Mice with a deficiency in Peroxisomal Membrane Protein 4 (PXMP4) display mild changes in hepatic lipid metabolism

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Peroxisomes play an important role in the metabolism of a variety of biomolecules, including lipids and bile acids. Peroxisomal Membrane Protein 4 (PXMP4) is a ubiquitously expressed peroxisomal membrane protein that is transcriptionally regulated by peroxisome proliferator-activated receptor α (PPARα), but its function is still unknown. To investigate the physiological function of PXMP4, we generated aPxmp4 knockout (Pxmp4−/−) mouse model using CRISPR/Cas9-mediated gene editing. Peroxisome function was studied under standard chow-fed conditions and after stimulation of peroxisomal activity using the PPARα ligand fenofibrate or by using phytol, a metabolite of chlorophyll that undergoes peroxisomal oxidation. Pxmp4−/− mice were viable, fertile, and displayed no changes in peroxisome numbers or morphology under standard conditions. Also, no differences were observed in the plasma levels of products from major peroxisomal pathways, including very long-chain fatty acids (VLCFAs), bile acids (BAs), and BA intermediates di- and trihydroxycholestanoic acid. Although elevated levels of the phytol metabolites phytanic and pristanic acid inPxmp4−/− mice pointed towards an impairment in peroxisomal α-oxidation capacity, treatment of Pxmp4−/− mice with a phytol-enriched diet did not further increase phytanic/pristanic acid levels. Finally, lipidomic analysis revealed that loss of Pxmp4 decreased hepatic levels of the alkyldiacylglycerol class of neutral ether lipids, particularly those containing polyunsaturated fatty acids. Together, our data show that while PXMP4 is not critical for overall peroxisome function under the conditions tested, it may have a role in the metabolism of (ether)lipids.

Abbreviations

ABC3D  ATP-binding cassette sub-family D member 3
ACOX1  Acyl-coenzyme A oxidase 1
AMACR  Alpha-methylacyl-CoA racemase
BAAT  Bile acid-CoA amino acid N-acyltransferase
BA  Bile acid
BCFA  Branched-chain fatty acid
CD1  Cluster of differentiation 1
CPT1A  Carnitine palmitoyltransferase 1a

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Peroxisomes play important roles in cellular detoxification but also in energy metabolism through oxidation and biosynthesis of lipids and bile acids. The importance of peroxisomes is highlighted by diseases in which peroxisomes are malfunctioning either due to a single peroxisomal enzyme deficiency or a defect in peroxisome biosynthesis. Mutations in genes encoding peroxisomal proteins lead to a diverse spectrum of disorders, which can be divided into peroxisomal biogenesis disorders and disorders of peroxisome function. The latter group can be further subdivided into enzymatic deficiencies and transport deficiencies. Peroxisomal disorders are characterized by a broad range of symptoms, which depend on the specific biological pathways controlled by the defective peroxisomal protein. Therefore, symptoms can vary from minimal clinical abnormalities in some patients to severe neurological features and early death depending upon the extent of the deficiency and the identity of the defect involved.

The functions of peroxisomes have been studied by manipulation of genes coding for peroxisomal proteins and by pharmacological manipulation of peroxosomal activity, such as peroxisome proliferators. Peroxisome proliferators act through activation of the peroxisome proliferator-activated receptors (PPARs), a subfamily of nuclear receptors, thereby stimulating specific peroxisome functions. Because PPARs can affect a plethora of metabolic processes, their potential role in the treatment of metabolic disorders has become subject to extensive research.

PPARα is a master regulator of lipid metabolism and is highly expressed in tissues with a high rate of fatty acid oxidation, including the liver, brown adipose tissue, (skeletal) muscle, and heart. Endogenous ligands for PPARα include polyunsaturated fatty acids and metabolites such as eicosanoids and leukotriene B4. PPARα plays an important role in the fasting response. Upon activation, PPARα increases fatty acid oxidation and ketogenesis in various tissues such as the liver and muscle and regulates glucose metabolism in the liver.

Many different genes encoding peroxisomal proteins have been identified over the last few years; however, several of the proteins involved have not yet been characterized. Knowledge about their function could be instrumental for the understanding of the pathogenesis of peroxisomal disorders. By analyzing transcriptome data using the Genevestigator database (genevestigator.com), we identified Peroxisomal Membrane Protein 4 (Pmp24, Pxmp4) as a strong target of PPARα. Similarly, Rakshandehroo et al. (2009) reported the upregulation of Pmp24, Pxmp4 by the PPARα agonist Wy14643 in human and mouse primary hepatocytes. PMP4 is an integral membrane protein of 212 amino acids and has a molecular mass of 24 kDa. The PMP4 gene is ubiquitously expressed and displays strong species conservation. Although various somatic mutations and hypermethylation resulting in the silencing of PMP4 in humans have been reported for several types of cancer, its role in tumor development as well as its physiological functions have remained unknown.

In the current study, we aimed to address the metabolic function of PMP4, using mice with a genetic deficiency in PMP4 in combination with pharmacological approaches to stimulate peroxisomal activity. 

Results

Generation and characterization of Pxmp4 knockout mice. By analyzing transcriptome data using the Genevestigator database (genevestigator.com), we identified PXMP4 as a strong target of PPARα (Fig. 1A). The mouse Pmp4 gene is located on chromosome 2 and contains four exons, encoding a protein of 212 amino acids (Fig. 1B). A 19 base pair (bp) deletion in exon 1 was introduced by targeted CRISPR/Cas9-mediated gene editing resulting in germline inactivation of Pmp4. This mutation causes a premature stop codon in exon 2, resulting in a loss-of-function protein. Levels of Pmp4 mRNA were not detectable in livers of Pmp4−/− mice (Fig. 1C). In line with this, endogenous PXMP4 protein levels, as determined by targeted proteomics, were below the detection limit in livers of Pmp4−/− mice, whereas PXMP4 was readily detectable in livers of wild type mice (Fig. 1D). Pmp4−/− mice were born according to the expected Mendelian ratio, were fertile, and showed no...
growth retardation or other visible abnormalities (data not shown). Under standard chow conditions, no differences were observed in body weight and fat mass between \( \text{Pxmp4}^{-/-} \) and wild type littermates (Supplementary Fig. 1A,B).

**Effect of PXMP4 deficiency on peroxisome morphology, numbers, and function under standard chow conditions.** Inactivation of various peroxisomal proteins has been shown to disrupt peroxisome proliferation, peroxisome morphology or function in many different organisms including mice and humans\(^{1,3-5}\). Ultrastructural analysis using electron microscopy showed that peroxisome number and morphology were not visibly altered in the livers of \( \text{Pxmp4}^{-/-} \) mice compared to wild type littermates (Supplementary Fig. 2A,B).

To assess whether PXMP4 deficiency affected peroxisomal α- and β-oxidation of VLCFA and other lipids, we measured VLCFA levels in plasma\(^{14}\). In \( \text{Pxmp4}^{-/-} \) mice, plasma levels of docosanoic acid (C22), lignoceric acid (C24), and hexacosanoic acid (C26) were not different as compared to wild type littermates (Fig. 2A). Hepatic expression of genes involved in mitochondrial and peroxisomal β-oxidation, respectively, which includes carnitine palmitoyltransferase 1a (\( \text{Cpt1a} \)) and acyl-coenzyme A oxidase 1 (\( \text{Acox1} \)), were not differentially expressed between \( \text{Pxmp4}^{-/-} \) mice and wild type littermates (Fig. 2B).

The α-oxidation of phytanic acid and β-oxidation of pristanic acid exclusively occurs in peroxisomes, and various peroxisomal disorders are therefore characterized by increased phytanic and pristanic acid levels\(^{1}\). In our study, \( \text{Pxmp4}^{-/-} \) animals displayed slightly elevated plasma levels of phytanic (respectively 0.56 vs. 0.65 µmol/L, \( p = 0.008 \)), but not of pristanic acid (respectively 0.05 vs. 0.08 µmol/L, \( p = 0.056 \)) (Fig. 2C). Hepatic expression of genes involved in mitochondrial and peroxisomal β-oxidation, respectively, which includes carnitine palmitoyltransferase 1a (\( \text{Cpt1a} \)) and acyl-coenzyme A oxidase 1 (\( \text{Acox1} \)), were not differentially expressed between \( \text{Pxmp4}^{-/-} \) mice and wild type littermates (Fig. 2B).

Because hepatic peroxisomes are involved in side-chain shortening of C27-bile acid intermediates and conjugation of C24-bile acid species\(^{33}\), we analyzed unconjugated and conjugated bile acid species in bile and plasma using LC–MS (Fig. 2E, Supplementary Figs. 3A, 4A, 5A). Biliary and plasma levels of unconjugated, conjugated, or individual BA species were not different between \( \text{Pxmp4}^{-/-} \) mice and wild type littermates under standard chow conditions. In line with this, hepatic gene expression of cholesterol 7 alpha-hydroxylase (\( \text{Cyp7a1} \)), sterol 27-hydroxylase (\( \text{Cyp27a1} \)), hydroxysteroid (17-beta) dehydrogenase 4 (\( \text{Hsd17b4} \)), and bile acid-CoA amino acid N-acyltransferase (\( \text{Baat} \)) which are all involved in BA metabolism, were also not changed (Fig. 2F). Taken together, our results indicate that PXMP4 deficiency has a minor effect on phytanic acid homeostasis whereas other tested peroxisome functions such as β-oxidation of VLCFA and BA synthesis including BA conjugation were not affected.
Effect of PXPM4 deficiency on peroxisome function upon PPARα-stimulation. As loss of PXMP4 did not alter peroxisome function under normal conditions, we next determined the relevance of PXMP4 after stimulating peroxisome activity by enhancing PPARα activity using fenofibrate (FF). Administration of FF (0.2% w/w) for two weeks in the diet resulted in significant decreases in the body weight of both Pxmp4−/− mice and wild type littermates compared to chow without affecting relative fat mass (Supplementary Fig. 1A,B). As expected, FF led to the increased hepatic expression of Pmp70 and the PPARα target genes Cyp4a10, Cyp4a14, and acetyl-CoA acyltransferase 1 (Acaa1) in both genotypes, whereas Pxmp4 expression was only increased in wild type mice (Supplementary Fig. 1C,F).

Plasma levels of VLCFAs or hepatic expression of PPARα target genes Cpt1a and Acox1 were not different between Pxmp4−/− and wild type littermates after FF administration (Fig. 3A,B). However, FF significantly decreased plasma levels of C22 and C24 in both genotypes compared to chow conditions (Supplementary Fig. 1D). FF decreased plasma levels of C26 only in Pxmp4−/− mice (0.40 vs. 0.34 µmol/L, \( p = 0.048 \)), but not in wild type mice (0.37 vs. 0.36 µmol/L, \( p = 0.969 \)). Although administration of FF did not change plasma levels of phytanic and pristanic acid between Pxmp4−/− mice and wild type littermates (Fig. 3C), FF did lower the levels of plasma phytanic and pristanic acid in Pxmp4−/− mice compared to standard chow conditions (Supplementary Fig. 1E). In wild type mice, FF decreased plasma levels of phytanic acid, but not pristanic acid, compared to standard chow. Hepatic expression of Pmp70, Fabp1, Scp2, Amacr, and Phyh were not altered (Fig. 3D).
Administration of FF did not result in different levels of unconjugated, conjugated, or individual BA species in bile or plasma between Pxmp4−/− mice and wild type littermates (Fig. 3E, Supplementary Figs. 3B, 4B, 5B). Also, the expression of genes involved in bile acid metabolism was not altered (Fig. 3F). Taken together, these results indicate that Pxmp4−/− mice do not have an overt phenotype under standard chow dietary conditions or after PPARα-dependent stimulation of peroxisomal activity by FF.

Effect of PXPM4 deficiency on peroxisome function after administration of phytol. Earlier studies investigating mouse models for peroxisomal disorders have used the chlorophyll metabolite phytol to study peroxisomal α- and β-oxidation pathways\(^7,31,33–37\). Phytol is converted into the branched-chain fatty acids (BCFAs) phytanic and pristanic acid stepwise through the concerted action of multiple enzymes distributed among different subcellular organelles in the cytosol, endoplasmic reticulum, peroxisome, and mitochondrion\(^14\). A deficiency in the oxidation of phytol, as in mice deficient in phytanoyl-CoA hydroxylase, can lead to a severe phenotype characterized by the accumulation of phytanic acid with or without elevated pristanic acid, resulting in liver failure, hepatic steatosis, infiltration of inflammatory cells, and peripheral neuropathy\(^7,31,38\). Because of the mildly elevated levels of phytanic acid in Pxmp4−/− mice under standard chow-fed conditions, we decided to study if dietary supplementation of phytol in the diet (0.25% w/w) for four weeks could induce a phenotype in Pxmp4−/− mice\(^35–37\). Phytol treatment was effective as indicated by increased hepatic expression of Pmp70 (Supplementary Fig. 6A) and the PPARα target genes Cyp4a10, Cyp4a14, and Acaa1 (Supplementary Fig. 6D) in all

**Figure 3.** Effect of PXMP4 deficiency on stimulation of peroxisome function by FF. (A) Plasma levels of the VLCFAs docosanoic acid (C22), lignoceric acid (C24) and hexacosanoic acid (C26); (B) Hepatic expression of genes involved in VLCFA oxidation; (C) Plasma levels of phytanic and pristanic acid; (D) Hepatic expression of genes involved in metabolism of phytanic and pristanic acid; (E) Biliary unconjugated and conjugated bile acid concentrations; (F) Hepatic expression of genes involved in bile acid metabolism in Pxmp4−/− mice and wild type littermates (n = 5–6). Scatter plots represent individual data with a median ± IQR and statistical significance was tested by a Mann–Whitney test. Bar plots represent mean ± SD and significance was tested by the non-parametric one-way ANOVA (Kruskal–Wallis) test, followed by Mann–Whitney U tests.
mice. Effective phytol treatment was also evidenced by a strong increase in peroxisome number observed in electron microscopic images (from 15 ± 6 to 53 ± 27 peroxisomes/hepatocyte in \( \text{Pxmp4}^{-/-} \) mice and 13 ± 5 to 54 ± 19 in wild type littermates) (Supplementary Fig. 2C,D). However, peroxisome numbers were not different between genotypes. Furthermore, except for mildly elevated levels of pristanic acid in \( \text{Pxmp4}^{-/-} \) mice, no overt toxicity was observed. Also, upon phytol supplementation, \( \text{Pxmp4}^{-/-} \) mice did not show differences in body weight, fat mass, and hepatic levels of phytanic acid compared to wild type littermates (Fig. 4A; Supplementary Fig. 6B,C). Moreover, we did not observe any differences in the expression of genes involved in phytanic or pristanic acid metabolism between \( \text{Pxmp4}^{-/-} \) mice and wild type littermates (Fig. 4B).

Effect of PXMP4 deficiency on the hepatic lipidome. As the loss of PXMP4 did not impair metabolism of VLCFAs or bile acids, we next explored its potential role in lipid metabolism by performing targeted lipidome analysis on the liver of \( \text{Pxmp4}^{-/-} \) mice and wild type littermates, with or without phytol supplementation. Partial Least Square (PLS) regression analysis showed that phytol supplementation explained most of the observed variance in the hepatic lipidome (Fig. 5A). The top 50 most changed lipid species based on PLS-derived VIP scores appeared to be strongly enriched in various branched-chain (BC), bismonoacylglycerophosphate (BMP), phosphatidylcholine (PC), and alkyldiacylglycerol (TG(O)) lipid species (Fig. 5B). Further stratification revealed that the increased levels of various branched-chain-, and BMP lipid species were largely explained by phytol supplementation and most likely reflect the incorporation of the branched chain fatty acid phytanic acid and its fatty acid metabolites, after initial conversion from phytol, into these complex lipids (Supplementary Fig. 7). The levels of these BC, PC, and BMP lipids, however, were not different between \( \text{Pxmp4}^{-/-} \) mice and wild type littermates (Supplementary Fig. 7) further underscoring that Pxmp4 is not involved in branched-chain fatty acid metabolism.

While the abundance of the majority of the >1000 analyzed lipids was not substantially affected by PXMP4 deficiency, we observed a moderate decrease in TG(O) in the liver of \( \text{Pxmp4}^{-/-} \) mice as compared to wild type littermates, and this lipid species was even further decreased upon phytol treatment (Fig. 5C). In contrast, loss of Pxmp4 or phytol treatment did not affect total hepatic TG levels (Fig. 5D). Closer examination of the effect of phytol on the TG(O) species profile showed that the levels of polyunsaturated TG(O) species were more strongly reduced as compared to the saturated, mono- di and tri-saturated species, which were present at comparable levels (Fig. 5E). On the other hand, species likely containing less unsaturated fatty acids (i.e. C18:2, C18:3, C20:4) were increased by phytol. This fatty acid effect was also present in TG species and occurred in both genotypes although more prominent in \( \text{Pxmp4}^{-/-} \) mice (Fig. 5B; Supplementary Fig. 8). There was no clear deficiency of...
polyunsaturated species in other major classes. Overall, these observations suggest that PXMP4 has a role in the metabolism of ether lipids, particularly those containing polyunsaturated fatty acids.

**Discussion**

In this study, we investigated the function of the peroxisomal PPARα target PXMP4 using a total body knockout mouse model. While *Pxmp4* deficient mice displayed no obvious defects in VLCFA and bile acid metabolism under standard chow conditions or after stimulation of PPARα, decreased hepatic levels of neutral ether lipids, particularly the ones containing polyunsaturated fatty acids suggest a role for PXMP4 in their metabolism. Although identified in 1999, the function of PXMP4 has remained unknown. The lack of an overt phenotype in knockout mice indicates that the function of PXMP4 is not essential or redundant under the tested conditions.

**Figure 5.** Effect of PXMP4 deficiency and phytol supplementation on the hepatic lipidome. (A) Partial Least Square (PLS) regression analysis; (B) Most important changed lipid species based on PLS-derived VIP scores; (C) Effect of PXMP4 deficiency and phytol supplementation on total hepatic TG levels; (D) Effect of PXMP4 deficiency and phytol supplementation on total hepatic TG(O) levels; (E) Effect of PXMP4 deficiency and phytol supplementation on the hepatic abundance of TG(O-60) species.
for the indicated metabolic pathways. In contrast to what has been reported for several other single peroxisomal protein deficiencies, no alterations in VLCFA levels or conjugated bile acids were found between Pxmp4−/− and wild type littermates46. Based on decreased PXMP4 expression in NOD mice, which have a deficiency in the numbers and function of natural killer T-cells (NKT) cells, Fletcher et al. proposed a role for PXMP4 in the adaptive and innate immune system by regulation of numbers of NKT type 1 (NKT1) cells. NKT1 cells are activated by the antigen-presenting molecule cluster of differentiation 1 (CD1) family, and ligands for CD1d include ether phospholipids, which are synthesized by peroxisomes48. Based on these findings, it has been proposed that PXMP4 is involved in ligand availability for CD1d. However, the role of PXMP4 and other peroxisomal proteins in the supply of ligands for NKT1 activation remains unclear40. Metabolic processes, including fatty acid oxidation, are aberrant during carcinogenesis, and due to their important metabolic function, a role for peroxisomes in cancer has been suggested. Several peroxisomal proteins such as α-methylacyl-CoA racemase (AMACR) are elevated in various tumour types, including prostate cancer (PCa) and patients with PCa often display increased plasma phytanic acid levels34. PXMP4 has been implicated in the development of PCa because it was transcriptionally silenced by the epigenetic process of DNA hypermethylation in a human PCa cell line45,46. Conversely, overexpression of Pxmp4 in LNCaP cells and the prostate cancer-3 (PC-3) cell line resulted in a decrease in the number of cancer cells (by 27% and 36%, respectively), indicating that Pxmp4 could function as a tumor suppressor gene40. However, the individual roles of many peroxisomal proteins, including PXMP4, in carcinogenesis are not conclusive and therefore remain to be elucidated. We did not investigate the incidence of PCa in our Pxmp4−/− mouse model because mice are relatively resistant to the development of PCa49.

PXMP4 is a target of PPARα and this nuclear receptor can be activated by fibrates as well as by the branched-chain fatty alcohol phytol and its metabolites51–47. PPARα activation resulted in decreased plasma VLCFA levels and changes in conjugated bile acids levels, in line with other studies48–50. PPARα activation was also supported by changes in hepatic gene expression of PPARα target genes both in the knockout mice and wild type littermates and reported in earlier studies51.

Despite the lack of an overt phenotype in Pxmp4−/− mice, increased plasma levels of phytic acid and hepatic levels of pristanic acid pointed towards an impairment in α-oxidation. Interestingly, the increase in phytic and pristanic acid in Pxmp4−/− mice compared to wild type littermates under standard chow disappeared after FF treatment. This was also reported for Phyh−/− mice that were treated with 0.1% FF for 2 weeks48. In that study, co-administration of FF and phytic acid decreased plasma phytic acid levels compared to administration to the diet of phytic acid alone. A possible explanation for this effect is that FF increases the α-oxidation of phytic acid in the liver, thereby decreasing levels in plasma.

Based on our finding that Pxmp4−/− mice have elevated plasma phytic acid levels under chow conditions, we hypothesized that PXMP4 could be involved in the peroxisomal oxidation of phytol. The branched-chain alcohol phytol is a precursor for phytic acid but usually is present at low amounts in the standard rodent diet. Therefore, dietary supplementation of phytol has often been used to challenge the peroxisomal metabolism of phytic acid and subsequently pristanic acid in mice51–55. Phytol mainly accumulates in the liver, where it is first converted in the ER into phytanic acid followed by uptake into the peroxisomes where it is converted into pristanic acid via α-oxidation51. In our study, phytol supplementation resulted in increased hepatic levels of phytic and pristanic acid, and this was slightly more pronounced in Pxmp4−/− mice compared to wild type littermates. In other mouse models in which phytol administration was used, hepatic levels of phytic and pristanic acid were higher than in our study, indicating that the breakdown of phytol is not limited by PXMP4 deficiency56,57,58. Together, these findings demonstrate that upon activation of PPARα by FF or by phytol, an evident phenotype was still absent in Pxmp4−/− mice. While our study did not identify a critical role for PXMP4 under standard dietary conditions or after stimulation of PPARα, we can not exclude that PXMP4 is important under other conditions where lipid metabolism is challenged, such as high-fat diet feeding or fasting.

Further analysis of the hepatic lipidome revealed reduced levels of TG(O), particularly species containing polyunsaturated fatty acids, in Pxmp4−/− mice, and this decrease was more pronounced after phytol treatment. Ether lipids have various biological functions including roles in membrane structure, and emerging studies suggest that they are involved in cell differentiation and signaling pathways59. Altered ether lipid production is also associated with several disorders including neurodegenerative diseases, cancer, and metabolic disorders48. The exact role, however, of PXMP4 in ether lipid metabolism and its relevance for physiology and disease remains to be further investigated.

The metabolic function of several peroxisomal proteins has not yet been elucidated. Several other studies investigating a single peroxisomal protein-deficient mouse model also reported no (evident) phenotype. Atsaves et al. reported that under chow conditions, both male and female Scpx deficient (Scpx−/−) mice had no altered body weight, liver weight, fat mass or lean mass compared to their wild type littermates. However, plasma and liver lipids were decreased in Scpx−/− mice and regulated in a gender-dependent manner59. Peroxisomal membrane protein 34 knockout (Pmp34−/−) animals, either under chow-fed conditions or after clofibrate administration, lacked a significant phenotype compared to control animals57. Mice with a deficiency in 2-hydroxycarboxylic-CoA lyase 1 (HACL1), a key enzyme in α-oxidation of phytic acid, also displayed no divergent phenotype under chow-fed conditions58. The enzyme AMACR is involved in the racemization of the bile acid synthesis C27-intermediates di- and trihydroxycholestanolic acid (DHCA and THCA respectively), as well as racemization of pristanic acid58,59. Amacr−/− mice had increased biliary and serum C27-intermediates and lower C24 bile acids, but no alterations in pristanic or phytic acid compared to wild type animals under chow-fed conditions60. Despite the changes in bile acid metabolism, no clinical phenotype was found in these Amacr−/− mice60. Under phytol-fed conditions, our mice only showed increased phytic acid levels in the liver, but no other peroxisome functions were affected, and no clinical phenotype was found.

Although various somatic mutations and hypermethylation resulting in the silencing of PXMP4 in humans have been reported for several types of cancer, its role in tumor development, as well as its physiological function,
has remained unknown. The gnomAD database v2.11 (gnomad.broadinstitute.org) lists more than 300 single nucleotide variants (SNVs) for human PXMP4, including 21 indels, 7 frame-shiftings, 11 splice variants and 10 loss/gain function. PXMP4 was predicted as a gene tolerant to variations, with the following constraint metrics: the probability of being loss-of-function (LoF) intolerant (pLI) = 0 and observed/expected (o/e) = 0.63. Further analysis of the common genetic variants in PXMP4 in the UK Biobank (ukbiobank.ac.uk) and FinnGen (finngen.fi/en) general population cohorts did not yield any lipid-related associated traits (data not shown).

Recently it has been shown that PXMP4 and the transmembrane protein 135 (TMEM135; PMP52) are both members of the Tim17 protein family showing 30% homology. TMEM135 has been associated with fatty acid metabolism and indicated as a PPARα target and could be functionally related to PXMP4. However, we did not find differences in hepatic Tmem135 expression between wild type and Pxmp4−/− mice after administration of chow, FF, or phyto (data not shown). To assess whether redundancy is caused by other peroxisomal proteins, future studies using double knockout of PXMP4 together with homologous proteins such as TMEM135 could give more insight into the metabolic function of PXMP4.

**Materials and methods**

**Animals.** A whole-body Pmp4 knockout mouse model (Pxmp4−/−) was generated by CRISPR/Cas9-mediated gene editing as previously described. Briefly, FVB females were super ovulated by injection with 5 IU Folligonan (0.2 ml i.p.) and 48 h later with 5 IU Chorulon (0.2 mL i.p.). The next day, zygotes were isolated from the infundibulum and injected with 100 ng/µL Cas9 RNA + 50 ng/µL sgRNA Pxmp4 (CGGTGCCGTCGGCGTG GATAA). Injected zygotes were incubated overnight at 37 °C and transferred to the infundibulum of pseudo-pregnant females. Offspring carrying targeted mutations resulting in a loss-of-function protein were selected for breeding with wild type C57BL/6j mice. Germline transmission of the mutation was verified and the mice carrying the mutation were backcrossed (N > 7) into the C57BL/6j background. During the experiments, male and female animals were housed individually, kept in a light- and temperature-controlled environment, and had ad libitum access to standard chow (RM1, Special Diets Services, Essex, UK) and drinking water. This study was carried out in compliance with the ARRIVE guidelines. All experimental procedures were approved by the local Ethics Committee for Animal Experiments of the University of Groningen. Experiments were performed in accordance with relevant guidelines and regulations (including laboratory and biosafety regulations and Directive 2010/63/EU).

**Animal procedures.** General characterization of male and female wild type and Pxmp4−/− mice did not show differences in plasma lipid levels, BAs levels, and the branched-chain fatty acids (BCFAs) phytanic and pristanic acid (data not shown). To investigate the function of PXMP4, male Pxmp4−/− mice and wild type littermates of 20–24 weeks old were studied under three different experimental conditions: standard chow, PPARα-activation by dietary fenofibrate (FF) administration, and overloading of the α-oxidation pathway by dietary phyto administration. Before every experiment, basal parameters, including food intake (24 h) and body composition, were measured. The body composition (total fat mass, lean mass, and fluid) was determined by nuclear magnetic resonance using the Bruker MiniSpec LF110 BCA-Analyzer (Bruker Optics Inc, Billerica, MA, USA). To determine basal blood parameters, blood was collected from the hind paw after 3 h of fasting. Food intake was monitored two times and bodyweight 2–3 times per week during the experimental period. Animals under chow conditions (n = 5–6) were followed for 2 weeks. For the PPARα-activation study, animals (n = 5–6) were treated with a diet containing 0.2% w/w FF (F6020-5G, Sigma-Aldrich, St Louis, MO, USA) for two weeks. Phytol is a precursor of phytanic acid and is often used to overload the α-oxidation pathway in peroxisomes. Accumulation of phytol and its metabolites phytanic and pristanic acid is toxic and can lead to severe damage to the liver. Because administration of a diet containing 0.5% w/w phytol has been shown to induce hepatotoxicity in mice, we administered a diet containing 0.25% w/w phytol (>97% sum of isomers, obtained as viscous liquid from Sigma-Aldrich, St Louis, MO, USA) for 4 weeks to Pxmp4−/− mice and wild type littermates (n = 6–8). The body composition of animals of the chow and FF experiment was monitored at the end of the experimental period. After 2 weeks of control chow diet or FF, or 4 weeks of phyto administration, animals were weighed in the morning, and food was taken away 3 h before gall bladder cannulation as described previously. Briefly, mice were anaesthetized by intraperitoneal injection using a mixture of Hypnorm (fentanyl/fluanisone; 1 mL/kg) and diazepam (10 mg/kg). Bile was collected for 20 min, and bile flow was determined gravimetrically (1 g = 1 mL bile secretion). Terminal blood was obtained through cardiac puncture and stored at −20 °C. The liver was harvested, flushed with ice-cold PBS and snap-frozen in liquid nitrogen before storage at −80 °C.

**Electron microscopy.** Electron microscopy was performed using standard protocols essentially the same as previously described. In the current study, primary fixation was in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). Samples were stored at 4 °C until further processing. Tissue was osmicated prior to embedding with 1.5% osmium tetroxide/potassium ferrocyanide. Ultrathin (80 nm) sections were spaced on single slot (2 × 1 mm) copper grids and contrasted with uranyl acetate. Acquisition was on a Zeiss Supra 55 ATLAS following procedures described in Ref. Acquisition of peroxisome numbers were counted in centrally cross-sectioned hepatocytes (according to a prominent nucleus).

**Gene expression analysis.** Snap-frozen livers were crushed using liquid nitrogen, and total RNA was isolated using TRI-reagent (Sigma-Aldrich, St Louis, MO, USA). RNA concentrations were quantified by NanoDrop (NanoDrop Technologies, Wilmington, DE, USA) and subsequently 1 µg used for cDNA synthesis (M-MLV reverse transcriptase, Thermo-Fisher Scientific, Waltham, USA). Real-time quantitative polymerase chain reaction (qPCR) was performed on QuantStudio 7 Flex machine (Applied Biosystems, Thermo Fisher,
Darmstadt, Germany) using Fast Advance Taqman Mastermix (Applied Biosystems, Foster City, CA, USA) or Fast Start SYBR Green (Roche, Mannheim, Germany). Taqman and SYBR Green primer sequences used in this study are listed in Tables 1 and 2. Gene expression levels were normalized using cyclophilin (Taqman) and TATA-Box binding protein (Tbp) (SYBR Green) as housekeeping genes (HKG).

**Lipidomics.** Lipidomics analysis was performed by the Core Facility Metabolomics of the Amsterdam UMC, as described.[71] The HPLC system consisted of an Ultimate 3000 binary HPLC pump, a vacuum degasser, a column temperature controller, and an auto sampler (Thermo Scientific, Waltham, MA, USA). The column temperature was maintained at 25 °C. The lipid extract was injected onto a “normal phase column” LiChrospher 2 × 250-mm silica-60 column, 5 μm particle diameter (Merck, Darmstadt, Germany) and a “reverse phase column” Acquity UPLC HSS T3, 1.8 μm particle diameter (Waters, Milford Massachusetts, USA). A Q Exactive Plus Orbitrap (Thermo Scientific) mass spectrometer was used in the negative and positive electrospray ionization mode. In both ionization modes, mass spectra of the lipid species were obtained by continuous scanning from m/z 150 to m/z 2000 with a resolution of 280,000 full width at half maximum (FWHM).

An in-house developed pipeline, written in the R programming language, was used for data processing. The RAW data files were converted to mzXML using MSconvert in centroided mode. Peak finding and peak group finding was done using the R package XCMS, with minor modifications to some functions for a better representation of the Q Exactive data. Annotation of the peaks was done based on an in-house database containing all possible (phospho)lipid species. Each combination of column (normal phase or reverse phase) and scan mode (positive or negative) was processed separately; after normalization, separate peak group lists were combined into two resulting lists, which were used for statistical analysis. Lipidomics data were analysed using Partial Least Square (PLS) regression analysis and PLC-derived Variable Importance in Projection (VIP) scores were used to identify the most important changed lipid species in the dataset.

**Targeted proteomics.** Livers from the mice were snap-frozen using liquid nitrogen and mechanically crushed in liquid nitrogen. A 10% liver homogenate was made using tissue powder together with NP-40 lysing buffer (400 mM NaCl, 1:1000 v/v NP40 (Igepal-640), 10 mM TRIS pH8, 1 mM EDTA pH8). Targeted proteomics was used to quantify PXMP4 in homogenized liver tissue via isotopically labeled peptide standards.

| Gene  | Forward sequence 5'–3' | Reverse sequence 3'–5' | Probe sequence                  |
|-------|------------------------|------------------------|---------------------------------|
| Acox1 | GCC AGC GAA CTC ATC TTC GA | CCA GCC CAC CAC TTA ATG GA | CCA CTG CCA CAT ATG ACC CCA AGA CCG |
| Baat  | TGT AGA GTT TCT CCT GAG ACA TCC TAA | GTC CAA TCT CTC CTA CAA TGC | CCT CGG CCC AGG TGT TGG CA |
| Cpt1a | CTC AGT GGG AGC GAC TCT TCA | GCC CTC TGT GGT ACA CGA CAA | CTT GGG GAG GAC GAC ACC ATC CCA C |
| Cyclophilin | CAG ATC GAG GGA TCG ATT CAG | TCA CCA GCT GAC ACC CTC ATT C | CTT CTC CAC ATT GGA GAC AAG AGA TCA |
| Cyp27a1 | GCC TGT CAC AAG GAA GTG ACT | CCG AGG GTC TCC TTA ATC ACA | CCC TGC GGG AAG GTG CCC CAG |
| Cyp7a1 | CAG GGA GAT GCT CTG TCA TGA | AGG CAT ACA CTT CTT CCG TGA | TGC AAA ACC TCC AAT CTG TCA TGA GAC CTC C |
| Dhp (Hsd17b4) | GAG GAA CAG AAG GAT GAG AAG TAC TG | TGG TTT CCT TTG GGT GTT CTT TGC | CTC GAC CTC TGG GCT TCA TGG TTC |
| Fabp1 | GAA CTT CTC CCG CAA GTA CCA A | TGT CCT CTT CTT CGA TGA G | CCA TCT ATG AAG GCA ATA GGT CTG GCC |
| Pmp70 | CGT GTG CGT GAG AAA TCA TCA AT | CCA GAT CGA ACT TCA AAA CTA AGG T | TGA TCA TGT TGC TTT AGC AAC ACC AAA TGG |
| Scp2  | GTG GCT CTC GAG CAC AAT CTA | CTG AAG GAG CTC TCA GCA GCT T | CAA CCA CAG CTC CTC CGA GGC C |

**Table 1.** Taqman primer sequences.

| Gene  | Forward sequence (5’–3’) | Reverse sequence (3’–5’) |
|-------|--------------------------|-------------------------|
| Acaa1 | ACA TCT CCG TGG GCA ATG TT | CTC AGA AAT TGG GCG ATG CG |
| Amacr | GAG AAT TTT CTG GCC CGA GG | AGT TCT TCC ATG ACA CGG CA |
| Cyp4a10 | CTT GGG GGA TCA GAT CCA AA | TGG GGT TAG CAT CCT GTT |
| Cyp4a14 | AGC AGC ACA CAG ATG GAG TG | TCT TCT TCC TGG OCT TCT GC |
| Phyh  | TAC TGC CTT CTC CCG GAG AT | CGG GAT GTC TTC TTG TCA AC |
| Pmp4  | CGC TGG CCG TGA TAA AGG | GAG AGT GGA TCG TGG CT |
| Tbp   | TTC ACC AAT GAC TCC TAT GAC C | CAA GAT TAC AGC CAA GAT TCA G |

**Table 2.** SYBR Green primer sequences.
(containing 13C-labeled lysine), derived from synthetic protein concatamers (QconCAT technology, PolyQuant GmbH Germany) using the workflow previously described. Briefly, in-gel tryptic digestion (1:100 g/g sequencing grade modified trypsin V5111, Promega) was performed on homogenized liver tissues containing 50 μg total protein after reduction with 10 mM dithiothreitol and alkylation with 55 mM iodoacetamide proteins, followed by solid phase extraction (SPE C18-Aq 50 mg/1 mL, Gracepure) for sample cleanup. Detection and quantification of PXMP4 were done targeting the peptide NLACFVFAYK within PXMP4. The isotopically labeled peptide standard was added at a concentration of 0.5 ng concanamer per μg total protein to the liver lysates. These peptides were targeted and analyzed by a triple quadrupole mass spectrometer (MS) equipped with a nano-electrospray ion source (TSQ Vantage, Thermo Scientific). Chromatographic separation was performed by liquid chromatography on a nano-UHPLC system (Ultimate UHPLC System, Dionex) using a nano-column (Acclaim PepMap100 C18, 75 μm × 500 mm 2 μm, 100 Å) with a linear gradient from 3 to 60% v/v acetonitrile plus 0.1% v/v formic acid in 110 min at a flowrate of 200 nL/min. The MS traces were manually curated using the Skyline software before the integration of the peak areas for quantification. The figures were created using Skyline.

**Biochemical analyses.** Determination of plasma VLCFA and the BCFAs phytanic and pristanic acid was performed using gas chromatography combined with mass spectrometry (GC–MS) as described. Individual bile acid species (unconjugated and taurine or glycine conjugated) in bile and plasma were determined using liquid chromatography-mass spectrometry (LC–MS) as described. Total bile acid concentrations were calculated as the sum of the individual bile acid species. The levels of (un)conjugated bile acids were calculated using the percentage of the total sum of bile acids in bile and plasma.

**Statistics.** The software GraphPad Prism 8.1 (GraphPad Software, La Jolla, CA, USA) was used for statistical analyses. Unless stated otherwise, graphs are presented as a scatter dot plot representing individual values with a median ± interquartile range (IQR). Gene expression bars are given as bar plots representing the mean ± standard deviation (SD). Statistical significance between two groups was tested by a non-parametric Mann–Whitney test, and in case of more than two groups by the non-parametric one-way ANOVA (Kruskal–Wallis) test, followed by Mann–Whitney U tests to assess statistical significance between experimental groups. Statistical significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

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Author contributions
M.B. designed and performed the experiments, analyzed and interpreted data; V.W.B. and M.I. performed lipidomics; D.S, R.J.A.W., F.V., F.K, B.S., A.K.G and H.J.V. interpreted data; J.C.W. performed targeted proteomics; F.V. and M.I. analyzed data; N.H. and N.K. generated the mouse model; J.C.W. performed targeted proteomics; F.V. and M.I. analyzed data; N.H. and N.K. generated the mouse model; J.C.W. performed targeted proteomics; F.V. and M.I. analyzed data; N.H. and N.K. generated the mouse model; J.C.W. designed the experiment, interpreted data and wrote the manuscript. All authors reviewed the manuscript.

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Competing interests
The authors declare no competing interests.

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