask, Human Subtelomeric WASH genes encode a new subclass of the WASP family, *PLoS Genet.* 3 (2007), e237, <https://doi.org/10.1371/journal.pgen.0030237>.
- [15] T.S. Gomez, D.D. Billadeau, A FAM21-containing WASH complex regulates retromer-dependent sorting, *Dev. Cell* 17 (2009) 699–711, <https://doi.org/10.1016/j.devcel.2009.09.009>.
- [16] V. Dostál, T. Humhalová, P. Beránková, O. Pácal, L. Libusová, SWIP mediates retromer-independent membrane recruitment of the WASH complex, *Traffic* 24 (2023) 216–230, <https://doi.org/10.1111/tra.12884>.
- [17] E. Derivery, E. Helffer, V. Henriot, A. Gautreau, Actin polymerization controls the organization of WASH domains at the surface of endosomes, *PLoS One* 7 (2012), e39774, <https://doi.org/10.1371/journal.pone.0039774>.
- [18] E. Derivery, C. Sousa, J.J. Gautier, B. Lombard, D. Loew, A. Gautreau, The Arp2/3 activator WASH controls the fission of endosomes through a large multiprotein complex, *Dev. Cell* 17 (2009) 712–723, <https://doi.org/10.1016/j.devcel.2009.09.010>.
- [19] M.N.J. Seaman, A. Gautreau, D.D. Billadeau, Retromer-mediated endosomal protein sorting: all WASHed up!, *Trends Cell Biol.* 23 (2013) 522–528, <https://doi.org/10.1016/j.tcb.2013.04.010>.
- [20] F. Ropers, E. Derivery, H. Hu, M. Garshasbi, M. Karbasiyan, M. Herold, G. Nurnberg, R. Ullmann, A. Gautreau, K. Sperling, R. Varon, A. Rajab, Identification of a novel candidate gene for non-syndromic autosomal recessive intellectual disability: the WASH complex member SWIP, *Hum. Mol. Genet.* 20 (2011) 2585–2590, <https://doi.org/10.1093/hmg/ddr158>.
- [21] P.N. Veldman, I.A. Meijer, A. Reynolds, A. Lei, P. MacLeod, D. Schlesinger, M. Zatz, E. Reid, P.A. Dion, P. Drapeau, G.A. Rouleau, Mutations in the KIAA0196 gene at the SPG8 locus cause hereditary spastic paraplegia, *Am. J. Hum. Genet.* 80 (2007) 152–161, <https://doi.org/10.1086/510782>.
- [22] S.T. de Bot, S. Vermeer, W. Buijsman, A. Heister, M. Voorendt, A. Verrips, H. Scheffer, H.P.H. Kremer, B.P.C. van de Warrenburg, E.-J. Kamsteeg, Pure adult-onset spastic paraplegia caused by a novel mutation in the KIAA0196 (SPG8) gene, *J. Neurol.* 260 (2013) 1765–1769, <https://doi.org/10.1007/s00415-013-6870-x>.
- [23] A.M. Elliott, L.R. Simard, G. Coghan, A.E. Chudley, B.N. Chodirker, C. R. Greenberg, T. Burch, V. Ly, G.M. Hatch, T. Zelinski, A novel mutation in KIAA0196: identification of a gene involved in Ritscher-Schinzel/3C syndrome in a first nations cohort, *J. Med. Genet.* 50 (2013) 819–822, <https://doi.org/10.1136/jmedgenet-2013-101715>.
- [24] P. Bartuzzi, D.D. Billadeau, R. Favier, S. Rong, D. Dekker, A. Fedoseienko, H. Fieten, M. Wijers, J.H. Levels, N. Huijckman, N. Kloosterhuis, H. van der Molen, G. Brufau, A.K. Groen, A.M. Elliott, J.A. Kuivenhoven, B. Plecko, G. Grangl, J. McGaughan, J. D. Horton, E. Burstein, M.H. Hofker, B. van de Sluis, CCC- and WASH-mediated endosomal sorting of LDLR is required for normal clearance of circulating LDL, *Nat Commun.* 7 (2016) 10961, <https://doi.org/10.1038/ncomms10961>.
- [25] M. Wijers, P. Zanoni, N. Liv, D.Y. Vos, M.Y. Jäckstein, M. Smit, S. Wilbrink, J. C. Wolters, Y.T. van der Veen, N. Huijckman, D. Dekker, N. Kloosterhuis, T.H. van Dijk, D.D. Billadeau, F. Kuipers, J. Klumperman, A. von Eckardstein, J. A. Kuivenhoven, B. van de Sluis, The hepatic WASH complex is required for efficient plasma LDL and HDL cholesterol clearance, *Jci Insight.* 4 (2019), e126462, <https://doi.org/10.1172/jci.insight.126462>.
- [26] L. Ding, L. Han, J. Dube, D.D. Billadeau, WASH regulates glucose homeostasis by facilitating Glut2 receptor recycling in pancreatic beta cells, *Diabetes* (2018), <https://doi.org/10.2337/db18-0189> db180189.
- [27] Y.-Y. Zhang, Z.-Y. Fu, J. Wei, W. Qi, G. Baituola, J. Luo, Y.-J. Meng, S.-Y. Guo, H. Yin, S.-Y. Jiang, Y.-F. Li, H.-H. Miao, Y. Liu, Y. Wang, B.-L. Li, Y.-T. Ma, B.-L. Song, A LIMA1 variant promotes low plasma LDL cholesterol and decreases intestinal cholesterol absorption, *Science* 360 (2018) 1087–1092, <https://doi.org/10.1126/science.aao6575>.
- [28] T.S. Gomez, J.A. Gorman, A.A.-M. de Narvajas, A.O. Koenig, D.D. Billadeau, Trafficking defects in WASH-knockout fibroblasts originate from collapsed endosomal and lysosomal networks, *Mol. Biol. Cell* 23 (2012) 3215–3228, <https://doi.org/10.1091/mbc.e12-02-0101>.
- [29] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol.* 37 (1959) 911–917, <https://doi.org/10.1139/c59-099>.
- [30] D.Y. Vos, M. Wijers, M. Smit, N. Huijckman, N.J. Kloosterhuis, J.C. Wolters, J. J. Tissink, A.C.M. Pronk, S. Kooijman, P.C.N. Rensen, J.A. Kuivenhoven, B. van de Sluis, Cargo-specific role for retriever subunit VPS26C in hepatocyte lipoprotein receptor recycling to control postprandial triglyceride-rich lipoproteins, *Arteriosclerosis Thrombosis Vasc Biology.* (2022), <https://doi.org/10.1161/atvbaha.122.318169>.
- [31] O.A.H.O. Ronda, T.H. Dijk, H.J. Verkade, A.K. Groen, Measurement of intestinal and peripheral cholesterol fluxes by a dual-tracer balance method, *Curr. Protoc. Mouse Biol.* 6 (2016) 408–434, <https://doi.org/10.1002/cpmo.16>.
- [32] A. Fedoseienko, M. Wijers, J.C. Wolters, D. Dekker, M. Smit, N. Huijckman, N. Kloosterhuis, H. Klug, A. Schepers, K.W. van Dijk, J.H.M. Levels, D.D. Billadeau, M.H. Hofker, J. van Deursen, M. Westerterp, E. Burstein, J.A. Kuivenhoven, B. van de Sluis, The COMMD family regulates plasma LDL Levels and attenuates atherosclerosis through stabilizing the CCC complex in endosomal LDLR trafficking, *Circ. Res.* 122 (2017) 1648–1660, <https://doi.org/10.1161/circresaha.117.312004>.
- [33] J.C. Wolters, J. Ciapaita, K. van Eunen, K.E. Niezen-Koning, A. Matton, R.J. Porte, P. Horvatovich, B.M. Bakker, R. Bischoff, H.P. Permentier, Translational targeted proteomics profiling of mitochondrial energy metabolic pathways in mouse and human samples, *J. Proteome Res.* 15 (2016) 3204–3213, <https://doi.org/10.1021/acs.jproteome.6b00419>.
- [34] I.P. van de Peppel, A. Bertolini, T.H. van Dijk, A.K. Groen, J.W. Jonker, H. J. Verkade, Efficient reabsorption of transintestinally excreted cholesterol is a

- strong determinant for cholesterol disposal in mice[S], *J. Lipid Res.* 60 (2019) 1562–1572, <https://doi.org/10.1194/jlr.m094607>.
- [35] C. Murphy, P. Parini, J. Wang, I. Björkhem, G. Eggertsen, M. Gåfvels, Cholic acid as key regulator of cholesterol synthesis, intestinal absorption and hepatic storage in mice, *Biochim. Biophys. Acta (BBA) - Mol. Cell Biol. Lipids.* 1735 (2005) 167–175, <https://doi.org/10.1016/j.bbalip.2005.06.001>.
- [36] L.A. Woollett, D.D. Buckley, L. Yao, P.J.H. Jones, N.A. Granholm, E.A. Tolley, P. Tso, J.E. Heubi, Cholic acid supplementation enhances cholesterol absorption in humans, *Gastroenterology* 126 (2004) 724–731, <https://doi.org/10.1053/j.gastro.2003.11.058>.
- [37] A.L. Ticho, P. Malhotra, P.K. Dudeja, R.K. Gill, W.A. Alrefai, Intestinal absorption of bile acids in health and disease, *Compr. Physiol.* 10 (2019) 21–56, <https://doi.org/10.1002/cphy.c190007>.
- [38] E. Bertaggia, K.K. Jensen, J. Castro-Perez, Y. Xu, G.D. Paolo, R.B. Chan, L. Wang, R. A. Haeusler, Cyp8b1 ablation prevents Western diet-induced weight gain and hepatic steatosis because of impaired fat absorption, *Am. J. Physiol.-Endocrinol. Metab.* 313 (2017) E121–E133, <https://doi.org/10.1152/ajpendo.00409.2016>.
- [39] W. Nihei, M. Nagafuku, H. Hayamizu, Y. Odagiri, Y. Tamura, Y. Kikuchi, L. Veillon, H. Kanoh, K. Inamori, K. Arai, K. Kabayama, K. Fukase, J. Inokuchi, NPC1L1-dependent intestinal cholesterol absorption requires ganglioside GM3 in membrane microdomains, *J. Lipid Res.* 59 (2018) 2181–2187, <https://doi.org/10.1194/jlr.m089201>.
- [40] A. Singla, A. Fedoseienko, S.S.P. Giridharan, B.L. Overlee, A. Lopez, D. Jia, J. Song, K. Huff-Hardy, L. Weisman, E. Burstein, D.D. Billadeau, Endosomal PI(3)P regulation by the COMMD/CCDC22/CCDC93 (CCC) complex controls membrane protein recycling, *Nat. Commun.* 10 (2019) 4271, <https://doi.org/10.1038/s41467-019-12221-6>.
- [41] B. Zhang, F. Kuipers, J.F. de Boer, J.A. Kuivenhoven, Modulation of bile acid metabolism to improve plasma lipid and lipoprotein profiles, *J. Clin. Med.* 11 (2021) 4, <https://doi.org/10.3390/jcm11010004>.
- [42] R. Li, A. Palmiotti, H.D. de Vries, M.V. Hovingh, M. Koehorst, N.L. Mulder, Y. Zhang, K. Kats, V.W. Bloks, J. Fu, H.J. Verkade, J.F. de Boer, F. Kuipers, Low production of 12 α -hydroxylated bile acids prevents hepatic steatosis in Cyp2c70^{-/-} mice by reducing fat absorption, *J. Lipid Res.* 62 (2021), 100134, <https://doi.org/10.1016/j.jlr.2021.100134>.
- [43] J. Li-Hawkins, M. Gåfvels, M. Olin, E.G. Lund, U. Andersson, G. Schuster, I. Björkhem, D.W. Russell, G. Eggertsen, Cholic acid mediates negative feedback regulation of bile acid synthesis in mice, *J. Clin. Investig.* 110 (2002) 1191–1200, <https://doi.org/10.1172/jci16309>.
- [44] H.R.D. Jr, L.M. Hoos, G. Tetzloff, M. Maguire, L. Zhu, M.P. Graziano, S. W. Altmann, Deficiency of Niemann-pick C1 like 1 prevents atherosclerosis in ApoE^{-/-} mice, *Arter. Thromb. Vasc. Biol.* 27 (2007) 841–849, <https://doi.org/10.1161/01.atv.0000257627.40486.46>.
- [45] M. Rutlin, D. Rastelli, W.T. Kuo, J.A. Estep, A. Louis, M.M. Riccomagno, J. R. Turner, M. Rao, The Villin1 gene promoter drives Cre recombinase expression in extraintestinal tissues, *Cell. Mol. Gastroenterol. Hepatol.* 10 (2020) 864–867.e5, <https://doi.org/10.1016/j.jcmgh.2020.05.009>.
- [46] F. Kuipers, H.H. Spanjer, R. Havinga, G.L. Scherphof, R.J. Vonk, Lipoproteins and liposomes as in vivo cholesterol vehicles in the rat: preferential use of cholesterol carried by small unilamellar liposomes for the formation of muricholic acids, *Biochim. Biophys. Acta (BBA) - Lipids Lipid Metab.* 876 (1986) 559–566, [https://doi.org/10.1016/0005-2760\(86\)90044-5](https://doi.org/10.1016/0005-2760(86)90044-5).
- [47] K.B. Halpern, R. Shenhav, O. Matcovitch-Natan, B. Tóth, D. Lemze, M. Golan, E. Massasa, S. Baydatch, S. Landen, A.E. Moor, A. Brandis, A. Giladi, A. Stokar-Avihail, E. David, I. Amit, S. Itzkovitz, Single-cell spatial reconstruction reveals global division of labour in the mammalian liver, *Nature* 542 (2017) 352–356, <https://doi.org/10.1038/nature21065>.
- [48] T. Yntema, D.P.Y. Koonen, F. Kuipers, Emerging roles of gut microbial modulation of bile acid composition in the etiology of cardiovascular diseases, *Nutrients* 15 (2023) 1850, <https://doi.org/10.3390/nu15081850>.