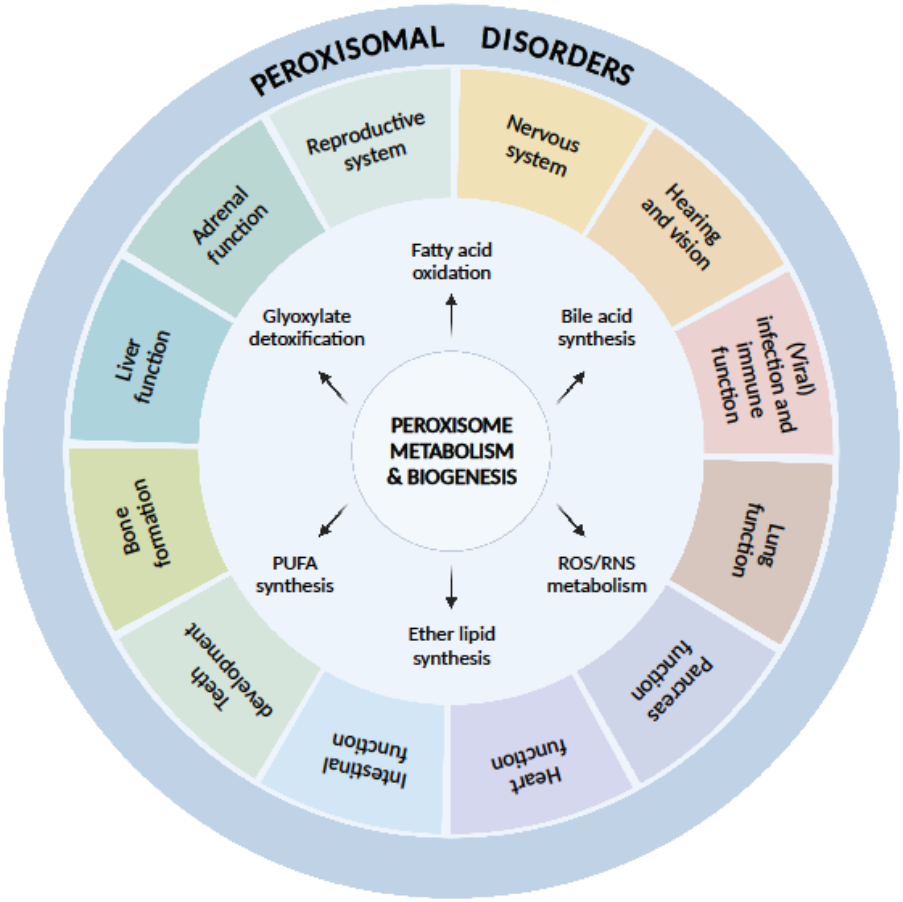
THE PHYSIOLOGICAL FUNCTIONS OF HUMAN PEROXISOMES

Ronald J.A. Wanders[1,2,3], Myriam Baes[4], Daniela Ribeiro[5], Sacha Ferdinandusse[1,3] and Hans R. Waterham[1,2,3]

[1]Laboratory Genetic Metabolic Diseases, Department of Clinical Chemistry, Amsterdam University Medical Center location University of Amsterdam, Amsterdam, The Netherlands
[2]Department of Pediatrics, Emma Children's Hospital, Amsterdam University Medical Center location University of Amsterdam, Amsterdam, The Netherlands
[3]United for Metabolic Diseases, The Netherlands
[4]Laboratory of Cell Metabolism, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, 3000 Leuven, Belgium
[5]Institute of Biomedicine (iBiMED) and Department of Medical Sciences, University of Aveiro, Aveiro, Portugal

Corresponding authors:

Ronald J.A. Wanders r.j.wanders@amsterdamumc.nl
Hans R. Waterham h.r.waterham@amsterdamumc.nl


PEROXISOMAL DISORDERS
Reproductive system
Nervous system
Hearing and vision
(Viral) infection and immune function
Lung function
Pancreas function
Heart function
Intestinal function
Teeth development
Bone formation
Liver function
Adrenal function
Fatty acid oxidation
Bile acid synthesis
ROS/RNS metabolism
Ether lipid synthesis
PUFA synthesis
Glyoxylate detoxification
PEROXISOME METABOLISM & BIOGENESIS

CLINICAL HIGHLIGHTS

- Peroxisomes are semi-autonomous subcellular organelles which can form de novo and/or by growth and division of preexisting organelles
- Peroxisomes play a unique role in metabolism in many different organisms including humans by catalyzing a number of different metabolic functions including fatty acid α-and β-oxidation, ether phospholipid biosynthesis, and glyoxylate detoxification
- The importance of peroxisomes in human physiology is exemplified by the existence of a group of genetic diseases in which there is an impairment in either the biogenesis of peroxisomes, i.e. the Zellweger spectrum disorders, or one of its metabolic pathways, i.e. the single peroxisomal enzyme deficiencies
- Zellweger syndrome is the prototype of the group of peroxisomal disorders, which now includes > 20 different disorders caused by pathogenic variants in >30 distinct genes
- Proper functioning of peroxisomes in metabolism requires the concerted interaction with other subcellular organelles including mitochondria, lysosomes, endoplasmic reticulum, lipid droplets and the cytosol
- Peroxisomes are essential for the functioning of many different organ systems as exemplified by the devastating consequences of defects in peroxisome biogenesis affecting multiple different organ systems in human patients as well as in different mouse models
- One of the more recently identified functions of peroxisomes involves their role in immune function and viral infections

OUTLINE

1. ABSTRACT

Peroxisomes are subcellular organelles which play a central role in human physiology by catalyzing a range of unique metabolic functions. The importance of peroxisomes for human health is exemplified by the existence of a group of usually severe diseases caused by an impairment in one or more peroxisomal functions. Among others these include the Zellweger spectrum disorders, X-linked adrenoleukodystrophy and Refsum disease. In order to fulfill their role in metabolism, peroxisomes require the continued interaction with other subcellular organelles including lipid droplets, lysosomes, the endoplasmic reticulum, and mitochondria. In recent years it has become clear that the metabolic alliance between peroxisomes and other organelles requires the active participation of tethering proteins to bring the organelles physically closer together thereby achieving efficient transfer of metabolites. This review intends to describe the current state of knowledge about the metabolic role of peroxisomes in humans with particular emphasis on the metabolic partnership between peroxisomes and other organelles and the consequences of genetic defects in these processes. We will also describe the biogenesis of peroxisomes and the consequences of the multiple genetic defects therein. In addition, we will discuss the functional role of peroxisomes in different organs and tissues and will include relevant information derived from model systems notably peroxisomal mouse models. Finally, we will pay particular attention to a hitherto underrated role of peroxisomes in viral infections.

2. INTRODUCTION

Peroxisomes are distinct subcellular organelles present in all major groups of eukaryotes and are involved in a variety of physiological processes which differ among species (1). Following their morphological identification by Rhodin in 1954 during his thesis work on proximal tubule cells from mouse kidney, peroxisomes were first isolated and biochemically characterized by de Duve and coworkers using differential and density gradient centrifugation techniques (2). The identification of several enzymes producing hydrogen peroxide together with the hydrogen peroxide degrading enzyme catalase in this newly identified organelle prompted de Duve to introduce the name "*peroxisome"* (2). Since then, peroxisomes have been isolated and characterized from different organisms, which revealed that the enzymatic properties of peroxisomes vary among different organisms and even between organs from the same organism. Indeed, whereas peroxisomes present in different cells and tissues from humans are all able to oxidize fatty acids and synthesize ether lipids, only hepatic peroxisomes catalyze the synthesis of bile acids and detoxify glyoxylate into glycine (3). Furthermore, the ability of peroxisomes to perform glycolysis is confined to a few species only, including the trypanosomatids of the genera *Trypanosoma* and *Leishmania* (4). These peroxisomes, also known as glycosomes, actually appear to lack catalase, which was one of the reasons to question whether these organelles were true peroxisomes. The issue whether a certain type of organelle did belong to the family of peroxisomes or not, was only resolved later when the principal features of peroxisome biogenesis became apparent, which includes the identification of the first peroxisome targeting signal at the carboxy terminal end of peroxisomal proteins (5, 6). The presence or absence of such peroxisome targeting signals in proteins allowed to build the peroxisome family with all its different members. This analysis revealed that the metabolic function of peroxisomes, which is virtually ubiquitous among all different species, is the oxidation of fatty acids rather than its role in hydrogen peroxide metabolism. Interestingly, fatty acid oxidation in mitochondria is much less conserved among different species and is, for instance, lacking in several yeast species including *Saccharomyces cerevisiae*. The identification of a peroxisomal β-oxidation system next to that in mitochondria by Lazarow and de Duve in 1976 (7) was confusing at first and led to the misinterpretation of the peroxisomal system being an auxiliary system helping the mitochondrial system in times of fatty acid overload. The realization that the peroxisomal and mitochondrial β-oxidation systems serve distinct purposes catalyzing the oxidation of different types of fatty acids, only came many years later. Indeed, it was the finding by Moser and coworkers in 1982 (8), who reported the accumulation of a specific set of fatty acids, notably C24:0 and C26:0, in plasma from Zellweger patients with no abnormalities in any of the other long-chain fatty acids, which pointed to the unique role of

peroxisomes in fatty acid homeostasis. Earlier work by Goldfischer et al (9) had shown the absence of morphologically distinguishable peroxisomes in tissues from Zellweger patients. One year later Heymans et al (10) reported the deficiency of a special subgroup of lipids, i.e. ether lipids. These two breakthrough discoveries provided convincing evidence for the major role which peroxisomes play in human physiology and led to detailed studies to resolve the metabolic role of peroxisomes as well as their biogenesis. Although these studies were initially rather descriptive, much knowledge has been gained in more recent years about many aspects of peroxisome physiology. This applies to the role of peroxisomes in cellular metabolism and the identification of tethering proteins required to bring peroxisomes in close physical contact with other organelles to allow transfer of metabolites from one organelle to the other. The importance of these interactions is once again strengthened by the identification of patients with a genetic deficiency of one of these tethering proteins. A second major development in recent years involves the knowledge gained about the differential role which peroxisomes play in different organs and the lessons learned from mutant mouse models with organ-specific disruption of specific peroxisomal genes. Finally, new physiological functions of peroxisomes have been discovered in recent years, including their role in antiviral signaling and importance for the infection cycle of different viruses. It is the purpose of this Review to document these new developments and describe the current state of knowledge about peroxisomes notably in humans.

3. METABOLIC ROLE OF PEROXISOMES

Peroxisomes catalyze a large number of metabolic functions ranging from the oxidation of fatty acids to the synthesis of ether lipids and the detoxification of glyoxylate among others as described in detail below. In order to perform these various metabolic roles, peroxisomes must be able to import metabolites from the cytosolic space and export the products of peroxisome metabolism across the peroxisomal membrane to the cytosol. The passage of metabolites across the peroxisomal membrane has long been disputed but there is now growing consensus for a model in which the peroxisomal membrane is selectively permeable with free passage of metabolites of a low molecular weight (Mw) but carrier-mediated transport of metabolites of a larger size as detailed below (Figure 1).

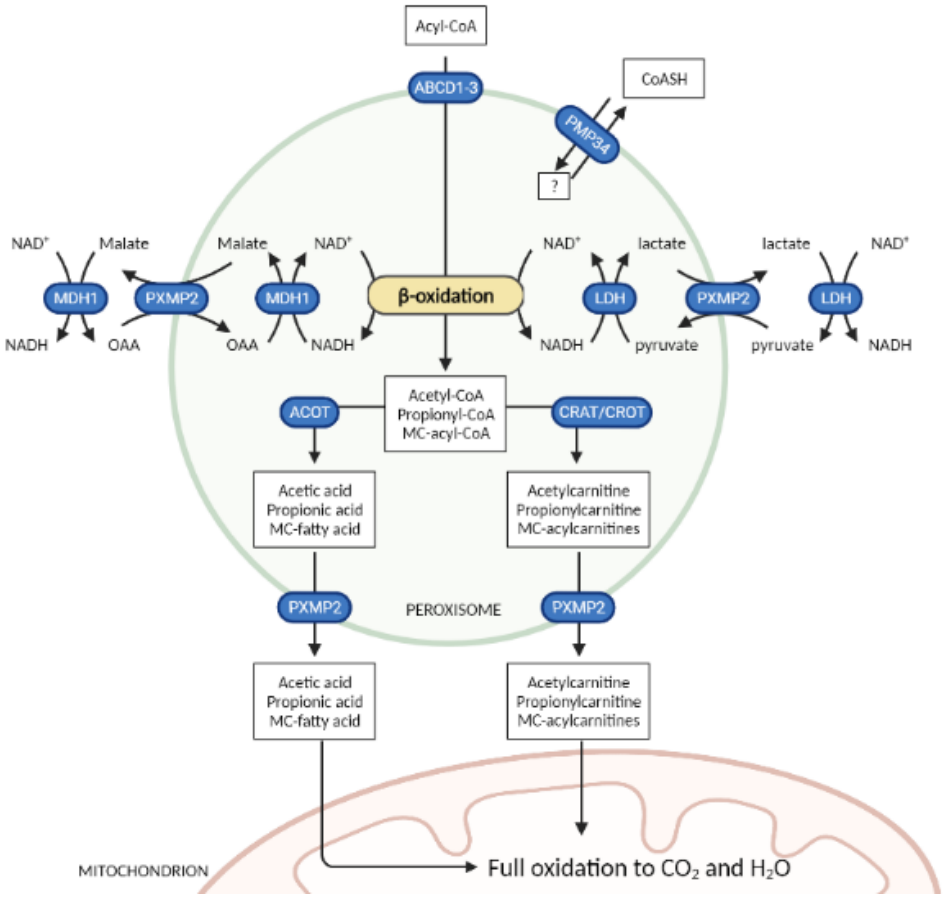


Figure 1. Consensus model depicting the permeability properties of the peroxisomal membrane in humans.

The peroxisomal membrane contains pore-forming proteins of which PXMP2 is best characterized, which allow the free passage of metabolites of low molecular weight (Mw) up to 600 Da whereas higher Mw-metabolites like ATP, and coenzyme A either acylated or not, require specific transport proteins to enter the peroxisome including the different half-ABC-transporters ABCD1, 2, and 3. The nucleotides NAD, NADH, NADP, and NADPH are also unable to traverse the peroxisomal membrane and require so-called redox-shuttles for their interconversion. Abbreviations used: OAA=oxaloacetate; MC=medium-chain; ACOT=acyl-CoA thioesterase;CRAT=carnitine acetyl transferase; CROT=carnitine octanoyltransferase; LDH=lactate dehydrogenase; MDH=malate dehydrogenase;CoASH=coenzyme A.

Figure prepared using Biorender.com.

3.1. Permeability properties of peroxisomes

Early work in the 1960s by De Duve and coworkers (2) revealed that, in contrast to mitochondrial enzymes-peroxisomal enzymes including D-amino acid oxidase, L-alpha-hydroxyacid oxidase and urate oxidase did not show structure-linked latency when analysed in postnuclear supernatants or isolated peroxisomes usually prepared from rat livers. Indeed, the activity of any of the three enzymes was found to be the same when measured in peroxisomes incubated in isotonic medium or in sonicated peroxisomes. These findings led to the conclusion that peroxisomes are freely permeable to low Mw

compounds like D-alanine, glycolate, and urate. In later years, Van Veldhoven et al. (11) performed more detailed studies in isolated peroxisomes and liposomes reconstituted with peroxisomal membrane proteins and proposed the existence of pore-forming proteins. Several of such pore-forming proteins have indeed been identified since then. PXMP2 was the first peroxisomal protein with pore-forming activity to be identified, has a diameter of 1.4 nm and allows passage of solutes up to 600Da (12). In order to resolve the physiological function of PXMP2 a mouse model was generated in which PXMP2 was deleted. Except from some abnormalities in mammary gland development, the mutant mice did not show much of a phenotype which was interpreted as evidence for functional redundancy caused by additional pore-forming proteins (13). Work by Mindthoff et al. (14) in the yeast *S. cerevisiae* has identified Pex11 as another pore-forming protein, which shares sequence similarity with TRPM cation-selective channels and has an estimated diameter of +/-0.6 nm. Mammals including humans possess three different PEX11 proteins named PEX11α, PEX11β, and PEX11γ. It remains to be established whether or not they are also pore-forming proteins in analogy to Pex11 in yeast (see (15) for discussion). In contrast to the free passage of low-Mw metabolites across the peroxisomal membrane, there is ample evidence showing that peroxisomes are impermeable to larger Mw-compounds including the various nucleotides NAD, NADH, NADP, and NADPH, as well as ATP, and coenzyme A either acylated or not. This has not only been established for yeast but also for mammalian peroxisomes. Through the years a number of integral peroxisomal membrane proteins (PMP) have been identified which would qualify as potential metabolite carriers but in most cases the evidence is either weak or circumstantial. Best characterized among these PMPs are the three PMPs which belong to the family of ATP-binding cassette (ABC) proteins named ABCD1, 2, and 3. Each of these ABCDs predominantly form homodimers in the peroxisomal membrane and catalyze the transport of different acyl-CoAs (16). Unfortunately, successful expression of these proteins in an active form followed by reconstitution in liposomes has not been described so far which implies that the evidence is indirect. The first evidence comes from the identification of human patients in whom either ABCD1 or ABCD3 is genetically deficient. In ABCD1-deficient patients, to be discussed later in this review, the oxidation of saturated very-long-chain fatty acids (VLCFA) is impaired despite the fact that the peroxisomal β-oxidation machinery itself is normally active. On the other hand, in ABCD3-deficient patients the oxidation of branched-chain fatty acids including the two bile acid synthesis intermediates di-and trihydroxycholestanoic acid (DHCA and THCA) is deficient whereas the oxidation of VLCFAs is fully normal. Based on these findings it has been proposed that ABCD1 and ABCD3 catalyze the transport of different acyl-CoA esters i.e. very-long-chain acyl-CoAs in case of ABCD1 and branched-chain acyl-CoAs in case of ABCD3. More direct evidence in

support of ABCD1 as a transporter of very-long-chain acyl-CoAs has been provided by Wiesinger et al. (17). Indeed, these authors reported the ATP-dependent uptake of C26:0-CoA into peroxisomes and its deficiency in ABCD1-deficient fibroblasts. Our own work using *S. cerevisiae* as a model system is fully in line with the notion that the three ABCDs are all acyl-CoA transporters each having its own substrate specificity (18, 19). The peroxisomal membrane has been reported to contain a variety of other carrier proteins belonging to different SLC families notably SLC16, SLC25, and SLC27 (15). In most cases, however, the situation is complicated by the fact that each particular SLC-protein is not unique to peroxisomes but is also localized in other organelles. This is true for the two SLC16 members MCT1 and MCT2 as well as the SLC27 member SLC27A2 also called ACSVL1 (see (15) for review). An exception to this rule is PMP34 encoded by the *SLC25A17* gene which belongs to the Mitochondrial Carrier Family (MCF) but has a strict peroxisomal localization as concluded from immunofluorescence microscopy studies (20) as well as proteomic analyses (21, 22). PMP34 was first thought to be the peroxisomal ATP-transporter (23) but in vitro reconstitution studies with the human PMP34 protein revealed that PMP34 prefers CoA, FAD, and to a lesser extent NAD as substrates and not ATP (24). The importance of PMP34 for normal physiology was recently stressed by the findings in mutant zebrafish (25) and mice (26). According to Kim et al. (25) PMP34 acts as a peroxisomal coenzyme A transporter but the results of Van Veldhoven et al. (26) are not fully in line with this proposal.

The fact that the peroxisomal membrane is impermeable to the various nicotinamide adenine nucleotides NAD, NADH, NADP, and NADPH has dictated the existence of so-called redox shuttles in analogy to the situation in mitochondria with the malate-aspartate shuttle being responsible for the re-oxidation of the NADH produced in the cytosol (27). Available evidence has shown that mammalian peroxisomes contain similar NAD(H)-redox shuttles including a lactate dehydrogenase-based shuttle and another one centered around malate dehydrogenase as discussed later (Figure 1; see also (15) for recent review).

3.2. Metabolic pathways in peroxisomes

Rather than covering all metabolic pathways in human peroxisomes, we will only describe the major metabolic pathways directly related to human peroxisomal disorders. This also applies to the role of peroxisomes in reactive oxygen and nitrogen metabolism (for reviews on these topics, see (28, 29)).

3.2.1. Fatty acid β-oxidation

Peroxisomes contain a fatty acid oxidation machinery (pFAO) which in chemical terms is identical to that in mitochondria (mFAO). This implies that fatty acids (FA) undergo four sequential steps of dehydrogenation, hydration, dehydrogenation again, and thiolytic cleavage. This mechanism ensures the cleavage of fatty acids at the carboxy terminal end to produce an acetyl-CoA unit in case of unbranched FAs and propionyl-CoA in case of 2-methyl branched-chain FAs. Despite the apparent similarities, the fatty acid oxidation systems in mitochondria and peroxisomes differ in many respects (30) and serve different purposes in mammalian cells which follows logically from the fact that the two systems catalyze the oxidation of different sets of FAs as detailed below. Indeed, the mitochondrial system is responsible for the oxidation of the bulk of FAs derived from our daily diet including the long-chain fatty acids palmitic, oleic, linoleic, and linolenic acid whereas peroxisomes only catalyze the oxidation of a set of minor FAs which cannot be handled by mitochondria. These FAs do need to be degraded not so much because of their role in energy provision, but because of their toxicity when they accumulate. The different physiological roles of the two β-oxidation systems are also clearly reflected in the widely different clinical signs and symptoms associated with defects in either system. Indeed, in case of mFAO defects, affected patients display cardiac abnormalities, muscular signs, including myopathy, fatigue, and rhabdomyolysis, hepatic dysfunction, and neurologic features, whereas patients affected by a defect in pFAO are in general much more severely affected with profound neurological abnormalities. Another major difference between the two types of β-oxidation disorders is the fact that patients affected by a mFAO defect usually show hypoketotic hypoglycemia as a consequence of the block in β-oxidation and the subsequent enhanced reliance on glucose oxidation for energy purposes (31) whereas this is rarely seen in pFAO-deficient patients (32, 33) which is in line with the notion that the pFAO system contributes little to energy provision.

3.2.1.1. Fatty acids which require peroxisomes for their β-oxidation

(a.) VLCFAs: fatty acids with a chain length of >22 carbon atoms occur in only minor amounts in most food components except in certain nuts (34). Instead VLCFAs are primarily derived by chain-elongation of shorter chain FAs to be incorporated into a diverse series of lipid species (35-38). Proper homeostasis dictates that the VLCFAs released upon degradation of these VLCFA-containing lipid species, are oxidized or recycled. Oxidation of VLCFAs notably C26:0 and longer-chain FAs occurs exclusively in peroxisomes. Oxidation of VLCFAs does not go to completion but stops at the level of medium-chain acyl-CoAs. The acetyl-CoA units produced during β-oxidation are then transferred to mitochondria together with the medium-chain acyl-CoAs for full oxidation to CO_2 and H_2O via one of two different routes (Figure 1). The

first one involves the hydrolysis of the different CoA-esters by one of the many acyl-CoA thioesterases known to be localized in peroxisomes (39) followed by transport of these free fatty acids across the peroxisomal membrane probably via one of the pore-forming proteins after which uptake into mitochondria can occur followed by oxidation. The second mechanism makes use of carnitine to carry the acetyl-CoA and medium-chain acyl-CoA across the peroxisomal membrane. To this end, peroxisomes are equipped with two different carnitine acyltransferases with specificity for short-chain acyl-CoAs (CRAT; carnitine acetyltransferase) and medium-chain acyl-CoAs (CROT; carnitine octanoyltransferase). Once across the peroxisomal membrane, the different acylcarnitines are imported into mitochondria via the mitochondrial carnitine acylcarnitine carrier (CACT) which is a member of the mitochondrial SLC25 carrier family encoded by the gene *SLC25A20*. The acylcarnitines are then reconverted into the corresponding CoA-esters followed by oxidation to CO_2 and H_2O (see (30) for details). Taken together, the chain-shortened end products of peroxisomal fatty acid oxidation exit the peroxisome either in their free acid form or as carnitine-ester. These two end products can then move to the mitochondria for full oxidation to CO_2 and H_2O but can also exit the cell to be metabolized in other organs. It is well known for instance that acetate is an important end product of peroxisomal β-oxidation in hepatocytes which is preferentially exported from the hepatocyte to the extracellular space to be used as respiratory substrate in other organs (40). Importantly, using isotopomer analysis (41, 42) it was shown that, at least in the heart, the acetyl-CoA units produced upon peroxisomal β-oxidation are not shuttled to mitochondria for full oxidation to CO_2 and H_2O but instead are preferentially used for the synthesis of malonyl-CoA in the cytosol as catalyzed by the enzyme acetyl-CoA carboxylase. This enzyme exists in two forms, one form is located in the cytosol and the other at the outer aspect of the mitochondrial outer membrane facing the cytosol. Since acetyl-CoA itself cannot exit the peroxisome, the mechanism behind the formation of malonyl-CoA from the acetyl-CoA generated in peroxisomes remains to be resolved.

(b.) Pristanic acid (2,6,10,14-tetramethylpentadecanoic acid): this fatty acid is synthesized endogenously from phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) by α-oxidation (to be discussed later in this review) and is also derived directly from dietary sources. Pristanic acid undergoes three cycles of β-oxidation in peroxisomes (43) after which the end products including 4,8-dimethylnonanoyl-CoA, propionyl-CoA, and acetyl-CoA are shuttled to mitochondria via the free-acid route or carnitine-mediated route just like the end products of VLCFAs discussed above (see Figure 2).

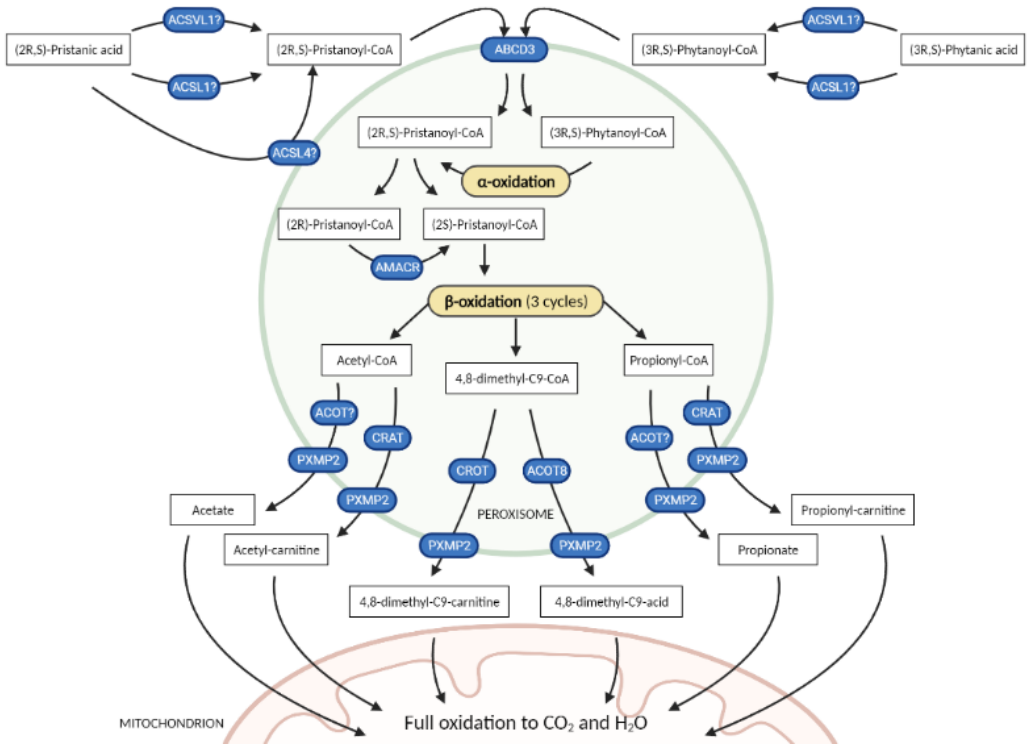


Figure 2. Oxidation of pristanic acid in peroxisomes.
Pristanic acid (2,6,10,14)-tetramethylpentadecanoic acid occurs in two different forms in which the (2)-methyl group either has the (2R)- or (2S)-configuration. Pristanic acid is derived from exogenous sources either as pristanic acid itself or from its precursor phytanic acid which is also solely derived from exogenous sources. Phytanic acid is a mixture of two different isomers as well including (2R, 7R, 11R, 15)-tetramethylhexadecanoic acid and (2S, 7R, 11R, 15)-tetramethylhexadecanoic acid which upon α-oxidation in the peroxisome (see Figure 5) yield (2R)-and (2S)-pristanic acid which after activation to (2S)-and (2R)-pristanoyl-CoA undergo three cycles of β-oxidation to produce 4,8-dimethylnonanoyl-CoA, propionyl-CoA, and acetyl-CoA which are subsequently shuttled to the mitochondria via one of two different mechanisms (see text) for full oxidation to CO2 and H2O. Figure prepared using Biorender.com.

(c.) Di-and trihydroxycholestanoic acid (DHCA and THCA): bile acid synthesis from cholesterol in the liver with chenodeoxycholic acid (CDCA) and cholic acid (CA) as end products involves the participation of multiple enzymes localized in different subcellular compartments including the peroxisome. The bile acid synthesis intermediates DHCA and THCA are generated from cholesterol via a complex series of enzymatic reactions, are subsequently activated to DHC-CoA and THC-CoA at the site of the endoplasmic reticulum and then travel to the peroxisome to be taken up into the peroxisomal matrix as mediated by ABCD3 (PMP70) (44). Once inside the peroxisome the CoA-esters of DHCA and THCA which both contain 27 carbon atoms, are subjected to one round of β-oxidation thereby generating the three carbon unit propionyl-CoA plus the CoA esters of CDCA and CA, respectively, which now contain 24 carbon atoms. Subsequently, the CoA moiety in the two CoA esters is replaced by either taurine or glycine to generate

tauro/glycochenodeoxycholic acid and tauro/glycocholic acid. The enzyme catalyzing this reaction is bile acid-CoA:amino acid acyltransferase (BAAT) which is a true peroxisomal enzyme ((45); see (46) for discussion). After export out of the peroxisome the tauro-and glyco conjugates of CDCA and CA are excreted from the hepatocyte via BSEP (ABCB11) to end up in bile.

(d.) Mono-and polyunsaturated fatty acids: in analogy to the oxidation of saturated FAs, peroxisomes and mitochondria are both able to catalyze the β-oxidation of mono-and polyunsaturated FAs. Oxidation of these FAs requires the active participation of auxiliary enzymes including 2-enoyl-CoA isomerases and 2,4-dienoyl-CoA reductases which have been identified in both mitochondria and peroxisomes (see (47) for review). Importantly, the two organelles are not each other's functional duplicate but instead catalyze the oxidation of distinct mono-and polyunsaturated FAs. In general, long-chain substrates are preferentially oxidized in mitochondria in contrast to very-long-chain mono-and polyunsaturated FAs which require the peroxisome for oxidation. Indeed, work by Christopherson et al. (48, 49) has shown that a series of C22-polyunsaturated FAs including erucic acid (C22:1 (n-3)), adrenic acid (C22:4 (n-6)), docosapentaenoic acid (C22:5 (n-3)), and docosahexaenoic acid (C22:6 (n-3)) all undergo chain-shortening by β-oxidation in the peroxisome. This conclusion was first derived from studies in rat hepatocytes but definitive evidence came from studies in peroxisome-deficient fibroblasts from Zellweger patients in which oxidation of all C22-containing polyunsaturated FAs turned out to be fully deficient. Another substrate unique to peroxisomes, is tetracosahexaenoic acid (C24:6 (n-3)) which is synthesized from linolenic acid (C18:3 (n-3)) via a series of chain-elongation and desaturation reactions at the ER. The C24:6 (n-3)-CoA synthesized in this way then moves over to the peroxisome for one round of β-oxidation to produce the CoA-ester of C22:6 (n-3), better known as docosahexaenoic acid (DHA). Rather than undergoing β-oxidation in the peroxisome, DHA is preferentially exported out of the peroxisome to be incorporated into different phospholipid species. The important role of peroxisomes in the synthesis of DHA was discovered by Sprecher and coworkers (see (50, 51) for reviews) following the realization that mammals do not contain a Δ-4 desaturase (52) and is since known as the "Sprecher-shunt". The same group also resolved the mechanism behind the preferential export of DHA compared to its degradation via β-oxidation in the peroxisome. This turned out to be due to the low 2,4-dienoyl-CoA reductase activity in peroxisomes which makes DHA a poor substrate for β-oxidation (53). Although not studied systematically as done for C22-polyunsaturated FAs, the C20-polyunsaturated FA arachidonic acid (C20:4 (n-6)) is also a unique substrate for peroxisomal β-oxidation (54).

(e.) Dicarboxylic acids: peroxisomes play a key role in the degradation of dicarboxylic acids which are not so much derived from exogenous sources but instead are produced endogenously from the corresponding monocarboxylic acids under conditions when fatty acid oxidation is increased like during starvation and diabetic ketosis. On the other hand, dicarboxylic acids are also formed under conditions when fatty acid oxidation-albeit in mitochondria or peroxisomes- is impaired as a consequence of a genetic deficiency especially of one of the enzymes involved in mFAO. In contrast to the oxidation of FAs by α- or β-oxidation which requires the obligatory activation of FAs to the corresponding acyl-CoA esters, the omega-oxidation pathway accepts only free, unconjugated fatty acids as substrate. The first step of the omega-oxidation pathway is catalyzed by members of the CYP4A subfamily of CYP450 enzymes followed by two subsequent oxidation reactions catalyzed by alcohol- and aldehyde dehydrogenases and/or another CYP450 enzyme, to generate the different dicarboxylic acids which are then activated by a specific microsomal dicarboxylyl-CoA synthetase (55). Early studies with isolated-mitochondria and peroxisomes have shown that the two organelles are both able to β-oxidize different dicarboxylic acids (56). Studies in intact cells and perfused livers and hearts, however, have shown that peroxisomes are the main, if not exclusive, site of oxidation of dicarboxylic acids, unless the peroxisomal oxidation of dicarboxylic acids is impaired (57). This applies to all dicarboxylic acids ranging from the C9-dicarboxylic acid azelaic acid (58) to the C12-dicarboxylic acid dodecanedioic acid (42, 58), the C14-dicarboxylic acid tetradecanedioic acid (59) and the C16-dicarboxylic acid hexadecanedioic acid (60). Available evidence indicates that the different dicarboxylic acids can be chain-shortened to shorter-chain dicarboxylic acids to generate the CoA-esters of succinic (C4), adipic (C6), suberic (C8), and sebacic (C10) acid which are subsequently shuttled to mitochondria either as carnitine ester or in its free acid form. The presence of different acyltransferases and thioesterases within peroxisomes which includes a specific succinyl-CoA thioesterase (61) allows this to proceed. Following earlier work using yeast as a model system (18), Houten and coworkers recently showed that the peroxisomal transporter ABCD3 plays a major role in hepatic dicarboxylic acid metabolism by catalyzing the uptake of dicarboxylyl-CoA esters into the peroxisome (57, 62). Interestingly, a pronounced dicarboxylic aciduria exacerbated by fasting was found in *Abcd3* $^{-/-}$ KO mice. Apparently, under conditions of a block in peroxisomal oxidation of dicarboxylic acids oxidation shifts to mitochondria which emphasizes once again the important interplay between the two organelles (57).

3.2.1.2. Enzymology of the peroxisomal β-oxidation system

The actual fatty acid β-oxidation machinery in peroxisomes consists of three different acyl-CoA oxidases (ACOX 1, 2, and 3), two multifunctional enzymes catalyzing the second and third step of β-oxidation, and two thiolases. Much of our knowledge about the physiological roles of each of these proteins has come from patients affected by a genetically determined defect in one of these proteins. Table 1 lists the genetic deficiencies in peroxisomal β-oxidation identified so far and the consequences in terms of the metabolites accumulating. In patients affected by ACOX1 deficiency for instance there is accumulation of VLCFAs but not of other fatty acids whereas in patients with ACOX2 deficiency there is only the accumulation of the bile acid intermediates DHCA and THCA. This provides direct evidence in favor of ACOX1 primarily reacting with the CoA-esters of VLCFAs including C26:0-CoA and ACOX2 handling the dehydrogenation of 2-methyl substituted acyl-CoAs including pristanoyl-CoA as well as DHC-CoA and THC-CoA. ACOX3 resembles ACOX2 in some respects including its preference for branched-chain acyl-CoAs but is not reactive with DHC-and THC-CoA. On the other hand, ACOX3 is more widely expressed than ACOX2 whose expression is confined to the liver and kidney. Taken together, ACOX3 is probably the primary oxidase handling 2-methyl branched-chain FAs in all organs except the liver and kidneys (63). With respect to the two multifunctional β-oxidation proteins, only patients with a deficiency of multifunctional protein 2 (MFP2) also called D-bifunctional protein have been described. In these patients there is accumulation of VLCFAs, pristanic acid as well as DHCA and THCA indicating that MFP2/DBP is the central enzyme catalyzing the second and third step in the oxidation of all these FAs. The physiological role of the other multifunctional enzyme (MFP1, also called L-bifunctional protein (LBP)) encoded by *EHHADH* is less clear but the enzyme does play a crucial role in the oxidation of long-chain dicarboxylic acids (60, 64). Among the two peroxisomal thiolases encoded by *ACAA1* and *SCP2*, respectively, the role of SCP2 has been worked out best thanks to the identification of patients with pathogenic variants in the *SCP2* gene (65-67). These patients accumulate pristanic acid as well as DHCA and THCA but not VLCFAs indicating that SCPx which is the product of the largest of the two transcripts derived from the *SCP2* gene, is the key enzyme involved in the oxidation of branched-chain FAs but not VLCFAs. It has been argued that ACAA1 and SCPx are both involved in the oxidation of VLCFAs. Human ACAA1 deficiency has not been described so far. Oxidation of 2-methyl branched-chain FAs requires the participation of yet another enzyme at least when the 2-methyl group has the (2*R*)-configuration. The reason is that acyl-CoA oxidases like ACOX2 only react with (2*S*)-acyl-CoAs. The enzyme required to convert (2*R*)-acyl-CoAs into the corresponding (2*S*)-acyl-CoAs is 2-methylacyl-CoA racemase encoded by *AMACR* which codes for a protein which is targeted to both the mitochondrion and peroxisome in line

with its physiological role in the two organelles (68-70). AMACR deficiency has been identified in humans and affected patients accumulate pristanic acid as well as DHCA and THCA.

Table 1. Disorders of peroxisomal fatty acid ß-oxidation deficiencies and their characteristics

Peroxisomal disorder			**Peroxisomal biomarkers (blood)**				
	OMIM	Gene	VLCFA	Pristanic acid	Phytanic acid	DHCA/THCA	Plasmalogens
X-linked adrenoleukodystrophy	300100	*ABCD1*	↑	N	N	N	N
D-bifunctional protein deficiency	261515	*HSD17B4*	↑	↑	↑	↑	N
Acyl-CoA oxidase 1 deficiency	264470	*ACOX1*	↑	N	N	N	N
Acyl-CoA oxidase 2 deficiency	601641	*ACOX2*	N	N	N	↑	N
2-Methylacyl-CoA racemase deficiency	604489	*AMACR*	N	↑	↑	↑	N
ABCD3 (PMP70) deficiency	170995	*ABCD3*	N	?	?	↑	N
Sterol carrier protein X deficiency	613724	*SCP2*	N	↑	↑	↑	N
ACBD5 deficiency	616618	*ACBD5*	↑	N	N	N	N

N: Normal; ↑: increased; ?: not determined

3.2.1.3. Peroxisomal ß-oxidation and the functional crosstalk with other organelles

As already alluded to above, peroxisomes are very much dependent on their interaction with other subcellular organelles in order to fulfill their role in metabolism. Exciting new data show that, at least in some cases, the interaction between peroxisomes and other organelles is mediated by tethering proteins which play a crucial role in the transfer of metabolites from one site to the other. We will describe these interactions here.

(a.) Peroxisome-lipid body interaction: by virtue of their role as neutral lipid storage organelles lipid droplets (LDs) transfer lipids to various organelles including peroxisomes. Recent work by Lippincott-Schwartz and coworkers (71) has shed new light on the transfer of FAs from LDs to peroxisomes. Indeed,

the authors' work revealed that the hereditary spastic paraplegia protein M1 Spastin, a membrane-bound AAA ATPase localized at the outer aspect of LDs, tethers lipid droplets to peroxisomes by binding to ABCD1 and directs fatty acid trafficking by recruiting the two ESCRT-III proteins IST1 and CHMP1B via its MIT domain to facilitate LD-to-peroxisome FA transfer.

(b.) Peroxisome-lysosome interaction: although still rarely studied, lysosome-to-peroxisome contact sites have been identified and it has been claimed that this contact is actively mediated by lysosomal synaptotagmin VII, which would then bind to the lipid PI(4,5)P2 at the peroxisomal membrane (72). These contact sites have been suggested to catalyze the transfer of cholesterol from the lysosome to peroxisomes (73).

(c.) Peroxisome-ER interaction: pFAO is also very much dependent on the interaction with the endoplasmic reticulum. This is immediately clear if it is realized that the VLCFAs oxidized in peroxisomes are not only derived from lipid bodies and lysosomes but also from the ER at which site VLCFAs are synthesized by chain elongation of shorter-chain FAs. Recent evidence suggests that the transfer of these very-long-chain acyl-CoAs from their site of synthesis to the peroxisome is mediated by another tethering complex composed of ACBD5 and VAPB (74, 75). ACBD5 is a peroxisomal tail-anchored protein with the bulk of the protein exposed to the cytosol and was first identified by Warscheid and coworkers as an exclusive peroxisomal protein as part of a large proteomics study of human peroxisomes (22). ACBD5 has different motifs including an acyl-CoA binding domain and a so-called FFAT motif (two phenylalanines [FF] in an acidic tract). ACBD5 was found to bind to the resident ER-protein VAPB (vesicle-associated membrane protein-associated protein B) which contains a major sperm protein (MSP) domain able to bind to FFAT-like motifs. The identification of patients with pathogenic variants in *ACBD5* in whom VLCFAs were elevated in plasma and oxidation of C26:0 in fibroblasts was deficient (76), strongly suggests that ACBD5 plays a crucial role in VLCFA homeostasis by promoting the transfer of very-long-chain acyl-CoAs from the ER to peroxisomes by bringing the two organelles together thereby facilitating the transfer of acyl-CoAs which compares well with the M1 Spastin-ABCD1 partnership. More recently, a close homologue of ABCD5, i.e. ABCD4 was identified which also binds to VAPB and mediates the contact between peroxisomes and the ER as well ((77); see (78) for review).

(d.) Peroxisome-mitochondrion interaction: whereas the interaction of peroxisomes with LDs, ER, and lysosomes is important from the perspective of input of substrates for β-oxidation, it is the interaction

with mitochondria which is crucial for the output of peroxisomal β-oxidation i.e. the end products of pFAO which include acetyl-CoA, propionyl-CoA, and medium-chain acyl-CoAs. As discussed above, these CoA-esters are shuttled to mitochondria either as free acids or as carnitine esters. Recent systematic mapping studies by Schuldiner and coworkers (79) in yeast have identified different peroxisome-mitochondrion tethering proteins including Fzo1, which is the yeast homologue of the human mitofusins 1 and 2 (MFN1 and MFN2) and Pex34, a PMP earlier shown to affect peroxisome growth and division. Pex34 but not Fzo1 was subsequently found to function in the transfer of acetyl-CoA as generated in peroxisomes to mitochondria. Apart from Fzo1 and Pex34 additional tethering proteins have been identified but their specific function in metabolite transfer has not been resolved yet. Another important functional association between peroxisomes and mitochondria concerns the reoxidation of NADH generated during β-oxidation. Current evidence holds that the reoxidation of intraperoxisomal NADH back to NAD is mediated by so-called redox-shuttles like the malate-aspartate shuttle in mitochondria (27). This has been worked out best in the yeast *S. cerevisiae* in which two different NAD(H)-redox shuttles have been identified including a malate dehydrogenase-based shuttle (80) and a glycerol-3-phosphate dehydrogenase-based shuttle (81). In mammalian peroxisomes reoxidation of NADH is catalyzed by a different redox shuttle involving lactate dehydrogenase as first shown by Baumgart et al. (82). Such a shuttle requires the presence of lactate dehydrogenase (LDH) activity at the two sites of the peroxisomal membrane (Figure 1). The identity of the peroxisomal LDH activity has long remained mysterious until Thoms and coworkers discovered that the peroxisomal LDH is not the product of a distinct gene but is instead produced from one and the same gene (*LDHB*) which generates two transcripts via a unique mechanism called translational readthrough (83). This mechanism allows production of two transcripts one being slightly larger (10 amino acids) than the other thereby creating a typical peroxisome targeting signal which targets this extended version of LDHB to peroxisomes. Work from the same group has shown that the same mechanism of translational readthrough is also observed for the *MDH1* gene which explains the presence of malate dehydrogenase activity in both the cytosol and peroxisomes as encoded by *MDH1* (84). The contribution of the two NAD(H)-redox shuttles to the reoxidation of the NADH generated within peroxisomes in mammals including humans remains to be established.

3.2.1.4. Physiological role of the peroxisomal β-oxidation system

The pFAO system is first and foremost a degradative machinery required for the oxidation of a range of FAs which cannot undergo β-oxidation in mitochondria as part of their homeostasis. This is true for FAs

like VLCFAs and pristanic acid for instance. Next to its degradative role, the peroxisomal β-oxidation system also has a biosynthetic role. This applies especially to the biosynthesis of the polyunsaturated FA docosahexaenoic acid (DHA) as well as the biosynthesis of the primary bile acids CA and CDCA as described above.

3.2.1.5. Inhibitors of peroxisomal β-oxidation

In the past, chemical inhibitors have been used to shed light on the involvement of specific enzymes and/or transporters in certain peroxisomal metabolic pathways including the peroxisomal β-oxidation system. Despite several claims to the contrary, however, there are at present no bona fide specific inhibitors of the peroxisomal β-oxidation system known with the possible exception of two acetylenic compounds (85, 86). Leighton et al (87) and Van den Branden and Roels and coworkers (88) previously claimed phenothiazines, including chlorpromazine and thioridazine, to be specific inhibitors of peroxisomal β-oxidation although the site of inhibition of these compounds was not established in these initial studies. Later work by Vamecq (89, 90) revealed that phenothiazines are in fact highly promiscuous inhibitors with inhibitory effects on multiple enzymes including the peroxisomal enzyme carnitine octanoyltransferase (CROT), but also other enzymes including microsomal palmitoyl-CoA synthetase, mitochondrial carnitine palmitoyltransferases and the mitochondrial respiratory chain enzyme cytochrome c oxidase. Importantly, work by Shin et al (91) in rat hepatocytes revealed that both thioridazine and chlorpromazine did not only inhibit peroxisomal β-oxidation but also mitochondrial β-oxidation to the same extent. Taken together these findings disqualify phenothiazines as specific inhibitors of peroxisomal β-oxidation and thus caution is warranted with respect to the interpretation of results obtained with these inhibitors (e.g. (92, 93)). The same applies to enoximone, another proposed selective inhibitor of peroxisomal fatty acid β-oxidation (94). In search of truly specific inhibitors of peroxisomal β-oxidation, oct-2-en-4-ynoyl-CoA was identified as a new, presumably specific inhibitor of acyl-CoA oxidase 1 (ACOX1)(85). Importantly, the authors resolved the mechanism of inhibition, which includes a nucleophilic attack of the amino acid glutamate at position 421 of ACOX1 by oct-2-en-4-ynoyl-CoA to generate a stable, covalently linked enzyme-inhibitor adduct. Unfortunately, this promising new inhibitor was not evaluated any further. Following up on this work, Zeng et al (86) screened a series of acetylenic acids and identified 10,12-tricosadiynoic acid (TDYA) as a highly specific inhibitor of ACOX1 acting as a suicide substrate for ACOX1 with no inhibitory effect on any of the other peroxisomal β-oxidation enzymes including ACOX2. Taken together, TDYA might well be the only specific inhibitor of peroxisomal β-oxidation at present although it should be noted that TDYA is only a partial inhibitor of β-

oxidation since the oxidation of all substrates handled by ACOX2 is not inhibited by TDYA. In general, however, one should be carefull with the use of so-called specific inhibitors, as they often turn out to be less specific than originally believed. Therefore, the CRISPR genome editing technology, which allows precise genetic disruption of selected genes in cells, should be the preferred method of choice for such studies.

3.2.1.6. Peroxisomal fatty acid β-oxidation and human disease

In the past decades, genetic deficiencies of many of the enzymes and transporters involved in pFAO have been identified. Table 1 lists these disorders which are all autosomal recessive except for X-linked adrenoleukodystrophy.

(a.) X-linked adrenoleukodystropy (ALD): ALD is most frequent among the pFAO disorders and in fact most frequent among the group of peroxisomal disorders altogether. Patients are normal at birth but most if not all patients become symptomatic although the clinical signs and symptoms may vary widely. Common to all individuals with ALD is adrenomyeloneuropathy with a penetration of 100% in males and 90% in hemizygous females (95). ALD used to be categorized into distinct phenotypes but this classification has now been abandoned. Indeed, at present ALD is considered as a progressive neurodegenerative disease that affects the cerebral white matter, spinal cord, peripheral nerves, and the adrenal glands (96). The age of onset and severity of symptoms may differ markedly, however, even within the same kindred. The most frequent phenotype in male patients is that of a progressive leukodystrophy (cerebral ALD) with a lifetime prevalence of +/- 60% with roughly 40% of the patients presenting before 18 years of age. MRI lesions usually develop (long) before symptoms become apparent. Initially symptoms are non-specific and include behavioral changes and cognitive deficits eventually causing problems at school or at work. As the disease progresses, overt neurological deficits develop including visual impairment, motor dysfunction, and sometimes epileptic seizures. Eventually, patients become quadriplegic and need complete care within 2-3 years after onset of symptoms. MRI scans of the brain usually display pathognomonic confluent white matter lesions with gadolinium enhancement just beyond the leading edge of the lesions (97). Lifetime prevalence of adrenal insufficiency has been found to reach 80% in male patients, and is often the first clinical sign in childhood (98). All adult patients eventually develop spinal cord disease characterized by degeneration of mainly the dorsal columns and corticospinal tracts (99). Main symptoms include a disordered gait due to spasticity and sensory ataxia as well as bowel and bladder dysfunction. Importantly, it has now

become clear that the majority of women heterozygous for ALD develop spinal cord disease and peripheral neuropathy with +/- 90% of women having symptoms and/or signs of spinal cord disease when 60 years of age (95). Compared to ALD males, symptoms start on average at a later age rarely before reaching the age of 40. Adrenal insufficiency is very rare among ALD women (<1%) and cerebral ALD has only been described occasionally.

The gene defective in ALD is *ABCD1* which codes for the peroxisomal half-ABC-transporter ABCD1/ALDP which plays a crucial role in the oxidation of VLCFAs notably C26:0. This explains why C26:0 accumulates in tissues and body fluids of ALD patients. Rather than analyzing C26:0-levels in plasma from patients as done for many years, this analysis is now superseded by the analysis of C26:0-lysophosphatidylcholine (C26:0-lysoPC) which has turned out to have superior sensitivity and is now regarded as the best biomarker for ALD and in fact all peroxisomal disorders in which VLCFA-β-oxidation is impaired. Importantly, whereas women heterozygous for ALD could not be identified with certainty using the original method of C26:0-analysis, C26:0-lysoPC analysis now allows identification of ALD women with no-false negatives (100). Finally, analysis of C26:0-lysoPC in bloodspots is also the method of choice to screen for ALD in newborns as currently performed in several USA states.

Cerebral ALD can be stopped by allogeneic haematopoietic cell transplantation (HCT) in boys and adult males but only at a very early stage of the disease when patients have just started to develop cerebral demyelination on brain MRI. For cerebral ALD an MRI severity score has been developed ranging between 0 to 34 (97). HCT is not recommended for patients with a Loes score of 9 or more since outcome is poor. *Ex vivo* lentiviral gene therapy first described by Cartier et al. (101) allows patients to be treated with cells derived from autologous rather than allogeneic bone marrow. The results of a phase III trial are encouraging and suggest that this will be the therapy of choice for ALD patients (102). Unfortunately, HCT does not appear to stop other manifestations of the disease including the spinal cord disease (103).

(b.) D-bifunctional protein deficiency: the clinical signs and symptoms of DBP deficiency are in general very severe and resemble those observed in patients affected by a defect in peroxisome biogenesis like Zellweger syndrome (to be discussed later) which explains why DBP deficiency has been included in the group of Zellweger Spectrum Disorders (PBD). In 2006 we published the clinical, biochemical, and genetic findings in 126 patients affected by DBP deficiency due to biallelic pathogenic variants in the *HSD17B4* gene (104, 105). Infants were found to be born full-term with no evidence of growth retardation and their clinical presentation dominated by neonatal hypotonia (98%) combined with

seizures within the first months of life (93%) and failure to thrive (43%). Patients usually show marked dysmorphic features including macrocephaly, high forehead, flat nasal bridge, low-set ears, large anterior fontanelle, and micrognathia. Virtually none of the 126 patients were found to achieve any significant milestones although, interestingly, several patients with a milder form of DBP deficiency have been described more recently as identified by whole exome sequencing (106).
The gene defective in DBP deficiency is *HSD17B4* which codes for the multifunctional peroxisomal enzyme protein involved in the oxidation of most of the fatty acids oxidized in peroxisomes. This explains why in most patients there is accumulation of VLCFAs, pristanic acid, as well as DHCA and THCA (see Table 1). Especially in milder affected patients, however, metabolite abnormalities may only be minimal and/or only restricted to either VLCFAs, pristanic acid, DHCA or THCA.

(c.) Acyl-CoA oxidase 1 (ACOX1) deficiency: ACOX1 deficiency first described in 1988 (107) has now been identified in some 30 patients at least as published in literature. An extensive analysis of 22 patients (108) revealed that most patients exhibited neonatal-onset hypotonia, seizures, failure to thrive, psychomotor retardation, sensorineural hearing loss, hepatomegaly, and visual loss with retinopathy. In 50% of the patients there were dysmorphic features resembling those observed in Zellweger Syndrome. Patients may show some early motor development but typically regress by 2-3 years of age. In more recent years milder, later-onset forms of ACOX1-deficiency have been described including two adult patients with normal early development who only developed progressive neurologic symptoms in later childhood (109).
The *ACOX1* gene codes for one of the three acyl-CoA oxidases with specificity for the CoA esters of VLCFAs notably C26:0. This explains why in ACOX1-deficient patients VLCFA levels are usually abnormal with normal values for pristanic acid as well as DHCA and THCA (see Table 1).

(d.) Acyl-CoA oxidase 2 (ACOX2) deficiency: ACOX2 deficiency has so far been described in only a few patients with different clinical signs and symptoms. The first patient is an 8-year-old male with intermittently elevated transaminases, liver fibrosis, mild ataxia, and cognitive impairment (110). The second patient was an adolescent boy with elevated transaminases which had persisted for more than 2 years without additional symptoms (111). The third patient identified with ACOX2 deficiency - a girl from a consanguineous marriage - had a much more severe clinical presentation but for reasons discussed in Ferdinandusse et al. (63) there is considerable doubt whether this severe presentation is caused by the deficiency of ACOX2. In all patients there was accumulation of the two bile acid intermediates DHCA and

THCA with normal values for other peroxisomal parameters including plasma VLCFAs which is in line with the functional role of ACOX2 in pFAO.

(e.) 2-Methylacyl-CoA racemase (AMACR) deficiency: after its first description in 2000 (70), AMACR deficiency has since been described in some 15 patients. Two distinct clinical presentations were initially recognized including a severe, early-onset form dominated by marked liver abnormalities culminating in liver failure (112) and a relatively mild form mimicking adult Refsum disease (70). Later, other variant forms have been described including a complex, adult-onset phenotype characterized by peripheral neuropathy, epilepsy, relapsing encephalopathy, bilateral thalamic lesions, cataract, pigmentary retinopathy, and tremor (113, 114). The enzyme AMACR plays a central role in the oxidation of 2-methyl branched-chain FAs which include pristanic acid, DHCA and THCA. Laboratory diagnosis of candidate patients can best be done by analyzing the two different stereoisomers of DHCA and THCA, i.e. (25*R*)- and (25*S*)-DHCA and -THCA using MS/MS since only the (25*R*)-isomer of DHCA and THCA accumulate in this disorder (115).

In addition to the disorders described above, a number of additional genetic deficiencies affecting pFAO have been identified including ACOX3 (116), ACBD5 (76, 117) and ABCD3 (PMP70) (118) deficiency. Since these defects have only been described in a few patients, we will not discuss these here in more detail and refer the reader to the original publications cited here.

3.2.2. Ether lipid synthesis

Ether lipids are a special class of lipids characterized by the presence of an ether linkage at the sn-1 position of the glycerol backbone and occur in a large variety of different molecular species. First of all, the ether bond in ether lipids may be desaturated to create a cis-vinyl ether bond usually with choline or ethanolamine as head group. The resulting phospholipids are called plasmenyl ether lipids and include plasmenylethanolamine (PlsEtn) and plasmenylcholine (PtsCho). Alternatively, the ether linkage is saturated and the ether lipids are named plasmanyl ether lipids. On top of this distinction based on the nature of the ether bond, ether lipids may differ in numerous other aspects. This includes: (a.) the 2-position of the glycerol backbone can either be free as in lyso-ether lipids or substituted; (b.) the 3-position of the glycerol backbone may also be either unsubstituted as in ether-linked diglycerides or occupied either by an acyl chain as in ether-linked triglycerides or by a phosphate group. In turn, the phosphate group may be unmodified as in the different alkyl-and alkenyl-(lyso)phosphatidic acid species or may be extended with a head group which includes choline, ethanolamine but also an atypical

headgroup like the sulphogalactosyl head group as present in seminolipid (see (119) for structural information).

3.2.2.1. Enzymology

Peroxisomes play an indispensable role in the formation of all kinds of ether lipids by virtue of the fact that peroxisomes contain the one and only enzyme able to generate this characteristic ether bond. This enzyme is 1-O-alkyl-dihydroxyacetone-3-phosphate synthase (ADHAPS) (alternative name: 1-alkyl-glycerone-3-phosphate synthase (AGPS) encoded by the gene *AGPS* and is strictly localized in peroxisomes. AGPS catalyzes the formation of 1-alkyl-dihydroxyacetone-3-phosphate from 1-acyl-dihydroxyacetonephosphate by replacing the 1-acyl chain with a long chain fatty alcohol (120). The importance of AGPS for ether lipid biosynthesis is exemplified by the fact that ether lipid biosynthesis is fully deficient in patients in whom AGPS is deficient due to bi-allelic pathogenic variants in the *AGPS* gene as discussed below. The two substrates of AGPS i.e. 1-acyl-dihydroxyacetone-3-phosphate (1-acylDHAP) and the long chain fatty alcohol are produced by two other peroxisomal enzymes including dihydroxyacetone-3-phosphate O-acyltransferase (DHAPAT; also known as glycerone-3-phosphate O-acyltransferase (GNPAT) and fatty acyl-CoA reductase (FAR). The two enzymes GNPAT and AGPS are membrane bound enzymes with their catalytic sites exposed to the interior of the peroxisome and form a heteromeric complex (121) whereas the acyl-CoA reductase enzyme of which there are two variants including FAR1 and FAR2 are both C-tail anchored proteins with their catalytic site exposed to the cytosol (122). It was originally thought that the product of the AGPS reaction, i.e. 1-O-alkyl-DHAP would be converted into 1-O-alkyl-glycerol 3-phosphate (1-O-alkyl-G3P) by the enzyme acyl/alkyl-dihydroxyacetone-3-phosphate reductase (ADHAPR) known to be present in peroxisomes. However, this enzyme has a bimodal distribution being targeted to both peroxisomes and the ER and according to recent work of Honsho et al. (123) it is the ER-localized form of ADHAPR which catalyzes the formation of 1-alkyl-G3P rather than the peroxisome-localized form of ADHAPR. The gene encoding this enzyme was identified by Lodhi and coworkers (124), renamed as “peroxisomal reductase activating PPARgamma (*PexRAP*)” and has now been assigned to the dehydrogenase/reductase SDR family as member 7B (in short: *DHRS7B*). These findings imply that 1-O-alkylDHAP is transported to the endoplasmic reticulum where all subsequent reactions involved in the formation of plasmanyl and plasmenyl (phospho) lipids take place (Figure 3).

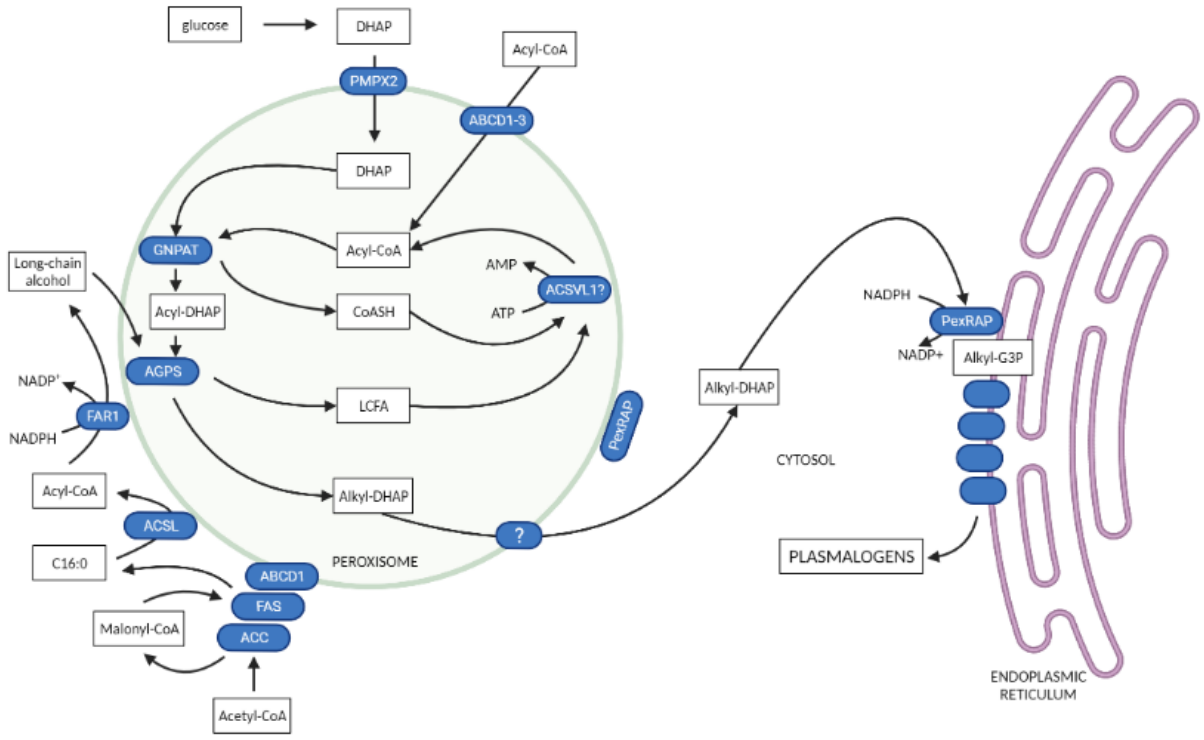


Figure 3. Role of peroxisomes in ether lipid synthesis notably plasmalogens.
Peroxisomes fulfill an indispensable role in ether lipid synthesis because the enzyme alkyldihydroxyacetonephosphate (alkylDHAP) synthase (AGPS) is strictly localized in peroxisomes. The two substrates required for the AGPS-reaction i.e. acylDHAP and a long-chain alcohol are provided by two other peroxisomal enzymes including GNPAT also called DHAPAT (acyl-CoA:dihydroxyacetonephosphate acyltransferase) and one of two different acyl-CoA reductases FAR1 and FAR2. The product of the AGPS-reaction i.e. alkylDHAP is then transferred to the site of the ER where all subsequent reactions occur to generate the different ether (phospho)lipids including plasmalogens (see text). Figure prepared using Biorender.com.

3.2.2.2. Ether lipid synthesis in peroxisomes and the functional crosstalk with other organelles

Overall, the contribution of peroxisomes to the synthesis of ether lipids is limited to the formation of 1-O-alkyl-DHAP from DHAP and a long-chain fatty alcohol. DHAP is generated in the cytosol either as one of the two products of the aldolase reaction of glycolysis or from glycerol as the product of a reaction catalyzed by the enzyme glycerol-3-phosphate dehydrogenase (G3P+ NAD—->DHAP+NADH). Interestingly, peroxisomes also contain glycerol-3-phosphate dehydrogenase activity which would allow peroxisomes to generate DHAP from G3P. The low molecular weight of both DHAP and G3P ensures free transfer of these two metabolites across the peroxisomal membrane via one of the pore-forming proteins. Apart from DHAP ether lipid biosynthesis requires the provision of long-chain fatty alcohols

which are generated from the corresponding acyl-CoA esters by one of two acyl-CoA reductases FAR1 and FAR2 which are both localized in the peroxisomal membrane. The importance of FAR1 has become clear thanks to the discovery of human FAR1-deficiency, which is characterized by a marked deficiency of plasmalogens and has therefore been assigned to the RCDP group as RCDP type 4 (125). More recently, Far2 has been disrupted in mice (126). These mutant mice showed sebaceous gland abnormalities resulting in cicatricial alopecia with lowered levels of a range of lipid species upon skin surface lipidomics. One unresolved aspect of ether lipid synthesis at least with respect to the role played by peroxisomes, is the source of the acyl-CoA required for the DHAPAT/GNPAT reaction. One possibility would be that the acyl-CoA which is required to generate acyl-DHAP in the DHAPAT/GNPAT reaction, and is then released in its free acid form in the AGPS reaction, is reactivated to the original acyl-CoA ester via an acyl-CoA synthetase present in peroxisomes. The enzyme ACSVL1 encoded by *SLC27A2* may catalyze this reaction as postulated before (30). Alternatively, the acyl-CoAs may be imported into the peroxisome interior either via ABCD1, 2, and/or 3. Another unresolved issue involves the source of the acyl-CoA esters which are used as substrate for reduction by FAR1 and FAR2 to generate the corresponding long-chain fatty alcohols. Indeed, these acyl-CoAs may be derived from exogenous dietary sources but could also be produced locally from acetyl-CoA units elongated by the FAS complex to generate palmitate (C16:0), which is the primary end product of the FAS complex. In this respect it is important to mention that previous work (127) has shown that FAS is partially localized to peroxisomes as part of a larger complex including acetyl-CoA carboxylase which converts acetyl-CoA into malonyl-CoA required in the FAS reaction (see Figure 3).

3.2.2.3. Regulatory aspects of ether lipid biosynthesis

Elegant work by Fujiki and coworkers has revealed that the synthesis of ether lipids is under feedback control as exerted by plasmalogens as end product of the pathway. This work was inspired by data obtained earlier by Rizzo and coworkers showing increased fatty acyl-CoA reductase activity in cells deficient in ether lipid biosynthesis (128, 129). This work was done at a time when the identity of the acyl-CoA reductase had not been established yet. The identification of FAR1 and FAR2 by Cheng and Russell (130) allowed Honsho and Fujiki and coworkers to study the mechanism of feedback inhibition of the ether lipid biosynthesis pathway (for review see (131)). The authors discovered that the protein levels of FAR1 but not of FAR2 were increased in plasmalogen-deficient cells and that levels were restored upon restoration of plasmalogen levels in plasmalogen-deficient cells. Furthermore, the authors discovered that the regulation is not at the transcriptional level but involves the stability of the

FAR1 protein (132). The authors further showed that the spatiotemporal regulation occurs through the sensing of plasmalogens localized in the inner leaflet of the plasma membrane (133). The key importance of this spatiotemperal control of ether lipid biosynthesis is stressed by recent findings by Ferdinandusse et al. (134) who identified heterozygous de novo variants in FAR1 in a group of patients presenting in early infancy with spastic paraparesis and bilateral cataracts. The causative variant in FAR1 affects the Arg480 residue of FAR1 which is in the heart of the regulatory region of FAR1 which determines the feedback control by plasmalogens as end product. As a consequence, FAR1 protein levels as well as the catalytic activity of FAR1 were found to be increased in cells from patients resulting in an enhanced rate of ether lipid synthesis as reflected in marked lipidomic changes including increased plasmalogen levels.

Plasmalogens have recently been shown to regulate other metabolic pathways as well including cholesterol metabolism. Again, earlier work had already pointed to the interconnection between the cholesterol and plasmalogen biosynthetic pathways, which includes the reduced synthesis of cholesterol in plasmalogen deficient cells (135) which turned out to be caused by plasmalogens regulating the stability of the key cholesterol biosynthesis enzyme squalene monooxygenase. Indeed, plasmenyl phosphatidyethanolamine enhances the degradation of squalene monooxygenase through the enhanced interaction of the E3-ubiquitin ligase MARCH 6 ((135); for review see (131)).

3.2.2.4. Physiological role of ether lipids

Ether lipids have been implicated in a large variety of physiological functions and there is a vast amount of literature on the topic. However, in many instances the effects reported involve in vitro experiments of which the in vivo relevance has not been verified. A good case in point concerns the proposed role of plasmalogens as anti-oxidants. Indeed, plasmalogens were shown to protect against reactive oxygen species (ROS) by Zoeller et al. (136) in plasmalogen-deficient CHO mutants. Using a pyrene-labeled fatty acid followed by irradiation with UV light to generate singlet oxygen and/or other ROS species plasmalogen deficient cells were found to be much more sensitive compared to wild type cells. Supplementation of chemically synthesized 1-O-alkylglycerol to the medium restored both the plasmalogen levels as well as cell viability. A number of follow-up studies-albeit all in vitro-have demonstrated the ability of plasmalogens to protect lipoproteins and model membranes from radical-mediated damage (137). An *ex vivo* study by Luoma et al. (138) in which use was made of whole sciatic and optic nerves and a hydroxyl radical generating system revealed that the myelin obtained from plasmalogen-deficient *Pex7*$^{-/-}$ mice was much more vulnerable to the ROS-induced compaction

compared to myelin from wild type mice. On the other hand studies by Brodde et al. (139) in the *Gnpat*$^{-/-}$ mouse have shown normal levels of thiobarbiturate-reactive substances (TBARS) and total reactive antioxidant potential (TRAP) which argues against a major role of plasmalogens at least at the whole organismal level. This conclusion is strengthened by work of Drechsler et al. in *C. elegans* (140). Taken together, the role of plasmalogens as anti-oxidants remains disputed. A similar argument concerns the proposed role of plasmalogens as source of signaling mediators as nicely discussed by Dorninger and coworkers (119).

The situation is different for the role of ether lipids notably plasmalogens in signal transduction. Indeed, there is not only a wealth of *in vitro* experiments showing that ether lipids and derivatives thereof play a major in role in signal transduction (see (119) for review) but at least for some of the reported *in vitro* effects more conclusive evidence has been derived from *in vivo* data notably derived from studies in mutant mice. Indeed, following earlier *in vitro* work, Brites and coworkers (141) showed that in the peripheral nervous system of *Gnpat*$^{-/-}$ mice, recruitment of PKB/AKT to the membrane as required for its phosphorylation and activation, was found to be impaired thus preventing the ability of phosphorylated AKT to inhibit GSK3β by phosphorylation at Ser9. Active non-phosphorylated GSK3β impairs Schwann cell differentiation resulting in disturbed radial sorting with detrimental effects on myelination. Supportive evidence for the proposed pathological sequence of events has come from additional work showing that supplementation of lithium chloride or TDZD-8-both inhibitors of GSK3β-rescued the phenotype (141). Importantly, the pathological effects observed in both the peripheral and central nervous system of the *Gnpat* $^{-/-}$ mouse were completely resolved by feeding the mice 1-O-tetradecylglycerol (C14-alkylgycerol) which in contrast to the C16-and C18-alkylglycerols 1-O-octadecylglycerol (batylalcohol) and 1-O-hexadecylglycerol (chimyl alcohol) appeared to be able to correct plasmalogen levels in all tissues of the mutant mice including the brain (142). The mitogen-activated protein (MAP) kinase ERK is another signaling cascade directly affected by plasmalogen levels as shown by Hossain et al. (143) using a mouse model in which *Gnpat* was targeted by shRNA.

Being important constituents of biological membranes plasmalogens contribute to the physiological characteristics of biomembranes especially since the properties of plasmalogens are different from their non-plasmalogen counterparts (see (144, 145) for reviews). One of the features which distinguish plasmalogens from other lipid species concerns their effect on membrane curvature and the propensity of plasmalogen-containing membranes to form non-lamellar inverse hexagonal structures (146). These findings were suggestive for a role of plasmalogens in membrane dynamics including the fission and fusion of membranes which is for instance important for the proper functioning of the synaptic vesicle

cycle. Work by the groups of Just (139) and Berger (147) both working with the *Gnpat* $^{-/-}$ mouse has provided evidence that the impaired neurotransmitter homeostasis in ether lipid deficient mice is due to the disturbed synaptic vesicle cycle caused by the deficiency of plasmalogens. Recent work by Lodhi and coworkers has pointed to yet another important role of plasmalogens in the dynamics of membranes including the mitochondrial membrane (148). The authors conclude that plasmalogens are present in mitochondria and play a crucial role in mitochondrial homeostasis by facilitating the fission of mitochondria. When deficient, mitochondrial fission is impaired possibly through the altered biophysical properties of the mitochondrial outer membrane thereby interfering with the Drp1-mediated membrane constriction with detrimental consequences for mitochondrial homeostasis including mtDNA loss, decreased expression of the mitochondrial DNA encoded components of the respiratory chain, and a reduced rate of coupled as well as uncoupled respiration (148). Recent work by Puigserver and coworkers (149) has also pointed to a functional alliance between mitochondria and ether lipids. These authors showed that lower levels of pyrimidines stimulate respirasome assembly thereby enhancing mitochondrial OXPHOS capacity by altering the mitochondrial lipid composition towards the increased abundance of ether lipids. Very recently, Zou and coworkers discovered a new, previously unrecognized role of ether lipids in ferroptosis which is an iron-dependent, non-apoptotic form of cell death caused by uncontrolled lipid peroxidation (150).

3.2.2.5. Inhibitors of ether lipid synthesis

At present no specific inhibitors of ether lipid synthesis have been described in literature.

3.2.2.6. Ether lipid synthesis and human disease

Ether lipid synthesis is deficient in a number of different genetic diseases either as a consequence of an isolated deficiency of one of the enzymes involved in ether lipid biosynthesis or due to a defect in the biogenesis of peroxisomes causing a secondary deficiency of these enzymes. The latter disorders will be discussed below (section III). The clinical phenotype of patients affected by a defect in ether lipid biosynthesis as in GNPAT, AGPS and FAR1 deficiency, is that of rhizomelic chondrodysplasia punctata (RCDP) characterized by severe growth retardation, proximal shortening notably of the upper extremities, contractures, spasticity, severe developmental delay, and cataracts together with a considerably reduced life expectancy. Indeed, classical RCDP patients do not survive beyond 12 years of age and do not acquire any developmental milestones. The majority of patients exhibit cardiac malformations, recurrent infections and have feeding difficulties (151). The identification of more mildly

affected patients has led to the concept of an RCDP spectrum in analogy to the Zellweger spectrum with at the mild end of the spectrum patients with mild to moderate developmental delay, cataracts, and bone dysplasia but no rhizomelic shortening. Importantly, many of these milder affected patients do reach adulthood. The clinical signs and symptoms of these milder affected patients have recently been described in detail in a series of 16 patients (152). Most patients had cataract, growth retardation, joint contractures, and developmental delay. Other features were: learning disability (87%), behavioral problems (56%), seizures (43%), and cardiac defects (31%). It may well be that FAR1 deficiency differs from GNPAT and AGPS deficiency in some aspects. Indeed, although the three original FAR1-deficient patients demonstrated several of the characteristic features of RCDP including profound growth retardation, developmental delay, pyrimidal track dysfunction, and seizures, all patients lacked the characteristic rhizomelic shortening of the long bones despite the marked deficiency of plasmalogens in erythrocytes.

Ether phospholipids have also been implicated in a variety of other more common diseases including various neurological disorders. Indeed, early studies dating back to the 1990s already showed reduced PlsEtn levels in brain samples of patients with Alzheimer's disease (AD) (153-155). Subsequent work revealed that this deficiency of PlsEtn was already discernable in the early stages of disease (156). Furthermore, Kou et al. (157) discovered that PlsEtn levels were lowest in brain tissue from patients with the highest grade of disease progression as reflected in the highest Braak score. Importantly, in the same study an inverse relation was found with respect to the VLCFA levels which points to a more generalized dysfunction of peroxisomes rather than an isolated deficiency in ether lipid synthesis. This conclusion is supported by work of Grimm et al. (158) who reported that increased β-amyloid levels give rise to enhanced oxidative stress in the brain which leads to peroxisome dysfunction, reduced AGPS activity and lowered ether lipid levels including plasmalogens. Later studies revealed that the PlsEtn levels were also lowered in serum of Alzheimer patients and correlate with disease progression (159, 160). Although less pronounced as compared to AD, deficiencies of plasmalogens have also been reported in a number of other neurological conditions including Parkinson's disease (161-163) and schizophrenia (164, 165). Taken together, there is now convincing evidence for an association between plasmalogens and neurodegenerative diseases especially in the case of AD. Rather than being specific for AD, it has been argued that the association between plasmalogens and neurodegenerative diseases is more general and is not just limited to AD only (166). The question which remains open for now, is whether the deficiency of plasmalogens is a mere bystander of neurodegenerative disease or contributes to the pathology observed. Resolution of this question is all the more important since it may

well be possible to correct the plasmalogen deficiency in the brain by administration of an alkylglycerol with a slightly reduced number of carbon atoms in the alkyl side chain (C14-alkylglycerol) as recently discovered by Brites et al. (142). Finally, plasmalogen deficiencies have also been reported in other non-neurological conditions including hypertension, obesity and diabetes (167-169).

3.2.3. Glyoxylate detoxification

Glyoxylate is a very toxic metabolite because it is a good substrate for the enzyme lactate dehydrogenase (LDH) which readily converts glyoxylate into oxalate. In the presence of calcium there is precipitation of oxalate in tissues notably in the kidneys giving rise to nephrocalcinosis and progressive kidney dysfunction ultimately leading to kidney loss. Given the ubiquitous presence of LDH which is a tetramer of LDHA and LDHB subunits, glyoxylate levels need to be kept as low as possible under all circumstances. Peroxisomes play a key role in this process by catalyzing the ultimate detoxification of glyoxylate via transamination of glyoxylate into glycine as mediated by the enzyme alanine glyoxylate aminotransferase (AGT). The pivotal role of peroxisomes is once again demonstrated by the identification of patients in whom AGT is deficient due to pathogenic variants in the encoding gene *AGXT* (170).

3.2.3.1. Glyoxylate metabolism in peroxisomes and the functional crosstalk with other organelles

Glyoxylate is not only produced in peroxisomes but also in other organelles including mitochondria and the cytosol (171). In peroxisomes glyoxylate is primarily derived from glycolate via one of the peroxisomal L-α-hydroxyacid oxidases of which there are three in humans (171). In human liver, the predominant oxidase is hydroxyacid oxidase A (HAOA) also named glycolate oxidase as encoded by the *HAO1* gene. Glyoxylate is also produced in mitochondria as obligatory end product of the oxidation of hydroxyproline whereas in the cytosol glyoxylate is produced from various sources notably different sugars (171). Current evidence holds that the glyoxylate produced in the cytosol and in mitochondria is readily detoxified by the enzyme glyoxylate reductase (GRHPR) which is localized in both mitochondria and the cytosol (our own unpublished work) and drives the NADPH-driven synthesis of glycolate from glyoxylate. Subsequently, the glycolate produced travels to the site of the peroxisome to undergo definitive detoxification into glycine via the glycolate oxidase-alanine glyoxylate aminotransferase system in peroxisomes (Figure 4) which illustrates beautifully how different subcellular compartments collaborate for the greater good of homeostasis (30).

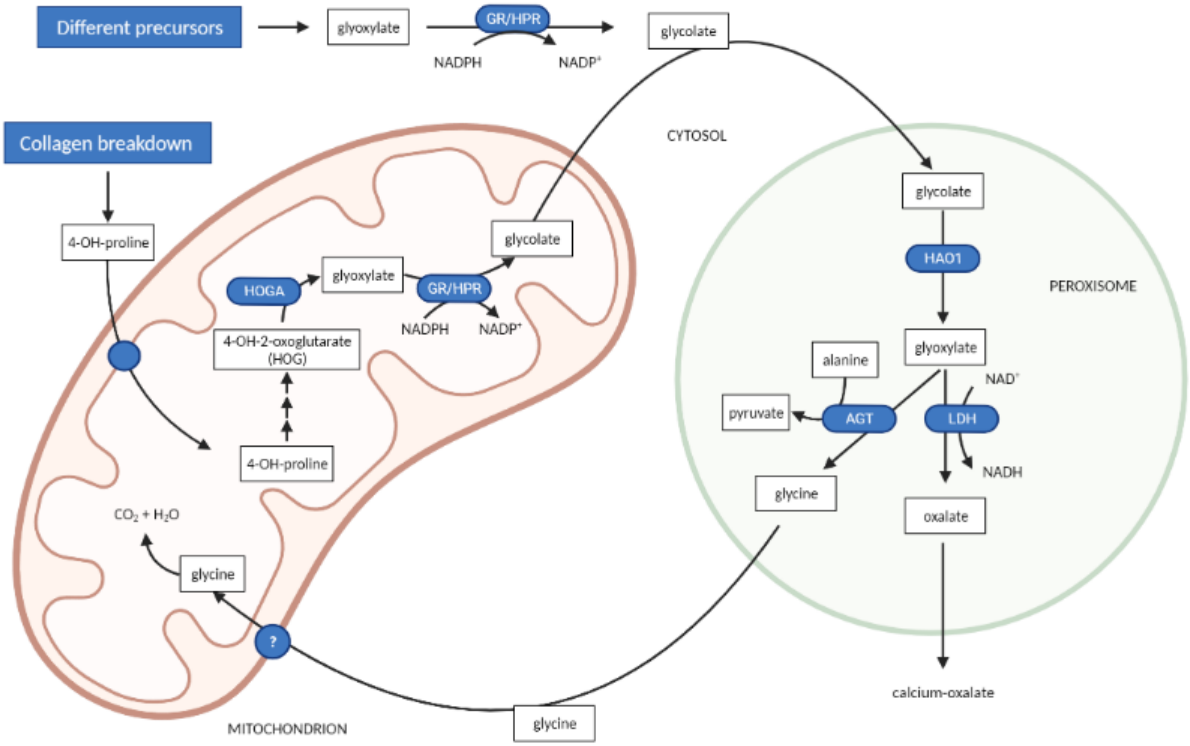


Figure 4. Glyoxylate homeostasis in human hepatocytes.
Glyoxylate is a potentially very toxic molecule not so much because of glyoxylate itself but because of its rapid conversion into oxalate by the ubiquitously present enzyme lactate dehydrogenase and it subsequent precipitation as calcium oxalate. In mitochondria glyoxylate is produced in the breakdown pathway of hydroxyproline in a reaction catalyzed by the enzyme 4-hydroxy-2-oxoglutaratealdolase (HOGA) after which glyoxylate is most likely reduced to glycolate. Glyoxylate is also produced in the cytosol from different precursors including several sugars. As in mitochondria glyoxylate is reduced into glycolate once again by the enzyme GRHPR which has a bimodal distribution in both the cytosol and mitochondrion. Ultimate detoxification of glycolate occurs in the peroxisome through the concerted action of the two enzymes glycolate oxidase and alanine glyoxylate aminotransferase (AGXT) (see text for more detailed information). Figure prepared using Biorender.com.

3.2.3.2. Inhibitors of glyoxylate metabolism in peroxisomes

At present no specific inhibitors of AGT have been described in literature. Aminooxyacetic acid (AOA) has been used to inhibit AGT but is an inhibitor of all transaminases, possibly with some selectivity for AGT (172). The other peroxisomal enzyme involved in glyoxylate detoxification is glycolate oxidase. In literature several potent inhibitors of glycolate oxidase have been described although their mono-specificity has rarely been determined (see (173) for recent review).

3.2.3.3. Glyoxylate detoxification in peroxisomes and human disease

The key role of the peroxisomal enzyme alanine glyoxylate aminotransferase (AGT) is exemplified by the autosomal recessive disorder primary hyperoxaluria type 1 (PH1) caused by pathogenic variants in *AGXT* coding for AGT. In most cases AGT is inactive due to pathogenic variants in *AGXT* which either affect the catalytic activity of the enzyme and/or its stability. However, in a substantial proportion of patients AGT is catalytically active when measured in homogenates prepared from liver biopsies of these patients but functionally inactive because of its mislocalization to the mitochondrion (174). Since most of the glyoxylate is generated within peroxisomes from glycolate, mislocalization of an in principle active form of AGT to the mitochondrion will give rise to massive production of oxalate in the peroxisome from the glyoxylate produced from glycolate via the peroxisomal enzyme glycolate oxidase. The clinical spectrum of PH1 patients is markedly heterogeneous ranging from infantile oxalosis progressing to end-stage kidney disease (ESKD) before two years of age to a mild late-onset phenotype at the other side of the spectrum characterized by occasional stones and first presentation during adulthood (170, 175, 176). Remarkably, a large proportion of patients (>60%) diagnosed in adulthood present with ESKD at the time of diagnosis. Some patients are only diagnosed with PH1 after a kidney-only transplantation and the finding of massive calcium oxalate crystals in the patient's kidney. It often turns out that the vast majority of these adult patients had a history of occasional to recurrent stone disease in retrospect. Importantly, subsequent genetic analysis has shown that many of these patients carry pathogenic variants in *AGXT* which allow the enzyme to be stimulated by pyridoxine (vitamin B6). This exemplifies the importance of timely diagnosis so that appropriate treatment which includes pyridoxine supplementation, can be initiated. The beneficial effect of pyridoxine on AGT has not been worked out in detail except for a few pathogenic variants but is probably the result of at least three different mechanisms (177) including: (a.) direct stimulation of the catalytic activity of AGT thanks to pyridoxal-5-phosphate as obligatory co-factor in the enzyme reaction; (b.) improved folding including dimerization of AGT induced by pyridoxal-5-phosphate and (c.) improved targeting of AGT to the peroxisome especially in case of those pathogenic variants which give rise to mistargeting of AGT to the mitochondrion.

In general, nephrocalcinosis or nephrolithiasis in any child or recurrent kidney stone formation at any age should raise suspicion for PH and should be followed up by urinary analysis of oxalate. Increased oxalate levels especially when combined with elevated glycolate levels is already strongly suggestive for PH1. It should be noted that there are two other forms of PH which are much less frequent (<10%) compared to PH1: PH2 as caused by pathogenic variants in *GR/HPR* coding for the mitondrial/cytosolic enzyme glyoxylate reductase, and PH3 caused by pathogenic variants in *HOGA* coding for the

mitochondrial enzyme 4-hydroxy-2-oxoglutarate aldolase (171). Discrimination between these three forms of PH can be done by metabolite analysis and/or molecular analysis. To date, the only available treatment option for patients with advanced systemic oxalosis and renal failure is a sequential or combined liver-kidney transplantation. Although curative, these procedures are associated with high rates of morbidity and mortality which explains why much effort has been directed towards the generation of alternative treatment options which includes substrate reduction therapy aimed at glycolate oxidase using siRNA (178).

3.2.4. Fatty acid α-oxidation

3.2.4.1. Enzymology of the peroxisomal fatty acid α-oxidation pathway

Branched-chain FAs with a methyl group at the 3-position cannot undergo β-oxidation but first need to undergo chain-shortening by one carbon atom to the corresponding 2-methyl FA which can be β-oxidized. This process of oxidative decarboxylation of 3-methyl branched-chain FAs occurs solely in peroxisomes by means of α-oxidation. Phytanic acid ((3,7,11,15)-tetramethylhexadecanoic acid) is the best-known FA undergoing α-oxidation. The enzymology of the α-oxidation machinery has been worked out in detail (see (47, 179) for review) and proceeds via 5 subsequent steps which include: (1.) activation of the 3-methyl FA to its CoA-ester; (2.) 2-hydroxylation of the 3-methylacyl-CoA to generate 2-hydroxy-3-methylacyl-CoA; (3.) cleavage of the 2-hydroxy-3-hydroxyacyl-CoA into formyl-CoA plus the corresponding (n-1)aldehyde; (4.) oxidation of the (n-1) aldehyde into the corresponding (n-1) acid; and (5.) activation of the (n-1) fatty acid to its corresponding acyl-CoA ester.

3.2.4.2. Fatty acid α-oxidation and the functional interaction with other organelles

Fatty acid α-oxidation in peroxisomes is also very much dependent on the interaction with other organelles. Phytanic acid is one of the most dominant 3-methyl branched-chain FA in humans, is solely derived from exogenous, dietary sources and cannot be synthesized via the chain-elongation system as is the case for VLCFAs. On the other hand, phytanic acid is readily incorporated into different lipid species including phospholipids as well as di-and triglycerides which immediately implies that phytanic acid α-oxidation requires interorganellar associations between peroxisomes and lipid bodies as well as peroxisomes and lysosomes. In analogy to the situation described above for fatty acid β-oxidation, α-oxidation is also very much dependent on the interaction with mitochondria. This applies to the first step of α-oxidation mediated by the enzyme phytanoyl-CoA 2-hydroxylase which is a dioxygenase with 2-oxoglutarate as second substrate and succinate as product. Efficient 2-hydroxylation of phytanoyl-CoA

requires the continuous provision of 2-oxoglutarate and removal of succinate. Regeneration of 2-oxoglutarate from succinate can best be done by mitochondria using the citric acid cycle to convert succinate into 2-oxoglutarate. Interaction with mitochondria is also required for step (4.) in the pathway in which NADH is generated and step (5.) in which ATP is required (see Figure 5 and Figure 4B in (179)).

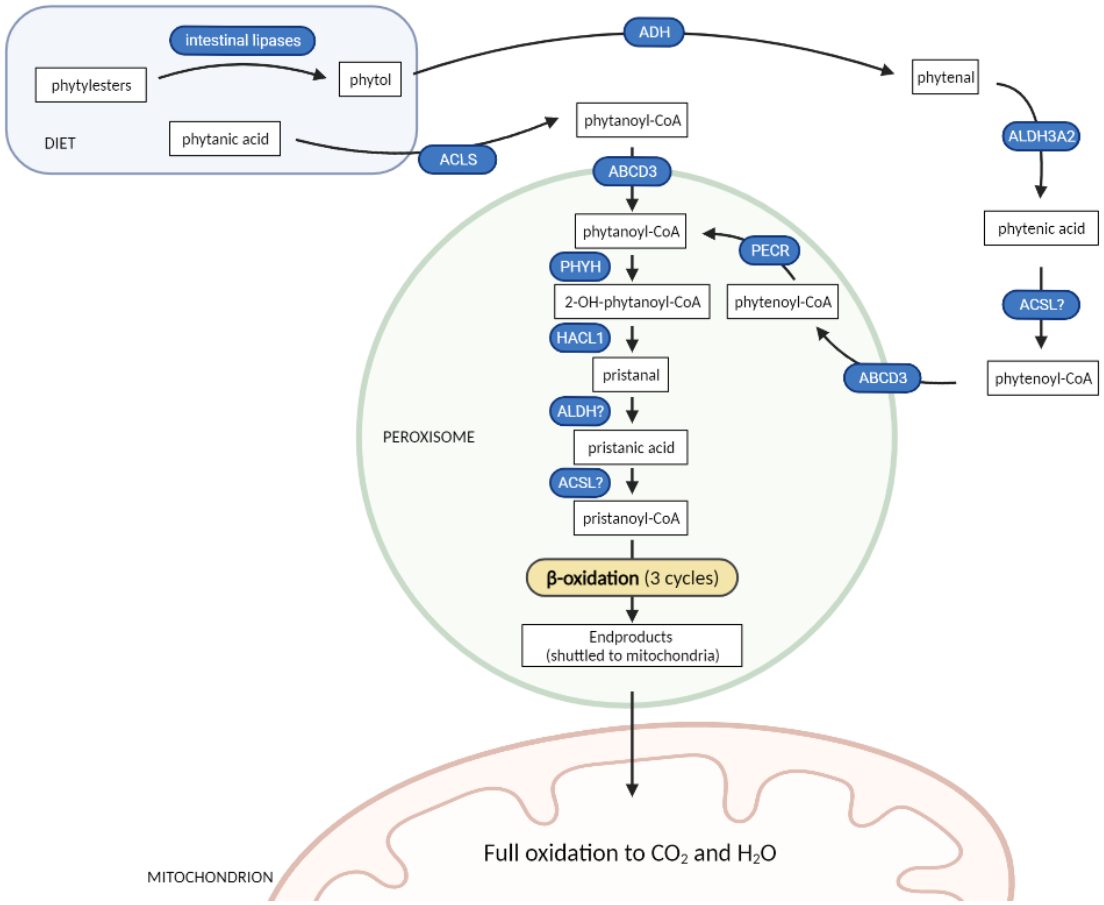


Figure 5. Phytanic acid oxidation in peroxisomes and its formation from different precursors including phytol and phytyl esters.
Phytanic acid occurs in many different food components notably of ruminant origin but in certain fish and nuts. Recently, phytyl esters were recognized as another dietary source of phytanic acid which upon cleavage by intestinal lipases generate phytol which is then converted into phytanic acid via a series of enzymatic reactions depicted in Figure 5 and described in the text. The phytanic acid α-oxidation pathway itself is fully confined to the peroxisomal matrix thus rendering peroxisomes indispensable for the oxidation of 3-methyl branched-chain fatty acids like phytanic acid. Figure prepared using Biorender.com.

3.2.4.3. Physiological role of the peroxisomal fatty acid α-oxidation system

The peroxisomal FA α-oxidation system is purely a degradative system required to control phytanic acid levels.

3.2.4.4. Inhibitors of peroxisomal fatty acid α-oxidation

Aminotriazole has been described as a potent inhibitor of α-oxidation but the exact target of aminotriazole in terms of which enzyme of the pathway is inhibited, has not been resolved (180). Furthermore, the inhibition observed could well be indirect since aminotriazole is known to be a very potent inhibitor of catalase.

3.2.4.5. Fatty acid α-oxidation and human disease

So far only a single genetic disorder of fatty acid α-oxidation has been identified. The disorder involved is adult Refsum disease (RD) caused by a deficiency of the peroxisomal enzyme phytanoyl-CoA 2-hydroxylase (181) caused by bi-allelic pathogenic variants in the *PHYH* gene coding for the enzyme phytanoyl-CoA 2-hydroxylase (for recent review see (182). Affected RD patients do not show symptoms at birth and only present in late childhood with loss of night vision as initial symptom followed by a progressive loss of visual capacity. Anosmia is also an early and frequently found feature in RD patients (182). Over the years RD patients develop additional abnormalities although not in every patient. Indeed, whereas retinitis pigmentosa and anosmia are found in virtually all RD patients, other features including deafness, ataxia, polyneuropathy, ichthyosis, fatigue, and cardiac conduction disturbances are not universally observed in all RD patients (183). The polyneuropathy in RD is of a mixed motor and sensory type which is asymmetrical, chronic, and progressive in untreated RD. Over the course of years muscular weakness can become widespread involving the limbs but also the trunk. Almost without exception patients exhibit peripheral sensory disturbances, most often impairment of deep sensation, particularly perception of vibration and position-motion in the distal legs. Cardiac features including cardiac arrhythmia and heart failure are frequent findings in RD which may be fatal especially in untreated patients (182). The laboratory diagnosis of RD patients usually starts off with the analysis of phytanic acid in plasma preferably in conjunction with the analysis of other peroxisomal biomarkers. If phytanic acid is the only parameter which is abnormal, the diagnosis RD is almost certain but requires molecular analysis of the *PHYH* gene especially since in a subset of RD patients, pathogenic variants are found in the *PEX7* gene (184) which codes for the PEX7 protein required for the import of phytanoyl-CoA 2-hydroxylase into peroxisomes. At present there is no curative treatment for RD. However, many symptoms can be resolved successfully by restricting the dietary intake of phytanic acid. This applies to the ichthyosis, sensory neuropathy, and ataxia in approximately that order whereas the progression of the retinitis pigmentosa, anosmia, and deafness can at best be halted (183). Phytanic acid is present in our daily diet in ruminant meat, krill, and other components but also as phytol, which is a precursor of phytanic acid readily converted into phytanic acid via a series of enzymatic reactions. Another source of

phytanic acid identified more recently (185) are phytylesters which are cleaved by intestinal lipases to generate phytanic acid. A mouse model mimicking Refsum disease in many respects at least when the mice were fed a diet high in phytol, has been generated (186).

4. PEROXISOME BIOGENESIS

Peroxisome biogenesis is the collective name used for the following processes 1) formation of peroxisomal membranes including the import of peroxisomal membrane proteins; 2) import of peroxisomal matrix proteins; 3) peroxisomal growth, division and proliferation; and 4) peroxisomal degradation (1, 187-190). Whereas the metabolic functions, protein content and number of peroxisomes can differ per organism, tissue and cell type, the biogenesis of the organelles and many of the proteins involved therein appear relatively well conserved among species, ranging from unicellular yeasts to multicellular plants and animals, including humans (1). Consequently, insight into human peroxisome biogenesis and identification of the mechanisms and key proteins involved have been aided greatly by genetic and molecular studies in different yeast species and Chinese hamster ovary (CHO) cells, which can be manipulated relatively easy, as well as by the resolution of the causes and consequences of the human peroxisome biogenesis disorders (PBD). The majority of PBDs are caused by a defect in the import of peroxisomal membrane or matrix proteins preventing the generation of metabolic functional organelles, commonly referred to as 'peroxisome deficiency'. These PBDs can be caused by defects in any of 13 different *PEX* genes, which encode proteins named peroxins that have specific functions in peroxisomal protein import and are denoted here as correspondingly numbered PEXs (1, 189, 191). In addition to these PBDs, more recently several PBDs with a genetic defect in peroxisome division have been recognized (reviewed in (190, 192, 193)).
In this section we will briefly discuss the current knowledge on the different aspects of human peroxisome biogenesis, including human disorders thereof.

4.1. Formation of peroxisomal membranes including transport and import of peroxisomal membrane proteins

Although not directly studied, the human peroxisomal membrane most probably consists primarily of phosphatidylcholine (50-60%) and phosphatidylethanolamine (25-30%), with less than 5% of each phosphatidylinositol, phosphatidylserine and sphingomyelin (based on data from rat liver peroxisomes (194)). These phospholipids most probably originate from the endoplasmic reticulum (ER) which is the

main site for their synthesis (1, 187, 195). In addition, there are indications that peroxisomes can be derived by budding from the ER (196-199).

Peroxisomes do not have their own DNA and thus all peroxisomal proteins are encoded by nuclear genes, synthesized on free cytosolic polyribosomes and subsequently transported to peroxisomes. There are 3 peroxins recognized with a specific function in the transport to and import of peroxisomal membrane proteins (PMPs): PEX19, PEX3, and PEX16. PEX19 is a cytosolic protein that functions as the receptor of newly synthesized PMPs (200) and which shuttles from the cytosol to the peroxisomal membrane and back. Binding of the PMPs to PEX19 depends on internal PEX19-binding motifs in the PMPs, which are composed of a short helical motif with a minimal length of 11 amino acids (201, 202). PEX3 is located in the peroxisomal membrane, does not require PEX19 for targeting, but functions as docking site for PMP-loaded PEX19 (203). Targeting via PEX19-PEX3 is the default import pathway for most PMPs, including several of the peroxins discussed below and PMPs with other functions such as metabolite transport (204). The role of PEX16, which also is located in the peroxisomal membrane, is less clear, but it was shown to be required for insertion of PEX3 into the peroxisomal membrane and thus functioning in peroxisome membrane assembly upstream of PEX3 and PEX19. Whether PEX16 only serves as receptor for PEX3 or also for other PMPs has remained unsolved (205, 206). PEX3, PEX16 and PEX19 are essential for the generation and propagation of peroxisomes: dysfunction or absence of any of the three proteins results in cells completely lacking peroxisomal membranes. This observation and the proposed functions of the three peroxins imply that insertion of PEX3 and PEX16 in the peroxisomal membrane occurs independently of PEX19. In accordance with this, PEX3 and PEX16 have been found also in the ER, based on which it was postulated that novel peroxisomes may derive from PEX3-PEX16-containing vesicles originating from the ER (196-199).

4.2. Transport and import of peroxisomal matrix proteins

The transport of the majority of matrix proteins to peroxisomes is mediated by one of two specific peroxisomal targeting sequences (PTSs). Most matrix proteins have a carboxy-terminal tripeptide PTS1 motif with consensus sequence (S/A/C)-(K/R/H)-(L/M) that is recognized by one of the two isoforms of the cytosolic receptor protein PEX5, i.e. PEX5S and PEX5L (6, 207, 208). A small subset of matrix proteins contains an amino-terminal octapeptide PTS2 motif with consensus sequence: (R/K)-(L/I/V)-X_5-(Q/H)-L/I/V) (6, 209) that is recognized by the cytosolic receptor protein PEX7. The PTS1 sequence remains part of the mature protein after peroxisomal import, but the amino-terminus including the PTS2 motif is removed via processing after import. A few peroxisomal matrix proteins have been identified that lack

both PTS motifs but still end up in peroxisomes by virtue of interaction and subsequent co-import with a peroxisomal protein containing a PTS, a mechanism also referred to as piggy-backing (210, 211). Furthermore, a few cytosolic proteins have been found to end up partially in peroxisomes as a consequence of translational stop codon read-through, which introduces a carboxy-terminal PTS1 motif encoded by the sequence 3' of the stop codon (83, 84). Finally, a few matrix proteins have been identified that contain two different organellar targeting signals as a consequence of which they can be targeted to both mitochondra and peroxisomes, including 3-hydroxy-3-methylglutaryl-CoA lyase (212) and α-methylacyl-CoA racemase (AMACR) (68-70).

After their synthesis, the matrix proteins are bound in the cytosol by their respective receptor proteins, after which they are transported to the peroxisomes (Figure 6). In humans, this transport is strictly dependent on PEX5; PEX7 needs to interact with PEX5L to get targeted to the peroxisomal membrane (207, 213). The matrix protein-loaded receptor proteins dock on to the peroxisomal membrane via interaction of PEX5S or PEX5L/PEX7 with PEX13 and PEX14, both located in the peroxisomal membrane (208, 214, 215). After docking, the matrix proteins are imported into the peroxisomal lumen after which the receptor proteins are released from the peroxisomal membrane for another import cycle or directed for degradation to the proteasome (215-218). The fate of the receptors is determined by the PEX2, PEX10 and PEX12 proteins, which form a E3 ubiquitin ligase complex located in the peroxisomal membrane that catalyse either the mono- or the poly-ubiquitination of PEX5S/L, which promotes their re-shuttling or degradation, respectively (218, 219). The actual release of ubiquitinated PEX5 from the peroxisomal membrane is mediated by the PEX1-PEX6-PEX26 complex (217, 219), which is composed of the cytosolic AAA-proteins PEX1 and PEX6 that form a heterohexamer with PEX6 anchored to the peroxisomal membrane through binding with the peroxisomal membrane protein PEX26. Mono-ubiquitinated PEX5 can be deubiquitinated by USP9X after which it becomes available for another round of matrix protein import (220).

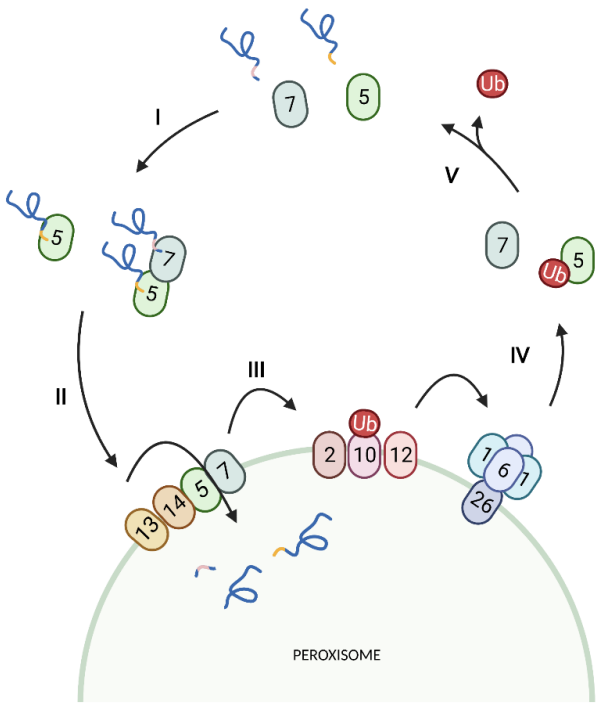


Figure 6. Peroxisomal matrix protein transport and import.
Roles of different PEX proteins (peroxins) in the human import machinery for peroxisomal matrix proteins. Peroxisomal proteins are encoded by nuclear genes and synthesized on free ribosomes. Newly synthesized proteins are recognized by the cytosolic receptor proteins PEX5S/L (PTS1 proteins) or PEX7 (PTS2 proteins) (I). Matrix protein-loaded PEX7 is targeted to the peroxisome via interaction with PEX5L. The matrix protein-loaded PEX5S/L receptors are recognized by the peroxisomal docking complex composed of PEX13 and PEX14 (II). The matrix proteins are imported into the peroxisomal lumen, after which PEX5S/L becomes mono-ubiquinated by the PEX2-PEX10-PEX12 E3 ligase complex (III). Mono-ubiquinated PEX5S/L is then released from the peoxisomal membrane by the PEX1-PEX6-PEX26 complex (IV). Mono-ubiquinated PEX5S/L is de-ubiquinated in the cytosol after which PEX5S/L and PEX7 become available for another round of protein import (V). See text for further background. Figure prepared using Biorender.com.

4.3. Growth, division and proliferation of peroxisomes

Peroxisomes are dynamic organelles that can adjust their number and protein content in response to metabolic needs and physiological conditions. This involves the interplay between de novo protein synthesis, peroxisome biogenesis, including peroxisome proliferation and fission, and peroxisome degradation. For humans, the regulatory mechanisms hereof at the gene level have not been resolved. The number of peroxisomes in human cells may range from 100 to more than 1000 per cell. They are most abundant in liver and several liver-specific peroxisomal enzymes and metabolic pathways have been described (see also above). In cultured skin fibroblast cells, the half-life of peroxisomes was shown to be approximately two days (221). Under normal conditions, new peroxisomes are assumed to be formed and propagated by budding, or fission, from pre-existing peroxisomes followed by growth due to import of matrix proteins (Figure 7; default pathway) (190, 192, 196, 222). However, the observation

that peroxisomes can also be formed when the corresponding gene is introduced in cells which entirely lack peroxisomal structures due to a defect of PEX3, PEX16 or PEX19, indicated that peroxisomes can also be formed *de novo* and then most probably form pre-peroxisomal vesicles that originate from the ER (Figure 7; Salvage pathway) (190, 192, 197, 198, 223). The formation and multiplication of novel peroxisomes via the 'growth-and-division' model can be subdivided in three subsequent stages, including elongation, constriction and fission (190, 192, 196). Several human proteins have been identified that play a role in these different stages, including PEX11β involved in elongation and constriction, and DLP1/DNM1L, FIS1 and MFF, involved in fission (Figure 7). Of note, although peroxisomes and mitochondria are not related organelles, DLP1/DNM1L, FIS1 and MFF are involved in the fission of peroxisomes as well as of mitochondria (192, 193).

Three different human PEX11 proteins are known, PEX11α, PEX11β and PEX11γ, encoded by different genes and with apparently distinct functions. Overexpression of PEX11α or PEX11β in mammalian cells causes an increase in peroxisome numbers, while PEX11γ overexpression has no effect (224). Moreover, in human PEX11β-deficient fibroblasts cultured at 37°C, elongated catalase-containing peroxisomes are observed (225). When these cells are cultured at 40°C, however, catalase is no longer present in peroxisomes, but cytosolic. This appears due to an additional deficiency of PEX11γ at this elevated temperature as was confirmed by transfection with PEX11γ, which restored the catalase import in the cells (225). Based on these findings, PEX11β appears to be involved in both peroxisome elongation and constriction in the 'growth-and-division' pathway, while PEX11γ was postulated to mediate the import of peroxisomal matrix proteins into the pre-peroxisomal vesicles that are derived from the ER in the *de novo* pathway (189, 225). PEX11α appears to play a role in the proliferation of peroxisomes rather than their fission: PEX11α overexpression results in increased peroxisome numbers, while its deficiency results in reduced peroxisome numbers (224, 226, 227).

PEX11 proteins have been shown to mediate the deformation and elongation of peroxisomal membranes prior to the fission but PEX11β was also found to recruit DLP1/DNM1L to the peroxisomal membrane via interaction with the FIS1 and MFF proteins (190, 192, 193).

DLP1 or DNM1L, which stands for dynamin-like protein 1, is a member of the dynamin superfamily, which includes multiple large GTPases that share structural homology and participate in various cellular membrane fission and fusion events (228). DLP1/DNM1L is involved in the fission of peroxisomes as well as mitochondria and, through binding with the membrane proteins FIS1 (i.e. Mitochondrial fission 1 protein) and MFF (i.e. Mitochondrial fission factor) and upon activation by PEX11β, oligomerizes in ring-like structures at the constriction sites resulting in the actual scission of the membranes (190, 192, 193).

FIS1, MFF and GDAP1 (i.e. Ganglioside-induced differentiation-associated protein 1) are membrane proteins that have been localized in both peroxisomes and mitochondria and also play an essential role in the fission of both organelles. Depletion of DLP1/DNM1L 1, FIS1, MFF or GDAP1 results in elongated peroxisomes and large, tubular mitochondria (190, 192, 193, 229-234). In DLP1/DNM1L-depleted cells, the elongated peroxisomes show a constricted morphology, indicating that DLP1/DNML1 is not required for the constriction but for fission of membranes (229, 230).

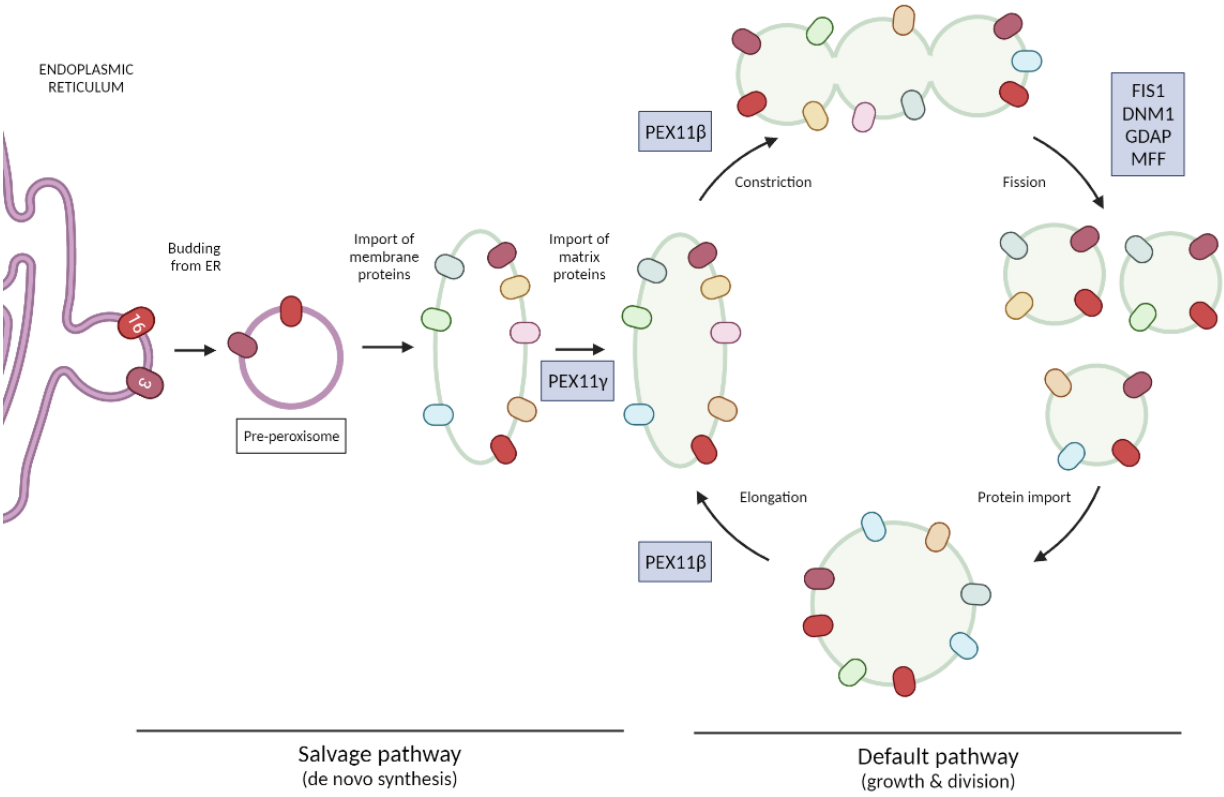


Figure 7. Model for peroxisome biogenesis and division.
Peroxisomes propagate primarily by growth and division of pre-existing peroxisomes (default pathway) but may also develop de novo from preperoxisomal vesicles that originate from the endoplasmic reticulum (salvage pathway). They subsequently develop into mature, metabolically active peroxisomes through the subsequent import of peroxisomal membrane and matrix proteins. Peroxisome division involves three sequential steps: elongation, constriction, and fission. Indicated are the different proteins involved in these processes. See text for further background. Figure prepared using Biorender.com.

4.4. Peroxisomal degradation (Pexophagy)

The dynamic nature of peroxisomes requires coordinated mechanisms for their propagation and proliferation but also for their degradation, which occurs by a selective autophagic process called pexophagy. Pexophagy can be induced in a ubiquitin-dependent and a ubiquitin-independent manner.

For mammals, the autophagy receptors NBR1 and SQSTM1/P62 have been shown to recognize ubiquitin-designated peroxisomes and target them to autophagosomes for degradation (235, 236). An important trigger for this pexophagy is the ubiquitination of PEX5. As mentioned above, mono-ubiquitination of PEX5 by the PEX2-PEX10-PEX12 E3 ligase complex is required for its release from the peroxisomal membrane by the PEX1-PEX6-PEX26 complex. In peroxisome-deficient cells with a defect in PEX1, PEX6 or PEX26, this release is blocked causing accumulation of mono-ubiquitinated PEX5 at the membrane, which triggers pexophagy (237). PEX5 was also found to become ubiquitinated as a consequence of the (over) production of reactive oxygen species (ROS), e.g. due to non/less-functional peroxisomes. The ROS (over)production promotes PEX5-mediated targeting of the 'ataxia-telangiectasia mutated' (ATM) kinase to peroxisomes during or after which the ATM kinase phosphorylates PEX5 which promotes ubiquitination of PEX5 by the PEX2-PEX10-PEX12 complex. The ubiquitinated PEX5 is then recognized by the autophagy adapter protein SQSTM1/P62 which targets the peroxisome to the autophagy machinery eventually resulting in its degradation (238, 239). Pexophagy can also be induced by amino acid starvation, which results in ubiquitination of PEX5 and the peroxisomal membrane protein ABCD3, and possibly additional peroxisomal membrane proteins, by PEX2, targeting the peroxisome for degradation (240). Finally, studies in Chinese hamster ovary (CHO) cells have suggested a ubiquitin-independent induction of pexophagy, in which the peroxisomal membrane protein PEX14, component of the PEX5 docking complex, can bind to autophagosome-bound MAP1LC3B-II which thus results in peroxisome degradation (241, 242).

4.5. Peroxisome biogenesis disorders (PBDs)

The PBDs form a genetically heterogeneous group of autosomal recessively inherited disorders with a generalized defect in peroxisome functioning due to a specific defect in one of the processes involved in peroxisome biogenesis.

When compared with the single enzyme deficiencies, where only one or few specific peroxisomal metabolic functions/pathways are defective, PBDs which are due to a defect in peroxisomal membrane and/or matrix protein import typically present with multiple defective peroxisomal functions, resulting in characteristic aberrant biochemical parameters, including specific metabolite profiles that can be used for laboratory diagnosis (3, 189, 243-245). In contrast, despite the distinctive elongated morphology of the peroxisomes in cells of patients with a PBD due to a defect in peroxisome division, the peroxisomal biochemical aberrations are often minimal or even absent (225, 229, 246). So far, no PBDs have been identified due to a defect in pexophagy. However, it has been reported that in cells of

certain ZSD patients, pexophagy is induced, most probably to remove non-functional peroxisomes (237, 247, 248).

4.5.1. PBDs with a defect in peroxisomal membrane and/or matrix protein import

The largest subgroup of PBDs includes the Zellweger spectrum disorders (ZSDs) and RCDP type 1 and type 5, which are due to bi-allelic pathogenic variants in any of the 13 different *PEX* genes encoding the different peroxins involved in the transport/import of peroxisomal membrane or matrix proteins as described above (188, 189, 249, 250). A smaller group of PBDs include the peroxisomal fission defects, due to pathogenic variants in genes involved in peroxisome maintenance, including division (189, 192, 193).

The ZSDs present with decreasing clinical and biochemical severity, ranging from severe, lethal multisystemic disorders to milder, late-onset progressive neurological disease or even isolated visual and/or hearing problems (32, 33, 251, 252). In general, the clinical presentation of ZSD patients is progressive in particular with respect to loss of hearing and vision, and neurological symptoms. There is currently no general curative treatment for ZSDs and patient management is mainly supportive and personalized, i.e. dependent on presenting symptoms (see (32, 253) for reviews).

The ZSDs include the previously reported disorders cerebro-hepato-renal syndrome or Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD) and Heimler syndrome (32, 249, 251, 254). Patients with the severe ZS presentation usually die early in life without any developmental progress. They typically present with severe hypotonia, ocular abnormalities, seizures, renal cysts, hepatic dysfunction and characteristic craniofacial features, including a large anterior fontanel, a prominent forehead, shallow orbital ridges, epicanthal folds, a high arched palate, a broad nasal bridge and a small nose with anteverted nares. Patients with the NALD and IRD presentation have a variable clinical presentation, which often includes developmental delay, hypotonia, liver dysfunction, sensorineural hearing loss, retinal dystrophy and/or vision impairment (32, 255, 256). Patients with the NALD presentation may reach their teens, while patients with the IRD presentation may even reach adulthood (257). Patients with the Heimler syndrome presentation represent the mildest patients of the ZSDs and usually don't show any developmental delay. They present with sensorineural hearing loss, enamel hypoplasia of the secondary dentition, nail abnormalities and/or occasional retinal pigmentation abnormalities (254, 258).

As discussed above, cells of patients with biallelic loss-of-function variants in the *PEX3, PEX16* and *PEX19* genes are affected in the import of both peroxisomal membrane and matrix proteins and, consequently,

completely lack peroxisomal remnants (187, 204, 259-262). These patients always present with the severe ZS presentation. It should be noted, however, that hypomorphic (i.e. milder) pathogenic variants in the *PEX16* and *PEX3* genes have been reported that resulted in less severe clinical presentations and cellular phenotypes with lower numbers of (enlarged) peroxisomes (263-265). Biallelic pathogenic variants in the *PEX1, PEX2, PEX5, PEX6, PEX10, PEX12, PEX13, PEX14, PEX26* genes only affect the import of peroxisomal matrix proteins (Figure 6). Cells from PBD patients homozygous or compound heterozygous for loss-of-function variants in these genes still have peroxisomal membrane vesicles lacking matrix proteins but containing (most of the) peroxisomal membrane proteins. Bi-allelic variants in the *PEX1* and *PEX6* genes are the most common cause of a ZSD with frequencies of 60 and 16%, respectively (188, 250). Improved laboratory diagnostic tests, the availability of genetic testing for the different *PEX* genes and exome sequencing, has led to an increased diagnosis of ZSD patients at later ages (254, 258, 263, 266-268). These patients are often relatively mildly affected and/or show only a few of the symptoms that are typically associated with ZSDs, including progressive loss of hearing and vision, and isolated ataxia, and previously had not been recognized. Furthermore, they often show mild to hardly recognizable biochemical aberrations pointing to a peroxisomal disorder, including a biogenesis defect.

Bi-allelic pathogenic variants in the *PEX7* gene only affect the import of PTS2-targeted peroxisomal matrix proteins. As a consequence, the PTS2-targeted peroxisomal enzymes 3-ketoacyl-CoA thiolase, AGPS and phytanoyl-CoA 2-hydroxylase are not imported into peroxisomes resulting in a defect in plasmalogen synthesis (AGPS deficiency as in RCDP type 3) and α-oxidation (phytanoyl-CoA 2-hydroxylase deficiency as in Refsum disease) (209, 269, 270). The clinical presentation of the vast majority of patients with defective *PEX7* is clearly distinct from ZSD and indistinguishable from the aforementioned patients with RCDP type 2 and type 3, explaining the name RCDP type 1 for this defect (271, 272). Hypomorphic *PEX7* variants have been found to give rise to milder phenotypes, including RCDP without rhizomelia (152, 271-274) or a Refsum disease-like presentation (182, 184).

RCDP type 5 has the same biochemical consequences as RCDP type 1, but patients are less severely affected (275, 276). In the first reported patients, the PEX5L isoform, which acts as the PEX7 co-receptor, was no longer expressed due to a frame shift variant located in the PEX5L-specific exon 9. Consequently, PEX7-mediated import of PTS2-targeted proteins is abrogated, while the import of PTS1-targeted proteins, mediated by PEX5S, is still functional (275). Recently, a second family was reported with a missense variant in PEX5L-specific exon 9, which prevents the interaction of PEX7 with PEX5L and

similarly abrogates PEX7-mediated import of PTS2-targeted proteins while the import of PTS1-targeted proteins, mediated by PEX5S, is still functional (276).

4.5.2. PBDs with a defect in peroxisome division

PBDs due to genetic defects in peroxisome division have been recognized more recently and currently include defects in PEX11β, DLP1/DNM1L, MFF and GDAP1. Despite the distinctive elongated morphology of the peroxisomes in cells with these defects, the peroxisomal biochemical aberrations are often minimal or even absent.

PEX11β deficiency has been reported in patients from 6 different families (225, 277-279). So far only bi-allelic loss-of function variants in the *PEX11B* gene have been reported, including different nonsense and splice-site variants, which thus appears a requisite for disease manifestation. All patients had congenital bilateral cataract, and furthermore presented with variable additional ZSD-like symptoms, such as mild intellectual disability, progressive hearing loss, sensory nerve involvement, skeletal abnormalities and short stature. Peroxisomal biochemical parameters were often normal, with occasional and transient small elevations of C26:0 levels. Patients' cells showed elongated peroxisomes, while mitochondria were unaffected.

Patients with genetic defects in DLP1/DNM1L and MFF present with a clinical phenotype that often is more severe than that of patients with defective PEX11β, most probably due to the additional if not predominant involvement of mitochondria in these defects. Based on the main clinical symptoms of the patients and the distinctive morphology of both peroxisomes and mitochondria in the patient's cells, DLP1/DNM1L and MFF deficiency are also referred to as Encephalopathy due to defective Mitochondrial and Peroxisomal Fission-1 and -2 (EMPF1 and EMPF2), respectively (www.omim.org).

Following the first report of a severely affected patient with a dominant negative DLP1/DNM1L defect (227) multiple additional patients with EMPF1 have been reported with pathogenic variants in the *DNM1L* gene, including *de novo* dominant negative variants and autosomal recessive variants, with variable severity (e.g. (280-287). Patients are characterized by delayed psychomotor development and hypotonia with variable ages of onset and survival and most patients developed refractory seizures, consistent with an epileptic encephalopathy, followed by neurologic decline. Some patients had increased serum lactate, while other patients did not show clinical or biochemical evidence of mitochondrial or peroxisomal dysfunction, although patients' cells always showed elongated peroxisomes and mitochondria.

MFF deficiency or EMPF2 has been reported in 6 different families (246, 288, 289). Patients presented with delayed psychomotor development, severe hypotonia, microcephaly, and abnormal signals in the basal ganglia. More variable features included early-onset encephalopathy, optic atrophy, and peripheral neuropathy. Reported patients were homozygous or compound-heterozygous for biallelic loss-of-function variants only. Although patients' cells showed elongated peroxisomes and mitochondria, no indication for mitochondrial or peroxisomal dysfunction have been reported for most patients; in few patients elevated serum lactate was found.

Patients with pathogenic variants in the *GDAP1* gene display a Charcot-Marie-Tooth (CMT) disease phenotype, characterized by peripheral neuropathy (233). For most CMT disease-associated variants, no effect on the morphology or function of peroxisomes was identified and the peroxisome elongation observed in cells with depleted GDAP1 levels was rescued by expressing GDAP isoforms containing these variants. Only a few C-terminal truncated GDAP1 proteins did not rescue the peroxisomal elongation, suggesting that these may cause a peroxisomal phenotype in patients in addition to the CMT presentation. So far, this has not been reported in patients.

5. ROLE OF PEROXISOMES IN DIFFERENT ORGANS (HUMAN AND MOUSE)

The diverse pathologies in patients with peroxisome deficiencies underscore the importance of peroxisomes in multiple tissues but few studies searching for pathological mechanisms have been performed. During the last 25 years, loss of function approaches in mice have generated a wealth of information with regard to the functional role of peroxisomes in different tissues. Almost every established peroxisomal enzyme and transporter as well as several proteins involved in peroxisome biogenesis were inactivated in mice either globally or in a cell type selective way, allowing mechanistic studies.

In the following paragraphs, current insights into the function of peroxisomes in different tissues will be discussed taking into account data from patients, from peroxisome distribution and from knockout mice. For brain and liver, topical elaborate reviews were published (290-292) which explains why we will primarily focus on novel insights that have become available more recently.

5.1. The nervous system

Neurological deficits are the most common pathology in peroxisomal diseases, illustrating that the nervous system strongly depends on intact peroxisomal metabolism for normal functioning. This encompasses both the central and peripheral nervous system and can involve purely developmental or

purely degenerative mechanisms although they most often coexist and cannot always be clearly distinguished.

5.1.1. The central nervous system

In the mouse brain, based on PEX14 staining, peroxisomal abundance strongly varies between different cell types, brain areas and developmental stages. For example, in the white matter, there is a surge of peroxisomes during the myelination period clearly indicative of their importance in the myelination process (293). Also peroxisomal enzymes and transporters are selectively expressed e.g. catalase is more enriched in astrocytes compared to neurons (293), and ABCD1 occurs mostly in astrocytes and microglia and in a subset of oligodendrocytes in certain brain regions (294).
Severe Zellweger patients almost always present with neonatal seizures and psychomotor retardation (32, 252). In longer surviving ZSD patients loss of developmental milestones due to leukodystrophy and cerebellar ataxia occur. The underlying neuropathological abnormalities involve developmental and wiring defects, white matter abnormalities, and degeneration and loss of axons and neurons (291).

5.1.1.1. Aberrances in CNS development

The hallmark of Zellweger syndrome is a cortical neuronal migration defect leading to an abnormal gyration of the neocortex that is distinct from other brain malformations (291). Also in patients with a mild ZSD an abnormal gyration pattern may well be more frequent than originally thought (295). Cytoarchitectonic abnormalities also occur in the cerebellum and inferior olives (291). The fact that MFP2/DBP- patients develop an indistinguishable migration deficit points to a major pathophysiological role of the peroxisomal β-oxidation system in this respect (296). However, the mechanism remains obscure and it is unresolved why neuronal migration defects are mimicked in all Zellweger syndrome mouse models (291), but not in the mouse model with MFP2/DBP deficiency (297). It should be noted that a severe deficiency of ether lipids can also give rise to abnormalities in brain development such as microcephaly (291).
Intricate studies rescuing PEX5 function selectively in either the brain or in the liver of *Pex5*$^{-/-}$ mice revealed that the cortical migration defect results from both a local and a liver-specific peroxisomal deficiency (298). In the opposite setting of a brain selective knockout of PEX5 only a delay in migration was noted, likely related to the late loss of peroxisomal function during brain development in *Nestin-Pex5* mice (299). It remains unexplored which metabolites, produced in the liver as a consequence of

the liver-specific elimination of peroxisomes, are involved and how they affect the migration of neurons over radial glial cells. Furthermore, given the major differences between mouse and human brain developmental stages including the maturation of the blood brain barrier, the molecular origin of the cortical malformation in Zellweger patients remains to be identified.

Dysfunction of serotonergic signaling was shown in juvenile mice with brain selective inactivation of PEX13 (300). This includes altered distribution of serotonergic neurons in the midbrain, dystrophy of axons and reduced levels of tryptophan hydroxylase-2, the rate-limiting enzyme in serotonin synthesis. The brain serotonergic system plays a broad role in brain development and neuronal circuitry formation. Because these anomalies were observed in a late stage of brain formation it is, however, not clear whether this contributes to the morphological brain abnormalities in mice with a global deficit in peroxisome biogenesis. Also the primary cause of these serotonergic changes was not elucidated.

An important confounding factor in brain developmental research of peroxin knockout mice is that they are born with a substantial growth retardation (301), in marked contrast to the situation in humans. This is exacerbated during the lactation period due to steatorrhea resulting from defective formation of mature conjugated bile acids in combination with the lipid-rich milk. It may well be that this growth impairment may impact on the formation of the brain in a non-specific way.

5.1.1.2. Cerebellar malformation and the role of BDNF signaling

In Zellweger syndrome patients, Purkinje cells are heterotopically localized in the cerebellar white matter and their relative position to granule neurons is altered (302).

With regard to cerebellar malformation, some insights have been gained from mouse model research. Only Zellweger syndrome mouse models on a specific genetic background survive in the postnatal period, which allowed to reveal multiple abnormalities in cerebellar morphogenesis, including foliation deficits, delayed migration of granule cells, impaired differentiation of Purkinje cells with blunted dentritic arborization, and axonal dystrophy (302, 303). Similar anomalies were observed in brain-selective *Pex5* (299) and *Pex13* knockouts (304), indicative of the local origin of these defects. In $Pex14^{\Delta C/\Delta C}$ mice, expressing a C-terminal–truncated mutant of PEX14, abnormalities in BDNF-TrkB signaling were shown to underlie the Purkinje cell defects (303). The Purkinje cell layer contained elevated levels of brain-derived neurotrophic factor (BDNF) that was suggested to be delivered by climbing fibers originating in the brain stem. In addition, the dominant negative truncated splice variant of the BDNF receptor, TrkB-T1 was overexpressed on the neuronal plasma membrane, thereby suppressing signaling via the TrkB-TK+ receptor. This leads to attenuated signal transduction by ERK and

AKT. Notably, this disturbed neurotrophin signaling is not responsible for the cortical migration defects as no alterations in levels of TrkB variants nor in BDNF were found in the cortex of newborn *Pex14*$^{\Delta C/\Delta C}$ mice (303), neither in the hippocampus. In contrast, partial deletion of peroxisomal function from the brain in adulthood, in tamoxifen inducible *Pex2* knockout mice, did elicit deregulated BDNF and TrkB-T1 expression in the hippocampus, leading to impaired memory (305). In additional *in vitro* studies the same authors showed that peroxisome deficient astrocytes augment their BDNF expression and secretion (306). When co-cultured with hippocampal neurons, the peroxisome deficient astroglia were found to cause increased axonal branching. These anomalies are not mediated by altered lipid metabolism but by the ROS imbalance due to catalase mislocalization.

In sum, the relevance of these findings in mice for the neurology of PBD patients remains incompletely understood and requires further studies. Questions which remain include: Does the imbalance of BDNF signaling and the increased axonal branching also occur in PBD patients? What is the connection between increased expression of BDNF and of the dominant negative variant of its receptor as they are expressed in different cell types? Why is the BDNF-signaling pathway affected in a specific brain region and in an age dependent manner? Does such a mechanism also underly the neurological features of MFP2/DBP- deficient patients, which are indistinguishable from PBD patients, although catalase is normally localized in peroxisomes?

5.1.1.3. Cerebellar degeneration in ZSD

In ZSD patients with a milder disease course, neuropathologies may only start during childhood, adolescence or even adulthood. In some cases, ataxia is the primary clinical symptom associated with clear cerebellar atrophy on brain MRI while intellectual abilities may be normal (266-268, 307, 308). Genetic analyses were required to identify these patients as suffering from a peroxisome deficiency disorder because the metabolic peroxisomal parameters measured in a blood sample from these patients were not or only borderline abnormal. This points to an exquisite vulnerability of the cerebellum for peroxisome dysfunction and questions the underlying mechanism. It becomes even more intriguing when considering that pathogenic variants are often located in one of the RING finger domain peroxins PEX2, PEX10 or PEX12 that act as ubiquitin ligase (E3)-like proteins (290). In contrast, mild PBD patients with pathogenic variants in *PEX1* or *PEX6* develop hearing and vision problems but no cerebellar degeneration.

5.1.1.4. Multiple peroxisomal metabolic pathways are required for cerebellar formation and maintenance

Patients with peroxisomal β-oxidation defects often show cerebellar pathologies including cerebellar malformation, leukodystrophy, and/or atrophy of the cerebellum, depending on the specific pathogenic variants (Figure 8) (290). Similar to the mild PBD patients, a few adult ACOX1- and MFP2/DBP- deficient patients who presented with progressive ataxia have been identified by genetic analysis (106, 109, 309, 310).

Ataxia and diverse cerebellar anomalies are also recapitulated in mouse models with MFP2/DBP deficiency caused by pathogenic variants in *HSD17B4*. Global and neural-selective *Mfp2/Dbp* mutants were already ataxic at weaning, indicative of a developmental origin (290). Associated pathologies were wiring defects of parallel and climbing fibers on Purkinje cells, impaired Purkinje cell firing frequency and axonal swellings at this young age. Importantly, Purkinje cell selective *Mfp2/Dbp* knockout mice also develop axonopathy starting in the deep cerebellar nuclei and proceeding to the proximal axon from the age of 6 months, evolving in loss of dendrites and Purkinje cell death (311, 312). These data unequivocally demonstrate that defective peroxisomal β-oxidation within neurons can cause a late onset decay but this is accelerated when the surrounding cell types also lack MFP2/DBP.

In adult, but not in young, mice with ACBD5 deficiency, Purkinje cells are affected in a similar way with axonal swellings followed by cell death (313). ACBD5 tethers peroxisomes to the ER and is required for the degradation of VLCFA by β-oxidation. Interestingly, lipidomics analysis of the entire cerebellum revealed a mild accumulation of C26:0- containing phospholipids but a more pronounced increase of phospholipids containing ultra-long chain fatty acids (> C30) that are polyunsaturated (VLC-PUFA). Although these lipid species occur at very low concentrations it may well be that they affect membrane properties.

Also, ether lipids are essential for cerebellar integrity as RCDP patients develop progressive cerebellar atrophy (Figure 8). This is confirmed in the *Gnpat* mouse model that shows deficits in cerebellar foliation, impaired migration of granule cells, axonal swellings, altered innervation of Purkinje cells by climbing and parallel fibers. It is striking that this largely overlaps with the pathology observed in *Mfp2/Dbp*$^{-/-}$ mice (314, 315).

Finally, peroxisomal α-oxidation is also required to safeguard the cerebellum (Figure 8). Cerebellar ataxia, which needs to be distinguished from sensory ataxia, is however not observed in all Refsum patients (290). The causative role of increased phytanic acid levels was demonstrated as reducing the dietary intake of this branched chain fatty acid improved gait disturbances. This was further confirmed

in the Refsum *Phyh*$^{-/-}$ mouse model. Supplementing the diet with phytol, a phytanic acid precursor, provoked the degeneration and loss of Purkinje cells (186).

Taken together, the three major peroxisomal lipid metabolizing pathways are essential for different aspects of cerebellar formation, maintenance and functioning.

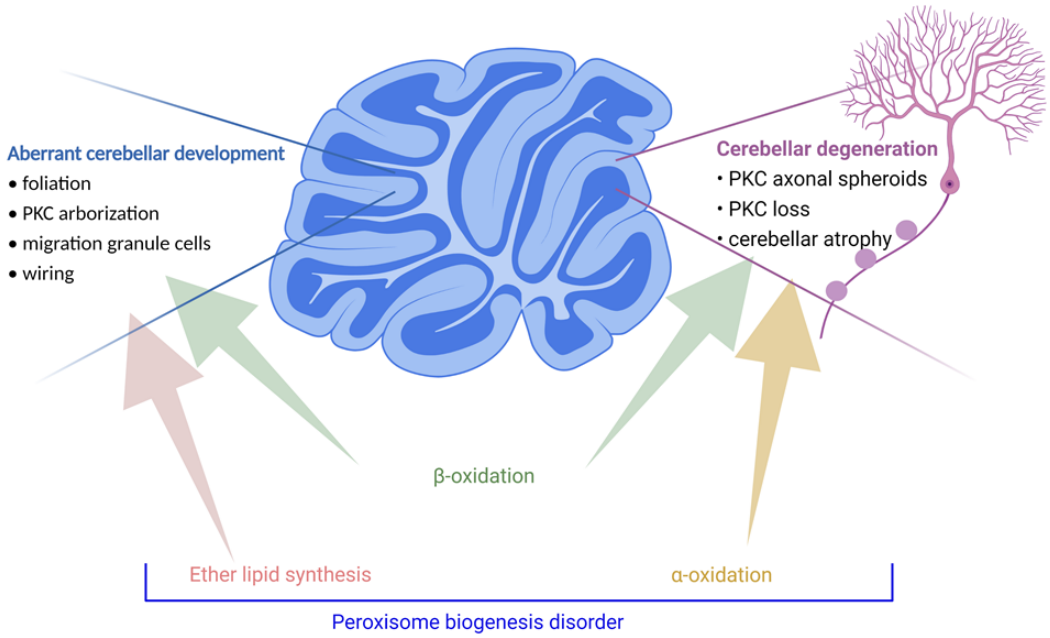


Figure 8. Cerebellar integrity depends on peroxisomal function in men and mice.
An array of abnormalities occur during the formation of the cerebellum when peroxisomes are dysfunctional, which are caused both by deficiency of ether lipids and impaired peroxisomal β-oxidation. In addition, defective peroxisomal β-oxidation and α-oxidation lead to cerebellar degeneration and atrophy.

5.1.1.5. The white matter

PBD and the peroxisomal fatty acid β-oxidation single enzyme deficiencies including X-linked adrenoleukodystrophy, DBP, ACOX1 and SCPx deficiency are categorized as leukodystrophies, underscoring the importance of peroxisomes for the formation and maintenance of the CNS white matter (316).

Myelin pathologies in ZSD are diverse (317) and may be developmental or regressive, with or without inflammation. At the severe end of the ZSD spectrum, reduction of white matter volume and delayed myelination occurs in Zellweger syndrome and in what was previously defined as neonatal adrenoleukodystrophy. In the longer surviving infants white matter degeneration is a constant pathology that is closely related with functional decline and loss of developmental milestones. The

myelin degeneration in ZSD is progressive and follows a typical pattern as revealed by MRI imaging (318). It starts in the hilus of the dendate nucleus and superior cerebellar peduncles followed by the cerebellar white matter and brainstem, and continues to the corpus callosum and the posterior limb of the internal capsule. In milder forms of ZSD leukoencephalopathy is observed but it remains clinically silent, whereas in other cases a late onset but rapid myelin loss develops. The distinguishing genetic or metabolic parameters have not been resolved. Importantly, MRI of DBP-deficient patients has shown patterns that are indistinguishable from those in ZSD patients (318), again pointing to the essential role of peroxisomal β-oxidation defects in the pathology (Figure 9). Although the accumulation of VLCFA may play an instrumental role, other metabolic deficits likely contribute to the early myelin abnormalities in ZSD, considering the later onset and different pathology in X-ALD/AMN patients, in which VLCFA are also increased.

The early death of global *Pex* knockout mice did not allow a longitudinal study of the myelin status. However, in brain selective *Pex5* knockouts (*Nestin-Pex5*), the ZSD leukodystrophy is mimicked (319, 320). This includes dysmyelination in juvenile mice and subsequent precipitous demyelination with a remarkably similar pattern as in ZSD, starting in the cerebellum and proceeding into the cerebrum. This was accompanied by loss of axons, expression of pro-inflammatory markers such as cytokines and complement factors and macrophage activation. Importantly, in a model in which *Pex5* deletion was induced after myelination was completed, a similar pattern of demyelination was observed, proving that the regression is not caused by an initial impairment of myelin formation (320). Inflammatory demyelination and axonal loss also develop in mice with oligodendrocyte restricted deletion of peroxisomes (*Cnp-Pex5*) (321). These findings emphasize the indispensable role of peroxisomes in oligodendrocytes for the maintenance of myelin.

As with the neuronal migration defect, the demyelination was not recapitulated in the *Mfp2/Dbp* mouse model (322). Still, a widespread neuroinflammation involving microglia and astrocytes developed. Although the CNS was not well analysed in other mouse models with peroxisomal β-oxidation defects such as *Acox1* and *Scp-x* knockouts, the long survival and lack of any description of behavioral abnormalities indicates that no major demyelination occurs. Therefore, the metabolic factors mediating the demyelination in PBD and β-oxidation patients remain obscure.

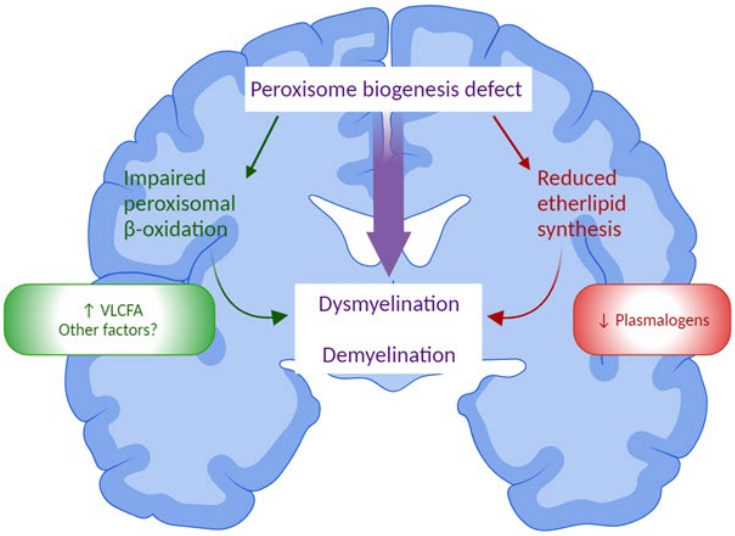


Figure 9. Peroxisomal dysfunction differentially impacts on the white matter in men and mice. *Impaired myelin formation during development and loss of myelin are hallmarks of severe peroxisome biogenesis disorders, which are recapitulated in Pex mouse models. Leukodystrophy also occurs in patients – but not in mice - with specific defects in peroxisomal β-oxidation, always linked with the accumulation of VLCFA but additional unknown factors are required. Reduced levels of plasmalogens due to ether lipid synthesis dysfunction also causes developmental and regressive myelin anomalies in men and mice, but these are less severe compared to the PBD.*

5.1.1.6. Cerebral demyelination in X-ALD

The relationship between a defect in peroxisomal β-oxidation and white matter becomes even more complex in X-ALD. This is the most common peroxisomal disease caused by dysfunction of the ABCD1 transporter with the accumulation of saturated VLCFA in plasma and tissues as hallmark (323, 324). The default clinical presentation is adrenomyeloneuropathy with a penetration of 100% in adult males and 90% in hemizygous females (95). This concerns a dying back axonopathy of the motor and sensory tracks in the spinal cord, leading to motor and other disabilities. In approximately 60% of male patients, a rapidly progressive inflammatory demyelination develops, usually initiating before the age of 10 years, but it can also happen in adulthood (323, 324). Demyelination typically starts in the genu of the corpus callosum proceeding into the parietooccipital lobes but alternative scenarios are also possible. In the initial phase, neurological deficits are minor which is associated with noninflammatory demyelinating lesions (325). Once the inflammation sets in, the demyelination and loss of cognitive and other functions strongly accelerates. This is associated with a breach in the blood brain barrier, infiltration of mononuclear cells, T helper and cytotoxic T cells, and B cells. In families both phenotypes of the disease may coexist. The levels of the diagnostic markers C26:0 and LPC-C26:0 in plasma are not predictive for

the outcome. Thus, elevated levels of C26:0 are a prerequisite but not sufficient to elicit the demyelination. Despite enormous efforts, the triggering factors or potential modifier genes causing the devastating cerebral demyelination are still elusive (326, 327). Similar as in the other β-oxidation mouse models, the demyelination is not recapitulated in *Abcd1*$^{-/-}$ mice, that rather represent the AMN phenotype, hampering the investigation of pathogenic mechanisms (324).

Some unexpected insights into childhood cerebral ALD (CCALD) were obtained by studying autopsy brain tissue. Altered expression of markers suggested that the blood brain barrier was compromised (328). On the other hand, advanced longitudinal brain perfusion studies in symptomatic and asymptomatic CCALD patients revealed alterations in microvascular physiology in the white matter that preceded white matter changes on MRI (329). By performing silencing in human brain microvascular endothelial cells *in vitro*, it was shown that the upregulation of adhesion molecules and suppression of tight junction proteins is a primary consequence of ABCD1 inactivation (328). Impairment in barrier properties was also seen after directed differentiation of iPSC derived from CCALD and control fibroblasts into brain microvascular endothelial cells (330).

The fact that demyelination can be halted with some delay after hematopoietic stem cell transplantation or *ex vivo* gene correction of autologous hematopoietic stem cells is a strong argument for immune cell involvement (101, 102, 331). It is assumed that the stem cells differentiate into microglia-like cells in the brain (332). This treatment does however not prevent the development of the myeloneuropathy. Although the precise role of microglia in the pathogenesis of CCALD is still obscure, it is remarkable that these cells disappear by programmed cell death from the perilesional white matter where myelin and oligodendrocytes are still largely intact (333). The few remaining microglia had lost the typical markers Tmem119 and P2ry12 suggesting that decay of microglia occurs early in the disease course and precedes the breakdown of myelin and loss of oligodendrocytes (333). Remarkably, microglia like cells of unknown origin reappear in the advanced gliotic scar region (333). The causal link with the only metabolic abnormality i.e. accumulation of saturated VLCFA was made by administration of LPC:C24:0 in the cortex, which resulted in microglia death (334). Further insights in the contribution of immune cells to the pathogenesis of AMN/CCALD is discussed in the section on the immune system.

The primary therapeutic option that is supposed to counteract the two phenotypes, AMN and CCALD, is to reduce saturated VLCFA levels. Historically, this was attempted by using Lorenzo's oil that contains erucic and oleic acid, which are mono-unsaturated VLCFA precursors, but this proved to be ineffective in patients (335). Recently, along the same rationale, saturated C16:0 and C18:0 were rerouted into mono-unsaturated fatty acids by inducing stearyl-CoA desaturase. This led to an increased ratio of mono-

unsaturated to saturated VLCFA, and to lower C26:0 levels in ABCD1 deficient fibroblasts, zebrafish and mice (336). Another avenue is the induction of ABCD2, the ABCD1 homolog that can also transfer C26:0 over the peroxisomal membrane but ABCD2 is expressed at low levels in many cell types (335). This could be achieved using the histone deacetylase inhibitor vorinostat, which enhanced peroxisomal ß-oxidation, reduced VLCFA accumulation, and reversed the pro-inflammatory skewing of X-ALD macrophages (337). In three advanced CCALD patients, compassionate vorinostat treatment improved the integrity of the BBB, but demyelination continued and patients had to be withdrawn from medication because of toxicity issues (337).

5.1.1.7. Myelopathy in X-ALD

Thanks to the *Abcd1*$^{-/y}$ mouse model, extensive investigations were performed on the pathogenesis of AMN, which were validated in patient samples (338). According to the current hypothesis, the axonal degeneration in the spinal cord is not a consequence of myelin loss. Rather, the accumulation of VLCFA causes oxidative stress, which impacts on mitochondrial respiration and biogenesis, and on protein quality control mechanisms including the proteasome and autophagy systems in neurons (338). Based on these insights in the pathogenic cascade, several clinical studies with compounds targeting ROS, mitochondrial biogenesis and autophagy were initiated (339). The potential involvement of immune cells is considered below.

5.1.1.8. Ether lipids and myelin

As myelin is strongly enriched in plasmalogens (315), it is not surprising that myelination deficits occur in RCDP patients and in the mouse models, but these are distinct from the ZSD pathology. Severely affected RCDP patients show both developmental and regressive myelin abnormalities on MRI situated in the supratentorial white matter and in the parietooccipital region (340).
Several mouse models with ether lipid synthesis defects show hypomyelination at a young age in different brain areas including the optic nerve, corpus callosum, cerebellum and spinal cord (Figure 9) (142, 314). This was accompanied by a 40% decrease in nerve conduction velocity of callosal myelinated fibers in *Gnpat*$^{-/-}$ mice (314). Subsequently, a late onset and slowly progressive demyelination accompanied with mild inflammation occurs (142), which was less drastic compared to the mice lacking PEX5 in the brain (320) and was for example not observed in the optic nerve, while overall axonal loss was minimal in the CNS. It was further shown that the differentiation of *Gnpat*$^{-/-}$ oligodendrocytes is

normal but that plasmalogens are crucial for oligodendrocytes to initiate the membrane wrapping process (142).

Several approaches to replenish ether lipid levels were examined. The ether lipid precursor 1-O-octadecyl-rac-glycerol (batylalcohol) could normalize plasmalogen levels in peripheral tissues but not in the CNS (341). In contrast, supplementation of *Gnpat*$^{-/-}$ mice with 1-O-tetradecyl glycerol, containing a medium chain fatty alcohol on sn-1, rescued plasmalogen levels in the brain as well as the myelination deficit (142).

5.1.2. The peripheral nervous system

5.1.2.1. Peripheral neuropathies in patients

Peripheral neuropathies are recurrent symptoms not only in patients with ZSD (32) but also in several single enzyme deficiency disorders. Similar to the CNS, peroxisomes are much more numerous in myelinating Schwann cells as compared to axons in peripheral nerves (342). They are mostly located in the uncompacted myelin close to the nodes of Ranvier.

In ZSD, sensorimotor axonal polyneuropathy is a late onset pathology that occurs in the adolescent – adult presentation of ZSD, often in combination with cerebellar ataxia (32, 253). Several peroxisomal metabolites might be involved in the pathogenesis. The inability to degrade phytanic acid by α-oxidation in Refsum disease also causes demyelinating polyneuropathy starting in adulthood (343). Phytanic acid levels are much higher in the peripheral compared to the central nervous system in these patients. Following dietary restrictions, plasma levels of phytanic acid can be lowered and the peripheral neuropathy can be halted and often reversed (343). ln line with the toxicity of increased levels of branched chain fatty acids for peripheral nerves, neuropathy also occurs in peroxisomal β-oxidation disorders in which the degradation of the branched chain fatty acid pristanic acid is impaired. These include MFP2/DBP, AMACR and SCPx deficiency. In all these diseases dietary restrictions are recommended to lower phytanic acid and pristanic acid levels, which were shown to reduce at least some of the clinical symptoms (343).

Peripheral neuropathy is a common feature and can be the first manifestation of AMN, indicating that elevated levels of saturated VLCFA not only affect the brain and spinal cord but also impair peripheral axons (323, 324). The impact of reduced plasmalogen levels on the PNS is less clear because neuropathies were only reported in a subset of RCDP patients (344).

5.1.2.2. Peripheral neuropathies in mouse models

Remarkably, *Pex10* knockout mice were identified in a mutagenesis screen based on locomotion defects in the prenatal stage (345). These mice exhibit several developmental defects of the PNS including a decreased number of Schwann cells that are misplaced along axons, axonal damage in sciatic nerves and motor neurons, and disorganization of the neuromuscular junction (NMJ). This may contribute to the motor impairment and weakness of ZSD mice but also of the patients. The depletion of plasmalogens likely plays a crucial role in this pathology as *Gnpat* knockout mice also show defects in Schwann cell differentiation, impairment of axonal sorting and deficits in myelination structure (141). The underlying signaling deficits were elucidated and consist of defective protein kinase B (AKT) phosphorylation resulting in overactivation of glycogen synthase kinase 3β (GSK3β) in *Gnpat*$^{-/-}$ nerves (141). Importantly, the Schwann cell defects could be rescued by treating mice with GSK3β inhibitors, without normalizing plasmalogen deficiency. With regard to the NMJ abnormalities in *Pex10* knockout mice (345), they seem to be only partially caused by plasmalogen deficiency because the phenotype of *Gnpat*$^{-/-}$ does not fully overlap (346). The increased branching of axons during development is a shared feature between the two mouse models. Whereas pre- and postsynaptic markers do not coincide in *Pex10*$^{-/-}$ mice, this aspect is normal in *Gnpat* knockouts but the endplate zone is broader. Functional electrophysiological tests are difficult to compare as they were performed at different ages. It is however important to note that the structural NMJ alterations in adult *Gnpat*$^{-/-}$ mice are accompanied by fewer spontaneous synaptic vesicle fusion events but normal end plate potentials after a presynaptic stimulus, indicative of normal transmission (346).

The selective loss of functional peroxisomes from Schwann cells (*Cnp-Pex5* mice) did not induce structural myelin deficits which is in sharp contrast with the demyelination in the brain of these mice (347). The cause of this discrepancy has remained unresolved. The preserved myelin structure in peripheral nerves of *Cnp-Pex5* mice also conflicts with the myelination abnormalities in *Pex10*$^{-/-}$ and in *Gnpat*$^{-/-}$ mice. This could however be related to the late inactivation of PEX5 in the conditional model and with compensatory mechanisms by surrounding cell types.

Despite intact myelin, the nerve conduction velocity of isolated sciatic nerves of *Cnp-Pex5* mice was diminished and compound action potentials were reduced *in vivo* although the number and size of the axons was unaltered (347). The proposed mechanism underlying the impaired conduction is that the inability to degrade VLCFA in peroxisomes secondarily causes lysosomal dysfunction in Schwann cells. Due to impaired turnover, gangliosides containing VLCFA accumulate in the juxtaparanodal membrane. In turn, this causes delocalization of Kv1.1 channels and their stabilizing proteins in the apposed axons from paranodal to internodal regions, perturbing current propagation. In agreement with this

hypothesis, mice with peroxisomal β-oxidation defects in Schwann cells (*Cnp-Mfp2/Dbp* mice) displayed the same pathology (347). This was also the case for *Abcd1*$^{-/-}$ mice albeit at later ages (347). These findings demonstrate that axons require intact peroxisomal lipid metabolism in the surrounding oligodendrocytes, although this is not related to the insulating function of myelin.

The destructive effect of branched chain fatty acids for peripheral nerves was confirmed in the Refsum mouse model by supplementing the diet with phytol, which increased phytanic acid levels in tissues (186). This elicited gait disturbances compatible with a neuropathy, which was confirmed by reduced motor nerve conduction velocity. This was not replicated in mice deficient in the second peroxisomal enzyme involved in α-oxidation, i.e. HACL1 (348), which may have to do with the existence of an alternative lyase localized in the ER (349).

5.2. Liver

5.2.1. Hepatocytes have a broad peroxisomal metabolic repertoire

Historically the liver has been the most studied organ with regard to the function of peroxisomes. Although many pathologies in ZSD patients are due to local peroxisome defects in diverse tissues, systemic defects that are the consequence of the impaired function of peroxisomes in hepatocytes may also contribute to a diseased tissue.

Hepatocytes contain the highest number of large-sized peroxisomes per cell in which all established peroxisomal metabolic pathways are active. In rodents, but not in humans, PPARα agonists induce peroxisome abundance in hepatocytes multifold (350). With regard to the other cell types of the liver, Kupffer cells, stellate cells and sinusoidal endothelial cells, not much is known about the expression and importance of peroxisomes.

Clinical features of severe ZS patients include hepatomegaly, and hepatic dysfunction as revealed by prolonged neonatal jaundice, coagulopathy, cholestasis and elevated transaminases (32, 253). In the milder ZSD patients, surviving into adulthood, liver pathology can be very diverse (351). It can go unnoticed because of normal transaminase levels up to liver failure as the cause of death. Different grades of fibrosis up to the stage of cirrhosis are common features (Figure 10). Other frequent pathologies are portal stenosis, inflammation and glycogenated nuclei, copper and iron storage. In a few patients premalignant dysplastic nodules and hepatocellular carcinoma were detected (351). Patients that showed peroxisomal mosaicism in the liver, in which a fraction of hepatocytes contained normal peroxisomes, had the mildest liver pathology. The fibrotic phenotype progresses in some patients. Conversely, there are also examples of patients who initially presented with strong liver involvement

with hepatomegaly and more than 10-fold increased liver parameters in the neonatal period, which subsequently spontaneously resolved after a few years (351, 352). Natural history studies in which metabolic parameters are associated with liver pathologies are badly needed.

At the ultrastructural level, besides the absence of catalase containing peroxisomes, angulate lysosomes containing trilammelar structures occur in Kuppfer cells, the liver macrophages. These can also be detected by light microscopy under polarized light as birefringent inclusions. In addition, lipid droplets accumulate in these cells that are however insoluble in acetone and n-hexane (353).

With regard to mechanisms underlying the liver pathology, evidence points to the dysfunction of the peroxisomal β-oxidation pathway. Liver fibrosis indeed also develops in patients with a genetic defect in *HSD17B4* encoding MFP2/DBP (105) and liver failure was the cause of death of the only known ABCD3 patient (118). These enzymes are involved in the degradation of pristanic acid and the bile acid intermediates D/THCA that all have 2-methyl branched-acyl-chains. Although branched chain fatty acids may cause hepatic pathologies when they accumulate in liver, the diet of peroxisomal patients is nowadays adapted to avoid exposure to these α- and β-oxidation substrates (292). Therefore, the most probable metabolites causing liver pathology are bile acid intermediates. This is also supported by increased transaminases in four recently identified ACOX2 patients, one of them developing liver fibrosis, but displaying normal pristanic/phytanic acid levels (63, 110, 111). In all these patients with peroxisomal β-oxidation deficiency the C27 bile acids di- and trihydroxycholestanoic acid, synthesized at the ER, accumulate because their chain shortening to the corresponding C24 bile acids cholic acid and chenodeoxycholic acid requires the active participation of the peroxisomal β-oxidation system as described above (III.2.1). Consequently, the mature C24 cholic and chenodeoxycholic bile acids are reduced, resulting in an increased C27/C24 bile acid ratio. In an *in vitro* study, C27 bile acid intermediates were shown to be more cytotoxic for a hepatocyte cell line than other bile acids (354). However, the fact that an increased C27/C24 bile acid ratio is not always associated with liver disease weakens this hypothesis. Indeed, out of 10 AMACR patients (355) only one developed liver fibrosis (112) although the C27/C24 ratio was elevated in all of them. Furthermore, whereas fibrosis develops in liver selective *Pex5* knockout mice (356), the liver histology of *Mfp2/Dbp* knockout mice is conspicuously normal (357) despite a similarly distorted C27/C24 bile acid ratio (358). Thus, the mechanisms underlying fibrosis and the precise toxicity of C27 bile acid intermediates for hepatocytes and other cell types need to be sorted out. Despite this uncertainty, bile acid therapy was advocated in patients with Zellweger spectrum disorders (359). The benefit is still disputed (360, 361) and although cholic acid

therapy may be harmless in most patients, serious adverse effects in patients with severe liver disease have been observed and need to be avoided (361).

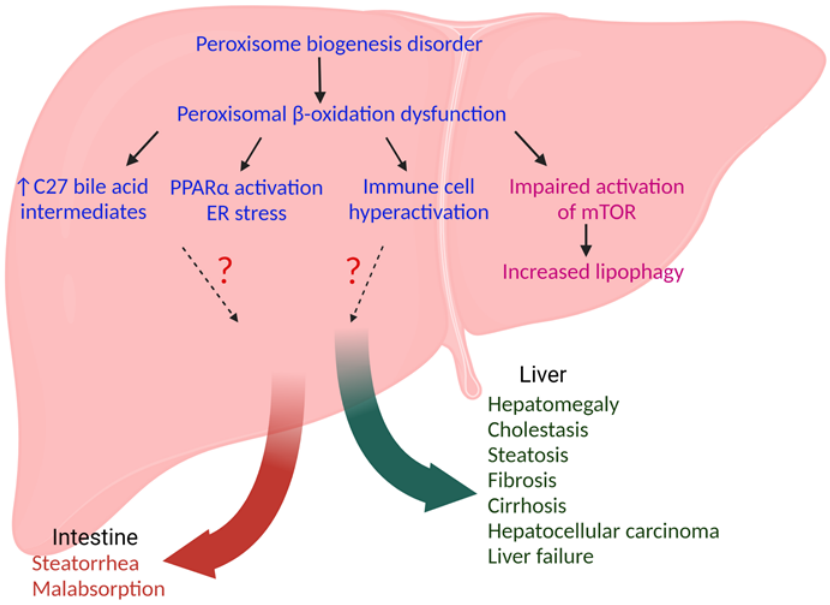


Figure 10. The liver is often affected in patients with peroxisome biogenesis dysfunction. *The liver pathology is extremely variable and strongly depends on the severity of the disease. The mechanistic underpinnings are still obscure but impaired β-oxidation plays a primary role because several but not all patients with β-oxidation deficiency also develop hepatic pathologies. A contributing factor may be the accumulation of immature C27 bile acids. According to findings in mouse models, PPARα activation and exacerbated immune cell activation are also involved. In disagreement with this model, hepatocyte selective Acox1 knockout mice were protected from hepatosteatosis induced by fasting or high fat diet through suppression of mTOR and enhanced lipophagy.*

5.2.2. The relation of peroxisomes with NAFLD

The potential involvement of peroxisomes in conditions similar to NAFLD has been the subject of recent investigations in mouse models. NAFLD is a spectrum of diseases starting with steatosis, evolving in steatohepatitis with inflammation and fibrosis, and eventually resulting in hepatocellular carcinoma. It is believed that both deregulation of lipid metabolism and inflammatory components play a role. First, global *Acox1* knockout mice spontaneously develop steatohepatitis and in a late stage hepatocellular carcinoma even when fed a regular diet (362). It was hypothesized that in *Acox1*$^{-/-}$ mice unmetabolized fatty acids activate PPARα (363) that in turn induce ER stress and the unfolded protein response (364). The same NAFLD phenotype was recapitulated in N-ethyl-N-nitrosourea (ENU) generated mice with an inactivating pathogenic missense variant in *Acox1* (365). These *Acox1* mutants displayed exaggerated

responses upon triggering the innate and adaptive immune system. Feeding these mice a brief obesogenic diet, exacerbated hepatocellular damage compared to wild type mice (366). Remarkably, multiple genes associated with hepatocellular carcinoma were already overexpressed after this short term in *Acox1* mutant livers. The contribution of hematopoietic cells to the hepatic disease was demonstrated in elegant bone marrow transfer experiments. ACOX1-deficient immune cells provoked hepatocellular damage in WT mice fed an obesogenic diet, albeit the severity was less pronounced than in whole body *Acox1* mutants. Likely, the normal hepatocytes in the hybrid model prevented strong lipid changes induced by the diet and thereby blunted the secondary inflammatory liver phenotype. Nevertheless, these data indicate that ACOX1 function in mice is crucial to prevent NAFLD development and progression through both metabolic and inflammatory actions.

Surprisingly, the outcome was completely opposite when mice with hepatocyte selective deletion of ACOX1 were exposed to HFD or subjected to starvation, as these mice were resistant to fat accumulation in the liver (367). This was unexpected given the fact that inhibition of mitochondrial oxidation under similar circumstances aggravated the lipid accumulation. The mechanism underlying this protection against hepatic steatosis was claimed to be mediated by reduced levels of cytosolic acetyl-CoA leading to reduced acetylation of RPTOR/RAPTOR, which impairs mTOR activity. This releases suppression of autophagy leading to enhanced degradation of lipid droplets by lipophagy (367). The mechanism behind the preferential use of acetyl-CoA derived from peroxisomal β-oxidation to acetylate RAPTOR and thereby activate mTORC1 requires further studies especially since peroxisomal β-oxidation contributes little to overall acetyl-CoA production compared to citrate and other sources. In older studies it was estimated that in standard conditions only 10 - 30 % of long chain fatty acids are degraded by peroxisomal β-oxidation in rat liver (368, 369). Such studies were however not performed in conditions of lipid overflow.

Other issues that need to be resolved include why the global *Acox1*$^{-/-}$ mice develop steatosis and steatohepatitis since absence of this enzyme causes resistance to lipid accumulation in hepatocytes (367), and thus the trigger for subsequent aberrant immune responses is abolished. The absence of a liver phenotype in *Mfp2/Dbp* knockout mice, that has a broader substrate specificity than ACOX1, also remains unexplained (357). Finally, it has to be investigated how these findings on liver pathology in mouse models can be translated to human disease.

5.3. The immune system

5.3.1. The innate immune system

No systematic study on the occurrence of peroxisomes or peroxisomal enzymes in the different cell types of the immune system was performed, but sporadic reports were comprehensively summarized recently (370). It is remarkable that the expression of the ABCD transporters, in particular ABCD1 and ABCD2 varies strongly between immune cell types (370). A pathognomonic feature in patients with PBD and with peroxisomal β-oxidation dysfunction is the occurrence of lipid-laden macrophages in different tissues (371, 372), likely due to their inability to degrade VLCFA.

As already mentioned in the section on AMN/CCALD, altered characteristics of inflammatory cells may be a vital part of the pathogenesis in these diseases. Monocytes from AMN patients, who did not have any signs of cerebral inflammation (373) are skewed in a pro-inflammatory state and cannot be converted to an anti-inflammatory phenotype. In line with this finding, compared with the other inflammatory demyelinating disease, multiple sclerosis, only few macrophages in CCALD brain lesions expressed anti-inflammatory markers, whereas in both diseases macrophages with pro-inflammatory markers were present (373). It was proposed that the inability of macrophages to convert into an anti-inflammatory repair state may underlie the fast progressive demyelination in CCALD, which contrasts with the relapsing-remitting course of multiple sclerosis (373).

In another study, spinal cord microglia of mice and humans with AMN were assessed (374). Although there were clear signs of microglial activation in *Abcd1*$^{-/-}$ spinal cord based on cell shape, pro-inflammatory markers were not increased. However, when cultured *in vitro*, microglia appeared to be primed as they reacted much stronger to a lipopolysaccharide challenge (374). Surprisingly, signaling molecules for phagocytosis (including MFGE8, Trem2) were upregulated both in spinal cord and *in vitro*. This occurred at an early stage, several months before synapse loss and axonal degeneration. Microglia in spinal cord of postmortem ALD patients displayed a similar pattern of increased activation and phagocytosis markers and without pro-inflammatory signs. This raised the question whether blocking phagocytosis may be a target to prevent neurodegeneration (374).

Besides ABCD1, several enzymes of the peroxisomal β-oxidation pathway were inactivated in innate immune cells *in vitro* and/or *in vivo*, which always resulted in phenotypic changes. Deletion of ACOX1 in BV-2 microglial cells upregulated pro-inflammatory markers as well as TREM2 (375). Inactivation of MFP2/DBP elicited a widespread and extensive neuroinflammation in the CNS that was limited to the gray matter (322, 376, 377). The microglia in these mice are chronically activated, highly proliferative, adopt a primed and mixed pro- and anti-inflammatory phenotype, and lose their typical homeostatic markers. Remarkably, the microglia remain in a reactive state without acquiring overt neurotoxic and phagocytic properties (376) which clearly differs from ABCD1 deleted microglia (374). Because

inflammation in the brain was much less pronounced in microglia-selective MFP2/DBP knockouts (378), this suggests that the interplay between peroxisomal β-oxidation deficient neurons and microglia, amplifies the neuroinflammatory cascade. It is at present not understood why the *Mfp2/Dbp*$^{-/-}$ mouse model does not mimic the human pathology in which rather the white matter is affected (104, 318).
In the periphery of MFP2/DBP knockout mice no signs of inflammation were discerned (376) and mice with myeloid-cell-specific deletion of MFP2/DBP mounted a normal response in the peritoneal cavity upon acute immune stimulation (379). Cultured MFP2/DBP deficient bone marrow derived macrophages, showed a mildly blunted response to a classical pro-inflammatory stimulus and normal anti-inflammatory polarization (379). Opposing data were obtained with RAW macrophages in which PEX14 or MFP2/DBP were inactivated as they produced increased pro-inflammatory markers upon lipopolysaccharide stimulation (380).
Summarizing, the metabolic alterations caused by impaired peroxisomal β-oxidation in innate immune cells affect the phenotype of these plastic cells but the outcome is determined by the specific enzymatic defect, the particular cell types tested and the experimental conditions. Metabolic factors that could play a role are anti-inflammatory lipid mediators that are derived from DHA, namely resolvins, protectins and maresins in addition to the accumulation of VLCFA that can affect membrane properties (370).
A serendipitous finding in mice in which fatty acid synthase (FAS) was acutely inactivated in adulthood under the control of a ubiquitously expressed promoter, uncovered a potential role of ether lipids in granulopoiesis (381). It was previously shown that ether lipids account for more than 50 % of the membrane glycerophospholipids in neutrophils (382). The inhibition of lipogenesis resulted in neutropenia without affecting the differentiation of granulocyte precursors (381). Lipidomic analysis revealed a mild reduction of plasmalogens in membranes of residual neutrophils. Mice with an induced inactivation of PexRAP, required for plasmalogen synthesis (see Figure 3), developed a similar phenotype with a near complete loss of neutrophils but with more pronounced depletion of ether lipids (381). To explain the relation between fatty acid and plasmalogen synthesis it was proposed that FAS associates with ABCD3 at the peroxisomal membrane (127) and delivers fatty acids required for plasmalogen synthesis (381).
The observations that *Gnpat*$^{-/-}$ mice that reach adulthood do not develop neutropenia and that RCDP patients have normal counts of neutrophils, cast doubt on the link between plasmalogens and neutrophil development (383). The discrepancy may be related to the chronic suppression of plasmalogen synthesis, which can induce compensatory mechanisms, versus the acute inactivation in

the FAS and PexRAP models (384). Additional questions that arise are why in FAS knockout mice, only choline ether lipids were reduced to a modest extent (30% less) whereas ethanolamine ether lipids were unaffected. Furthermore, the specificity for neutrophils is unclear as lymphocyte numbers were also reduced in the FAS and PexRAP models. To clarify these issues, it would be instructive to inactivate plasmalogen synthesizing enzymes in the granulocyte lineage both in a chronic and in an acute way.

5.3.2. The acquired immune system

The functional importance of peroxisomes in the acquired immune system has not been investigated in cells of human origin, but was recently established in mice (385). Unexpectedly, deletion of functional peroxisomes from T-cells, using *Cd4-Cre* and *floxed Pex5* mice, did not affect T-cell development nor responses to viral infections that require T cell activation. Furthermore, similar targeting of B-cells, only impacted on a subclass, known as innate-like B cells. Although they were formed normally, the homeostatic maintenance of these B1 and marginal zone B cells was impaired, which resulted in a defective antibody response to *Streptococcus pneumoniae*. In contrast, follicular B-cells deficient in PEX5 behaved normally with regard to development, maintenance and function (385).
Earlier, a role for plasmalogens in the functioning of natural killer cells was reported. Natural killer cells, which express a semi-invariant T cell antigen (iNTK) have an important immunoregulatory role. Upon activation, iNKT cells perform several activities such as secretion of cytokines, killing of target cells, and interactions with B cells and dendritic cells. Plasmalogens were identified as lipid antigens that drive the selection and maturation of iNKT cells in the thymus and their expansion in the periphery (386). The importance of plasmalogens as self antigens for iNTK was further demonstrated by examining *Gnpat*$^{-/-}$ mice in which fewer iNTK cells in the thymus and in peripheral organs were detected (386). Taken together, the necessity for peroxisomes varies considerably between the different lymphocyte subtypes.

5.4. Eye and ear

Vision and hearing problems are among the most common clinical symptoms that not only occur in Zellweger syndrome but also in mild PBD patients and in some of the single peroxisomal enzyme deficiencies (32, 104, 253). Because these can be the primary manifestations in the milder patients in combination with normal peroxisomal metabolic parameters, this has led to misdiagnosis such as Usher syndrome (258, 387, 388), which is the most common cause of inherited combined deaf-blindness. The multiple ocular pathologies of severe ZS patients including corneal opacification, cataract, glaucoma,

nystagmus, optic atrophy and retinopathy are indicative of the diverse functions of peroxisomes in the vision process (389).

5.4.1. Multiple ophthalmological pathologies

The crucial role of plasmalogens for the lens is supported by the clouding of the lens, known as cataract, in all patients with reduced levels of these ether lipids (390). It can be the primary manifestation of plasmalogen deficiency as recently exemplified in a family who upon genetic diagnosis proved to have a pathogenic variant in the exon encoding the long form of the import receptor PEX5 (276). As explained in the section on peroxisome biogenesis this protein domain is required for the interaction with PEX7, and thus for the import of PTS2-targeted AGPS, which is involved in peroxisomal ether lipid synthesis. Also, all mouse models with defects in the biosynthesis of plasmalogens display cataracts already at eye opening, regardless of having full or partial reductions in plasmalogens levels (391). Histological studies of the lens both in patients (271) and in GNPAT deficient mice (392) revealed that true lens fibers were not formed and that undifferentiated precursor cells lacking parallel orientation accumulated, pointing to a developmental defect. It should be noted that not only the deficiency of plasmalogens, but also the defects in peroxisomal β-oxidation induces cataracts in men (104) and in mice (393), but this occurs at later age.

In demyelinating peroxisomal diseases the optic tract is also affected, which is the cause of blindness for example in X-linked adrenoleukodystrophy (390). Glaucoma was reported in the neonatal and childhood presentation of ZSD but the pathogenesis was not studied yet. In view of the abundance of peroxisomes in retinal ganglion cells (394), which are located at the inner surface of the retina and which are frequently the cause of glaucoma, it can be speculated that dysfunction of peroxisomes in these cells may provoke pathology.

Currently, more information is being gathered on the role of peroxisomes in the other retinal layers, as recently reviewed (389). Peroxisomes are distributed in all retinal layers including photoreceptor inner segments, interneurons and in the retinal pigment epithelium. MFP2/DBP, the central enzyme of peroxisomal β-oxidation displays a similar pattern (394). However, there is clear cell type selectivity for other peroxisomal enzymes, indicative of differential peroxisomal functions. For example, catalase is enriched in the RPE, which may relate to its protective role to cope with the large exposure to oxygen in the choroid plexus (394).

5.4.2. The retina

ZSD patients often develop retinopathy with abnormal retinal pigmentation and impaired ERG responses (390). In a few old histopathological reports on severe Zellweger syndrome patients photoreceptor outer and inner segment degeneration, photoreceptor loss, bi-leaflet inclusions in the RPE, RPE atrophy and macrophage infiltration in the retina have been described (395, 396). Retinal defects are also common in mild to very mild ZSD patients often carrying the PEX1-G843D pathogenic variant (397). A mouse model with the analogous homozygous PEX1-G844D pathogenic variant developed impaired function of rods and cones, resulting in reduced ERGs and visual acuity but not in loss of photoreceptors (398). These mice were subsequently used as a preclinical model to test gene therapy with an adenoviral vector. In the initial studies, subretinal injection of AAV8.CMV.HsPEX1.HA improved the cone-mediated photopic ERGs and to a lesser extent the rod-mediated scotopic ERGs, but retinal function remained far below wild type levels (less than 33%) and visual acuity was not enhanced (399). Underdosing of the AAV vector, limited targeting to several cell types in the retina and competition with the endogenous mutant protein for integration in the PEX1-PEX6 exportomer complex may underly the low efficacy of this gene augmentation trial. The latter mechanism may be less prominent in humans because the mutant PEX1-G843D was shown to be less stable in human versus rodent cells (399). In ongoing studies with an optimized viral vector, the efficiency of the gene transfer and the rescue of visual function was much more efficient in the mutant *Pex1* mice (C. Argyriou, personal communication).

Specific failure of α-oxidation as occurs in Refsum disease patients is usually diagnosed because of night blindness (343). The inability to degrade the branched chain fatty acid phytanic acid is the causative factor as the retinal decay can be halted by restricting the intake of phytanic acid from the diet. Increased levels of phytanic acid were proposed to impede the regeneration of 9-cis retinal, to distort membranes or to impair mitochondrial functioning but this still needs to be addressed (343). Surprisingly, the retina of *Phyh* knockout mice supplemented with phytol, did not show obvious structural abnormalities but retinal function was not investigated (186).

Several patients with a defect in an enzyme of the peroxisomal β-oxidation pathway, develop retinal pathologies that were not always accompanied with functional visual deficits (400). Besides the further breakdown of the branched chain fatty acid pristanic acid, peroxisomal β-oxidation plays a crucial role in the metabolism of PUFA and VLC-PUFA that are abundantly present in the discs of photoreceptor outer segments. This also includes the acquisition of DHA, but it is unclear whether the peroxisomal β-oxidation step in the Sprecher shunt takes place locally in the retina or in the liver. In addition, the breakdown of these VLCFA likely depends on peroxisomal β-oxidation (47). Inactivation of the central β-

oxidation enzyme MFP2/DBP in mice elicits a complex retinal phenotype compatible with both developmental and degenerative anomalies and affecting several retinal cell types (401). Already at 2 weeks, the time of eye opening, the outer segments are shortened, which is followed by apoptotic cell death of photoreceptors, both rods and cones. Also the RPE is affected with accumulation of lipid droplets and loss of visual cycle genes and of its typical hexagonal pattern. The protrusion of RPE cells in the photoreceptor layer is analogous to the description of RPE in Zellweger syndrome patients (395, 396) and points to a dedifferentiation process. In the lipidome of the retina a severe shortage of DHA containing phospholipids and an increase of phospholipids containing ultra long chain PUFA (> C32) were prominent findings (401). In view of the tight interdependence of photoreceptors and RPE, the cause - consequence relationship needed to be elucidated, as well as the role of peroxisomal β-oxidation in other retinal cell types. Quite surprisingly, selective deletion of MFP2/DBP from photoreceptors and bipolar cells, the latter are interneurons that transmit signals from photoreceptors to ganglion cells, provoked a late onset and mild retinal phenotype (402). Mice with complete inactivation of peroxisomal function (through loss of PEX5) in the same cells developed the same phenotype, with preserved phototransduction but with impaired signaling between photoreceptors and bipolar cells and bipolar cell degeneration (402). This was unexpected given the abundance of peroxisomes in the inner segments of photoreceptors (394). On the contrary, ongoing studies in our lab indicate that peroxisomal β-oxidation plays a crucial cell autonomous role in RPE cells (M.B. unpublished). Additional support for the essential role of peroxisomal β-oxidation in the retina is that loss of ACBD5 in men causes cone-rod dystrophy (117, 400). This was associated with increased levels of VLCFA in plasma (76) but in fibroblasts also phospholipid species containing VLC-PUFA were detected (117). It was speculated that ACBD5 is required for the retinal homeostasis of the VLC-PUFA (117). This is plausible given the fact that VLC-PUFA accumulate in the cerebellum of *Acbd5*$^{-/-}$ mice, as mentioned above. Notably, the retina of ALD patients is not affected, indicating that the accumulation of saturated VLCFA is not the cause of retinal degeneration (390).

5.4.3. Hearing

Sensory deafness is also associated with both ZSD and peroxisomal β-oxidation deficiencies, but the pathological mechanisms have not been investigated. In a recently identified mild *PEX26* patient, nonsyndromic sensorineural hearing loss was the single pathological feature (403). More information was recently gathered on the potential involvement of peroxisomes in noise induced hearing loss. Noise overexposure induces a fast degradation of peroxisomes by pexophagy in auditory hair cells which is

followed by proliferation of peroxisomes, supposedly to cope with the oxidative stress that is generated by loud noise (404). The protein Pejvakin that was shown to localize in peroxisomes (405), presumably in the membrane, is thought to play a crucial role in these processes but its apparent contradictory actions in both pexophagy and in peroxisome proliferation remain to be clarified. Furthermore, the subcellular localization is still contested as other researchers could not colocalize Pejvakin with ABCD3, and rather found a cytosolic localization in the rootlets at the base of the stereocilia in hair cells (406).

5.5. Heart

In adult mouse heart, peroxisomes are differentially distributed between the 4 chambers with the left ventricle being most enriched (407). Several enzymes including catalase, peroxisomal β-oxidation enzymes and ether lipid synthesis are also more abundant in the left ventricle but other peroxisomal proteins are more evenly expressed.

Similar to the brain, cardiac tissue is markedly enriched in plasmalogens (271). In RCDP patients with severe deficiency of plasmalogens, a high incidence (> 50%) of congenital cardiac defects is observed (151). This includes atrial and ventricular septal defects, and tetralogy of Fallot. Mitral valve prolapse also occurs and it is not clear yet whether this is a developmental or a degenerative feature. These structural defects were associated with electrocardiogram (ECG) and haemodynamic abnormalities. In an RCDP mouse model, reduced cardiac conduction velocity was observed in adulthood (408). This could be rescued by supplementing a plasmalogen precursor likely reversing the abnormal biophysical parameters of membranes and associated dysfunction of ion channels and/or connexins.

Mitochondrial fatty acid oxidation is well known to be the primary energy source of cardiomyocytes. Unique fatty acid oxidation studies in the intact heart were performed (41). Similar to liver, these studies revealed that C22:0 and dicarboxy-C12:0 are only degraded by peroxisomal β-oxidation, whereas oleic acid is primarily and short chain fatty acids are solely metabolized in mitochondria. Intriguingly, in contrast to the liver, the formed acetyl-CoA was not hydrolyzed to acetate but incorporated in malonyl-CoA (35). Whether this pathway is essential for the functioning of the heart was thus far not investigated. It is interesting to note that the content of the peroxisomal β-oxidation enzyme, ACOX1 was only 1% compared to liver.

In adult Refsum disease, the inability to metabolize phytanic acid has an important impact on the heart (407). If the dietary intake of phytanic acid is not reduced, cardiomyopathy often gives rise to heart failure. Sudden death can occur after phytanic acid release from adipose stores in fasting conditions.

Little is known on heart pathology in PBD patients, although in severe cases the depletion of plasmalogens can be expected to give rise to similar anomalies as in RCDP. The early postnatal death of peroxin knockout mice did not allow to assess heart function, but in newborn *Pex5*$^{-/-}$ mice abnormalities in mitochondrial structure and function were observed in cardiomyocytes (409). This was, however, not recapitulated in mice with selective deletion of PEX5 from striated muscle (*Mck-Pex5*) (358), indicating that the early defects had a non- cell autonomous cause. Investigations on cardiac function in the *Mck-Pex5* mice are lacking. Overall, peroxisomal metabolism is essential for the formation and functioning of the heart but details on the mechanisms are unresolved.

5.6. Lung

Despite the fact that pulmonary hypoplasia is a constant feature in Zellweger syndrome patients (372) this was not further investigated until now. In an extensive study on the distribution of peroxisomes in the human and mouse lung, peroxisomes were found to be present in all cell types of the lung and of the bronchial epithelium but with a striking difference in abundance, enzyme content and shape in the different cell types (410). In both species, they are most enriched in AECII cells, in Clara cells and in alveolar macrophages, whereas they are sparse in AECI cells. The anti-oxidant enzyme catalase was also highly expressed in AECII, Clara cells and macrophages but was hardly detectable in AECI cells. In the ciliated AECII cells peroxisomes often appear elongated or tubular (410).

5.7. Kidney

Besides hepatocytes, proximal kidney tubules contain numerous full-sized peroxisomes although PEX14 positive peroxisomes are also abundant in other cell types of the nephron (411).

Kidney pathology was recognized early-on in Zellweger syndrome patients as reflected in the original name cerebro-hepato-renal syndrome. Renal micro- to macrocysts of glomerular or tubular origin occur in almost all severe ZS patients at birth (372). These cysts were not reported in milder phenotypes but they do occur in MFP2/DBP patients, albeit at lower frequency (33%) (104).

A mouse model with selective depletion of functional peroxisomes from renal tubular cells was recently phenotyped (412). A number of adaptive changes at the transcript level were noticed for example of several transporters that are expressed in the nephron. In addition, transcriptome and metabolome alterations pointed to reprogramming of metabolic pathways related to lipid metabolism and redox homeostasis. Given the abundance of peroxisomes in the renal tubule, it was quite surprising that no functional kidney deficits developed under basal conditions. This led to the conclusion that peroxisomes

are dispensable from the renal tubule and that the renal pathology in ZS patients has an extrarenal origin (412).

Also, in Pex11α knockout mice the kidney phenotype was investigated in more detail (413). Although no overt pathology was reported in basal conditions, when challenging the mice with either deoxycorticosterone acetate salt or with fatty acid-bound albumin, two models in which proximal tubule cells are overloaded with fatty acids, *Pex11α*$^{-/-}$ mice exhibited larger interstitial lesions than wild type mice (413).

Another cause of kidney malfunction in relation to peroxisomal disorders is the deposition of calcium oxalate crystals as a consequence of pathogenic variants in the gene encoding peroxisomal alanine:glyoxylate aminotransferase (414). The inability to convert glyoxylate into glycine in hepatocytes leads to the formation of oxalate and massive hyperoxaluria (414). This also occurs when the enzyme is mislocalized to the cytosol in PBD.

5.8. Bone

Developmental skeletal pathologies are a constant feature of severe RCDP and ZSD patients (272, 391). This includes shortening of the proximal limbs, premature calcifications in the knee and hip, and abnormalities in mineralization of cartilage, resulting in severe growth retardation. In patients with milder disease presentations containing high residual plasmalogen levels, the rhizomelia does not occur, strongly indicating that the lack of ether lipids is the causative factor. Still, in patients with intermediate to mild ZSD, bone density scans revealed poor bone mineralization, leading to fractures (415).

The importance of peroxisomes for ossification is supported by the increased numerical abundance in hypertrophic chrondrocytes, osteoblasts and osteoclasts compared to precursor cells and mature osteocytes (416). This applies both to areas with endochondral (e.g. in long bones) and intramembranous (e.g. in calvaria) ossification. In mouse models of RCDP, rhizomelia was not observed but endochondral ossification defects were shown in newborn pups (417).

Several issues are unexplained including the selective shortening of the proximal limbs in humans, and the discrepancies between mice and men.

5.9. Teeth

Dentition abnormalities form together with sensorineural hearing loss and nail abnormalities the hallmark of a condition that was first designated as Heimler disease and was subsequently genetically

identified as being caused by pathogenic variants in *PEX1, PEX6 or PEX26* (254, 418, 419). The pathology may also encompass retinal dystrophy. This is the mildest end of the ZSD spectrum in which the typical metabolic peroxisomal abnormalities are often not apparent. Dental anomalies include amelogenesis imperfecta, i.e. deficient mineralization of enamel, failure of tooth eruption and displacement of teeth. The impaired amelogenesis is more prominent in the secondary dentition and it is rather difficult to recognise in the primary dentition (258). Dental problems also occur in teenage or adult ZSD patients who present with additional symptoms such as mental retardation.

Peroxisomes are already present in the early stages of dental development and they increase in abundance during the differentiation of ameloblasts and odontoblasts (420). The localization in these cells near the mineralized areas of teeth suggests that peroxisomes may play a role in this process. Interestingly, the peroxisomal enzyme content appeared to change during the differentiation process with an upregulation of lipid metabolizing enzymes and a down regulation of catalase in mature ameloblasts (411). The latter may explain the susceptibility to oxidative stress of these cells but the precise importance of peroxisomes for tooth development remains to be settled.

Dental problems have not been reported in any of the mouse models, but no dedicated studies were performed yet.

5.10. Intestine

In the intestine, peroxisomes are most abundant in epithelial cells throughout the tract where they reside at the apical side of enterocytes (421, 422). However, the distribution along the longitudinal axis varies pointing to differential peroxisomal functions in the different intestinal segments. While some proteins show constant expression from proximal jejunum to large intestine, other enzymes are more enriched in the jejunum (catalase and some β-oxidation enzymes) whereas plasmalogen synthesizing enzymes occur more abundantly in the large intestine (421). This is in accordance with high plasmalogen levels in the lower end of the tract. With regard to the crypt-villus axis, the number of peroxisomes increases as intestinal epithelial cells differentiate (423). Whereas catalase activity was higher in the crypt cells than in the mature enterocytes, peroxisomal oxidases, i.e. fatty acyl CoA oxidase, D-amino acid oxidase and polyamine oxidase increased during differentiation.

It is plausible that the peroxisomes play a role in the processing of dietary lipids that are taken up in gut epithelial cells. In addition, in view of the exposure of enterocytes to metabolites generated by the intestinal flora, this might be a hitherto unrecognized novel role of peroxisomes. In this respect it is not surprising that the volume that they take up in enterocytes is comparable to hepatocytes (421).

However, peroxisomal β-oxidation activity is several fold lower in intestinal compared to liver homogenates based on immunoblotting and enzyme activity measurements (421). Peroxisomes may also play a role in common diseases of the intestine. Indeed, they are markedly upregulated in the injured crypts of IBD patients (424).

5.11. Adipose

To our knowledge there is no evidence for disturbances in white or brown adipose tissue homeostasis in patients with peroxisomal disorders. Feeding difficulties and early death in the most severe cases may obscure a potential role of peroxisomes in these tissues. Yet, *in vitro* and mouse model studies indicate that peroxisomes do play a crucial role in adipocytes.

The differentiation of 3T3-L1 preadipocytes into adipocytes is accompanied with an increase in peroxisome abundance (425, 426). This was more than a mere association as inhibiting peroxisome biogenesis by silencing *Pex16* suppressed adipocyte differentiation, triglyceride storage and adipose specific gene expression (425). Blocking catalase, either genetically or chemically, during the differentiation process also prevented lipid accumulation, suggesting that the peroxisomal redox balance is involved in adipogenesis (426).

The lack of plasmalogens is an additional factor hampering adipocyte maturation as illustrated by the extreme reduction of all white adipose depots (epididymal, inguinal, retroperitoneal and subscapular) in *Pex7* knockout mice (341). Although the size of dorsal brown fat pads was normal, their adipocytes contained unusually small lipid droplets in the cytosol similar to adipocytes in WAT. Importantly, lipid droplet size could be restored by supplementing *Pex7* knockout mice with the ether lipid precursor 1-O-octadecyl-rac-glycerol, which replenished plasmalogen levels (341).

Cold exposure induces peroxisome biogenesis in brown and beige adipose tissue in mice (148). It was further demonstrated that this is necessary for thermogenesis in these tissues. Absence of PEX16 in adipocytes indeed impaired cold tolerance and energy expenditure. This was mediated by a block in cold-induced mitochondrial fission also impacting mitochondrial respiration. Whereas silencing of the β-oxidation enzyme ACOX1, did not affect adipogenesis, suppression of GNPAT, the ether lipid synthesis enzyme, mimicked the effects of the overall loss of peroxisome function in *Pex16* knockdowns (148). The assumption that reduced levels of plasmalogens in mitochondria underlie the impaired mitochondrial dynamics, was substantiated by the finding that supplementing *adiponectin-Pex16* mice with plasmalogen precursors, rescued mitochondrial numbers and thermogenesis.

Besides their function in adipose development and lipid storage, it was recently shown that peroxisomes are critical for lipolysis. In fasting conditions, physical contacts between LDs and peroxisomes increase both in intestinal cells of *C. elegans* of live worms and in white adipocytes of mice (427). This promotes the translocation of adipose triglyceride lipase (ATGL) onto LDs in a PEX5 dependent way - although ATGL does not contain a C-terminal PTS1 sequence- and the induction of lipolysis. This was confirmed in adipocyte specific-*Pex5* knockout mice, in which fasting did not induce the transfer of ATGL onto LDs and lipolysis was impaired. Although more detailed investigations are needed, these findings exemplify the pivotal interorganellar communications of peroxisomes with mitochondria and lipid droplets to support adipocyte functioning.

5.12. Pancreas

Except for a few reports mentioning fibrosis of the pancreas and islet cell hyperplasia in ZSD, not much information is available on the importance of peroxisomes in the exocrine and endocrine pancreas. Pex14 immunoreactivity is much higher in the endocrine compared to the exocrine pancreas, suggesting a specific role of peroxisomes in hormone secreting cells (428). It was already noted several decades ago that catalase belongs to a group of 'forbidden' proteins of which the gene expression is strongly suppressed in islet α- and β-cells (429). The importance of low catalase levels remains obscure as overexpression of catalase in either the cytosol or mitochondria did not provoke a pathological phenotype in mice (430). However, reconstitution of catalase in peroxisomes was not attempted. It was further postulated that the well described lipotoxicity of fatty acids for β-cells is due to hydrogen peroxide generated by peroxisomal β-oxidation of fatty acids (431). A mouse model with tamoxifen-induced deletion of PEX5 in pancreatic β-cells in early adulthood, displayed glucose intolerance (428). This was accompanied with reduced insulin secretion and reduced total pancreatic insulin content, decreased density of mature insulin granules, vacuolization of β-cells and signs of apoptotic cell death. These data clearly demonstrate that complete absence of peroxisomal function negatively impacts β-cell structure and function in mice (428).

5.13. Reproductive organs

5.13.1. Diverse functions of peroxisomes in the testis

There are two cell types in the germinal epithelium of the male reproductive system. The large and highly polarized Sertoli cells function as supportive cells for the developing sperm that undergo meiosis and differentiation into mature spermatozoa. In the human and mouse testis, peroxisomes were

detected in all cell types, except in mature spermatozoa, with a marked variation in abundance and enzyme content (432, 433). The testosterone producing Leydig cells, situated between the tubuli seminipheri, are strongly enriched in catalase. Cells of the spermatogenic lineage express both the enzymes of the peroxisomal β-oxidation and of the ether lipid synthesis pathway (432). The differential distribution of peroxisomal lipid transporters in Sertoli cells (ABCD1 and ABCD3) and Leydig cells (ABCD2) suggests that peroxisomal metabolism plays specific roles in certain cell types. During the end stage of spermatogenesis, peroxisomes are clustered and subsequently shed into residual bodies after release of the mature spermatozoa (432).

The crucial role of peroxisomes in different testicular cell types for male fertility was demonstrated by investigating several knockout mouse models. Selective deletion of peroxisomal function in premeiotic germ cells (*Stra8- Pex13* knockout) caused an interruption of differentiation whereby instead of single round spermatids multinucleated giant cells (MNCs) were formed (434). As a consequence, the testis was devoid of mature spermatozoa. In addition, anomalies in the tight junctions that form the blood-testis barrier between Sertoli cells were found. Marked lipid and transcriptome changes were detected but the vast difference in cellular content between wild type and mutant testis did not allow to draw a causal relationship (434).

5.13.2. Multiple peroxisomal metabolic paths are needed in the mouse testis

Most likely, the impaired synthesis of ether lipids is an important contributing factor to the infertility of *Stra8-Pex13* mutant mice as the disruption of ether lipid synthesis in *Gnpat*$^{-/-}$ mice led to an analogous phenotype (392). The spermatogenic cycle was arrested between the late pachytene and the spermatid stage with the formation of multinucleated giant cells. In the supportive Sertoli cells, neutral lipids accumulated.

The dependence of spermatogenesis on ether lipids is not surprising given the fact that two thirds of the ethanolamine and choline glycerophospholipids in germ cell membranes contain ether bonds on sn-1, either in the form of an alkyl or alkenyl moiety, as extensively discussed by Gorgas et al. (435). This includes seminolipid that is highly specific for germ cells, and consists of 1-O-alkyl-2-O-acyl-3-O-β-D-(3'-sulfo)galactosyl-sn-glycerol (SGalAAG) inserted into the outer leaflet of the sperm plasma membrane. These ether lipids might be crucial for the remodeling of the plasma membrane during spermatogenesis as well as for the integrity and maintenance of the clonal spermatid syncytium.

Remarkably, also peroxisomal β-oxidation was shown to be essential for germ cell maturation. *Mfp2/Dbp* knockout mice produced few mature spermatozoa in early adulthood, but subsequently

spermatogenesis was interrupted (393). Both pre- and postmeiotic germ cells disappeared from the germinal epithelium leaving a Sertoli-only phenotype of the tubuli seminipheri and complete testicular atrophy by the age of 4 months. Already at the age of 10 days the Sertoli cells accumulate cholesterylesters and triglycerides in huge lipid droplets. The assumption that Sertoli cells play a major role in the testicular degeneration in *Mfp2/Dbp* knockouts was supported by the phenotype of a Sertoli-selective *Pex5* knockout model (393). These mice developed similar lipid droplets in Sertoli cells and a progressive loss of mature spermatogonia albeit with a slightly later onset. Germ cells and Sertoli cells are strongly interdependent, which bears many similarities with the interrelationship of photoreceptors and the RPE, as already mentioned. Furthermore, similar to photoreceptors, germ cells are enriched in poly-unsaturated fatty acids (DHA in human and C22:5ω-6 (DPA) in rodents) and the VLC-PUFA derivatives that are often hydroxylated (436). Both Sertoli cells and the spermatogenic lineage express the enzymes needed for the synthesis of C22 PUFA, including the peroxisomal β-oxidation enzymes. C22:5ω-6 was depleted in *Mfp2/Dbp*$^{-/-}$ testis but this was analysed in degenerated testis and can be the consequence of the disappearance of germ cells (393). Also the origin of the lipid droplets in Sertoli cells remains unexplained. Because the lipids already accumulate in the prepubertal age, this cannot be due to the resorption of residual bodies by Sertoli cells which only occurs from the end of the first spermatogenic cycle on.

Also male *Acox1* knockout mice are infertile (362) but in contrast to *Mfp2/Dbp*$^{-/-}$ mice no abnormalities of Sertoli cells were reported. Rather, the hypospermatogenesis was accompanied by a reduction in the Leydig cell populations. The remarkable differences in pathology seen in the testis of *Acox1* and *Mfp2/Dbp* knockout mice may be due to the differences in the substrate specificity of the two enzymes. In human peroxisomal β-oxidation disorders the impact on male fertility is poorly documented. In AMN patients, testicular lesions do occur including birefringent inclusions in Leydig cells, although the consequences for male fertility are debated as they mostly develop normal sexual characteristics (437, 438).

5.13.3. Peroxisomes in the female reproductive tract

Less information is available on the role of peroxisomes in the female reproductive tract. PEX14 immunoreactive peroxisomes are present in all ovarian cells including thecal cells and granulosa cells and to an increasing extent in maturing oocytes in developing follicles (411). It is remarkable that both in male and female reproductive organs, catalase is expressed at extremely high levels in endocrine cells, i.e. in Leydig cells and in theca and granulosa cells (411).

Female *Gnpat*$^{-/}$ and *Mfp2/Dbp*$^{-/-}$ mice are subfertile whereas *Acox1*$^{-/-}$ mice are infertile (362, 392, 393). Further evidence that peroxisomes play a role in female reproduction comes from the finding that pathogenic variants in *HSD17B4* and in *PEX6* are two of the six currently identified genetic causes of Perrault Syndrome (439, 440), which is characterized by premature ovarian insufficiency in addition to sensorineural hearing loss.

5.14. Adrenal gland

Peroxisomes are not evenly distributed in the three layers of the adrenal cortex, being clearly more abundant in the zona glomerulosa and reticularis than in the zona fasciculata (411). Adrenocortical insufficiency is one of the major hallmarks of X-ALD and occurs in 80% of male patients but only rarely in females (98). In some patients, denoted as Addison-only, adrenal failure is the only clinical manifestation. The zona fasciculata and zona reticularis where glucocorticoids are produced are particulary affected whereas the zona glomerulosa that is involved in mineralocorticoid production is often largely preserved (98, 438), thus not coinciding with the abundance of peroxisomes. Saturated VLCFA accumulate in the adrenal cortex that are predominantly esterified with cholesterol (441). This corresponds with the occurrence of lamellae and needle like trilamellar inclusions in the cytosol, similar to those in Leydig cells and in macrophages. Oxidative stress and mild oxidative damage were observed (441). Adrenal cortex atrophy is also prevalent in other peroxisomal diseases in which β-oxidation is defective. This includes MFP2/DBP- deficient patients (104), and severe and mild ZSD patients (442). However, the pathophysiological role of VLCFA is unclear. In X-ALD patients the plasma levels of VLCFA did not correlate with the occurrence of adrenal dysfunction, neither with the age of onset (438). In contrast, in a small cohort of ZSD patients, those who developed adrenal dysfunction had higher C26:0 levels than those who were asymptomatic (442).

The study of the *Abcd1*$^{-/-}$ mouse models did not shed further light on the pathological mechanisms in the adrenal cortex (443). VLCFA accumulated in the adrenals, which was amplified in double *Abcd1/Abcd2*$^{-/-}$ mice, but this did not result in adrenal insufficiency. Lamellae and lamellar-lipid profiles similar to those found in human patients occurred in cortical cells, but this did not progress with age (441).

In conjunction with the anomalies in reproductive organs, it is remarkable that steroid producing cells are particularly vulnerable to the accumulation of VLCFA. However, the lack of a clear correlation with VLCFA levels, and the differences between mice and men render the link at the level of adrenal pathologies enigmatic, similar to the cerebral pathologies.

Taken together, the investigations on patients and on mouse models with peroxisomal disorders have unveiled that peroxisomes in mammals are enormously flexible and can play a diversity of functions in cells, tissues and organs. Yet, many of the mechanistic details remain to be resolved in order to understand the pathologies that arise when peroxisomes are dysfunctional and to expand therapeutic options.

6. PEROXISOMES IN VIRAL INFECTIONS

Viruses are completely dependent on their host cells' machinery to replicate and propagate. Upon infection, the viral genome is replicated, transcribed and translated into viral proteins, which then assemble, together with the genome and other viral components, into new virus particles. Some viruses also acquire a lipidic envelope, derived from the host cell organelles' membranes or the plasma membrane. Throughout these different phases, viruses extensively modulate host cell metabolism to their own benefit and, depending on the virus, specific host cell organelles are exploited to favor the proper formation of new virus particles and the spread of infection to the neighboring cells. Exciting recent results have demonstrated that peroxisomes play important roles in viral infections and that different animal and plant viruses modulate peroxisome biogenesis and metabolism to favor virus particle formation and the smooth progression of their life cycle.

Peroxisomes have been established as key signaling platforms in cellular antiviral immunity. In response to viral infections, host cells activate a robust network of signaling events that lead to the expression of interferons (IFNs), IFN-stimulated genes (ISGs) and pro-inflammatory cytokines, which suppress viral replication and restrict infection. This antiviral response is triggered through the recognition of different pathogen-associated molecular patterns (PAMPs) by a set of host membrane-bound or cytoplasmic pattern-recognition receptors (PRRs) (444). Depending on the virus and the recognized PAMP, infection may trigger Toll-like receptor (TLR) signaling, cytosolic DNA sensors signaling and/or cytosolic RNA sensors signaling (444). The role of peroxisomes within the cellular antiviral response emerged with the discovery that MAVS (mitochondrial antiviral signaling protein, also known as IPS-1, Cardif, and VISA) localizes at this organelle (445), in addition to its localization at mitochondria (446-449) and mitochondria-associated membranes (MAMs) (450). MAVS is activated by the interaction with the retinoic-inducible gene-I (RIG-I)-like receptors (RLRs), such as RIG-I and MDA-5, which travel to these organelles upon recognition of viral RNA at the cytoplasm of the infected cell (Figure 11). Activated MAVS trigger a series of further signaling events that culminate in the production of IFNs and ISGs (451). Although the mitochondrial pathway has been extensively studied, not much is known concerning the

downstream signaling events initiated by peroxisomal MAVS. However, key differences regarding kinetics and end products have been identified between these two pathways. Dixit et al have shown that, while peroxisomal MAVS activates a fast but short-termed type I IFN-independent expression of ISGs, the mitochondrial MAVS triggers a delayed but sustained ISG production that is dependent on type I IFNs (452). Peroxisomes have further been identified as the key signaling platform from which the expression of type III IFNs, a class of IFNs with tissue-specific roles in antiviral immunity, is stimulated (453). Another study has reported the expression of both type I and III IFNs upon peroxisomal MAVS signaling (454). Although further studies should be performed to uncover the basis for such different peroxisome-dependent IFN responses, these may reflect adaptations to distinct infectious contexts, cells and/or tissues. Additional studies are also necessary to define the common and distinct pathways activated by peroxisomal and mitochondrial MAVS, as well as the specific organelle moieties that dictate the observed differences.

Viruses have a tremendous capacity to mutate and adapt to cellular responses. As such, it is not surprising that several viruses have evolved specific strategies to subvert the peroxisome-dependent antiviral response. Some of these involve the targeting of viral proteins to the peroxisomal membrane and the consequent interference with downstream signaling events, while others involve the specific modulation of peroxisome biogenesis and/metabolism. Although some of these strategies involve viral proteins that affect both peroxisomal and mitochondrial MAVS signaling, the specific mechanisms involved are not always similar for the two organelles. This section describes and discusses the current knowledge on how mammalian viruses interact with peroxisomes and interfere not only with the antiviral signaling pathways (Figure 11) but also with peroxisome biogenesis (Figure 12) and peroxisomal metabolism (Figure 13).

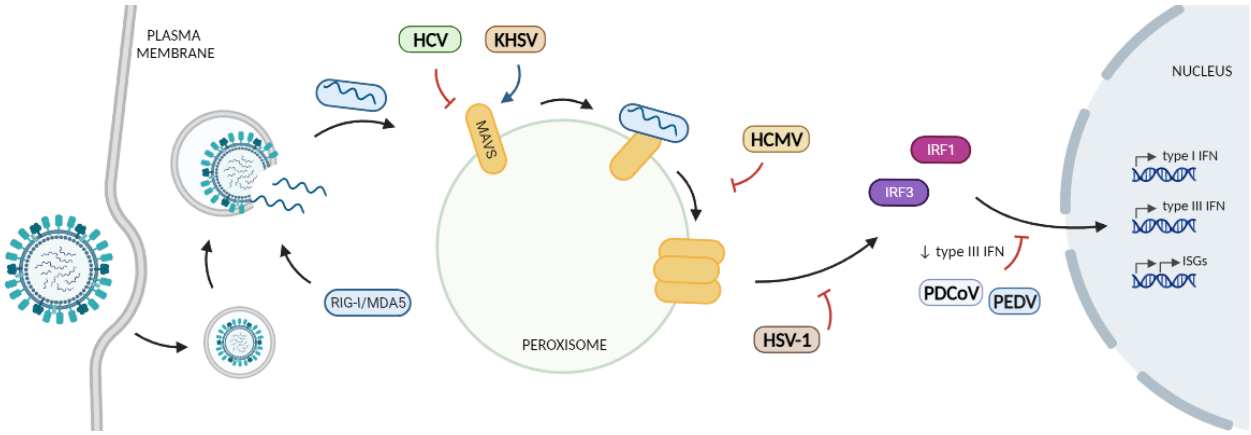


Figure 11. Schematic representation of the peroxisome-dependent antiviral signaling and how this pathway is subverted by different viruses.
Upon infection, viral RNA is released into the cytosol and recognized by the retinoic acid-inducible gene I (RIG-I)-like receptors, such as RIG-I or melanoma differentiation-associated protein 5 (MDA5). These proteins interact with the mitochondrial antiviral signaling protein (MAVS) at mitochondria and peroxisomes, which then oligomerize and trigger a signaling cascade that activates the transcription factors interferon regulatory factors (IRF) 1 and 3. Translocation of IRF1 and IRF3 to the nucleus promotes the expression of type I and type III interferons (IFNs) and IFN-stimulated genes (ISGs). Different viruses have developed specific strategies to counteract this antiviral response. The human cytomegalovirus (HCMV) protein, viral mitochondrial inhibitor of apoptosis (vMIA), interacts with the peroxisomal MAVS and inhibits its oligomerization, impeding downstream signalling. The herpes simplex virus (HSV-1) also impairs the peroxisome-dependent antiviral signaling, via its protein VP16, although the specific mechanisms remains unknown. The hepatitis C virus (HCV) cleaves MAVS at peroxisomes, impeding downstream signaling. Both porcine epidemic diarrhea virus (PEDV) and porcine deltacoronavirus (PDCoV) have been shown to disrupt the IFN regulatory factor 1 (IRF1)-mediated type III IFN via a not yet fully discosed mechanism. Black arrows represent host-related processes; blue and red arrows represent virus-related processes; red arrows indicate inhibition mechanisms. Figure prepared using BioRender.com.

6.1. Herpesviruses: human cytomegalovirus, herpes simplex virus 1 and Kaposi's sarcoma-associated herpesvirus

Herpesviruses are complex DNA viruses with slow infection cycles and the capacity to establish balanced interactions with the host cell in order to persist, even in a latent form, and successfully spread without much damage to the host (455). Different studies have addressed the importance of peroxisomes in the context of infection by the human cytomegalovirus **(**HCMV). A spatial-temporal proteomics study, which addressed the expression and localization of HCMV and host proteins during infection, initially showed the accumulation of peroxisomal proteins throughout infection (456). Later studies revealed that HCMV infection induced the upregulation of PEX3, PEX16, PEX13 and PEX14 expression (457), corroborating a previous study that reported the upregulation of PEX3 upon HCMV infection and a decrease in viral titers upon PEX3 knock-down (458). HCMV infection was also shown to increase the number of peroxisomes and alter the organelle's morphology, mainly at later stages of infection (Figure 12) (457).

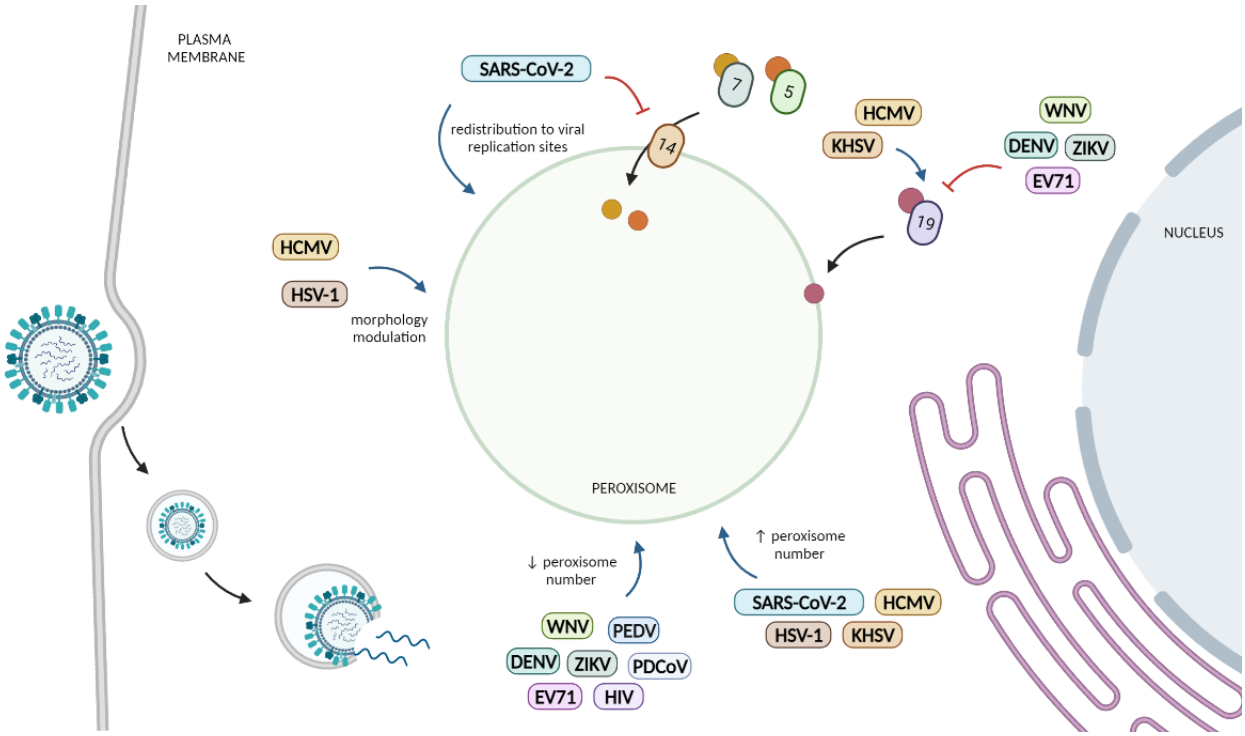


Figure 12. Schematic representation of the modulation of peroxisome biogenesis by different viruses. *Different viruses have been shown to modulate peroxisome morphology and biogenesis to promote virus particle formation and dissemination. An increase in peroxisome number has been shown to occur upon infection with herpes simplex virus 1 (HSV-1), human cytomegalovirus (HCMV), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and Kaposi's sarcoma-associated herpesvirus (KSHV). Infection with human immunodeficiency virus (HIV), Zika virus (ZIKV), West Nile virus (WNV), dengue virus (DENV), porcine epidemic diarrhea virus (PEDV), porcine deltacoronavirus (PDCoV), and EV71, however, induce a decrease in peroxisome number. Infection by SARS-CoV-2 induces the redistribution of peroxisomes to sites of active viral replication. HCMV and HSV-1 have also been shown to modulate peroxisomes morphology during infection. The KSHV's vFLIP protein localizes at peroxisomes, upon interaction with PEX19, where it is stabilized by the peroxisomal mitochondrial antiviral signalling protein (MAVS), a process that is essential for the establishment of viral latency. HCMV's protein vMIA protein also interacts with PEX19 to localize at peroxisomes, and induces the organelle fragmentation. The capsid proteins of WNV, DENV, and ZIKV have been shown to form a stable complex with PEX19, inducing its sequestration and/or degradation. EV71 infection has been shown to induce the downregulation the expression of PEX19. Black arrows represent host-related processes; blue and red arrows represent virus-related processes; red arrows indicate inhibition mechanisms. Figure prepared using BioRender.com.*

This increase in peroxisome biogenesis was found to accompany a boost in peroxisomal metabolic functions (Figure 13), mainly associated with the increased production of plasmalogens, required for HCMV virion assembly (457). HCMV was also shown to rewire host cell metabolism leading to an increased synthesis of VLCFAs, required for the production of infectious virus particles (459). Thus, it may be interesting to study whether stimulation of the peroxisomal β-oxidation of VLCFAs or inhibition of fatty acid elongation to lower the VLCFAs concentration could act as a strategy to combat not only

HCMV but also other viruses that show this dependency.

Given its slow replication cycle, HCMV depends on sustained cell viability and, accordingly, has developed several mechanisms to block apoptosis and evade the antiviral immune response (460). One of these mechanisms involves the viral protein vMIA which, besides being anti-apoptotic, is responsible for the inhibition of MAVS signaling at the level of peroxisomes and mitochondria (461, 462). vMIA is targeted to the peroxisomal membrane via PEX19, where it interacts with MAVS and inhibits the downstream antiviral response (Figure 11) (461). Although also responsible for the inhibition of MAVS signaling at mitochondria, important differences have been observed between the mechanisms occurring at both organelles: while at mitochondria vMIA inhibits MAVS signaling through the induction of the organelle's fragmentation (462), peroxisome fragmentation is not required for an effective inhibition of the antiviral response (460, 461). vMIA has also been shown to inhibit antiviral signaling at both organelles by directly interfering with MAVS oligomerization. This process was found to be dependent on Mff at the peroxisomes, but independent of MFF at mitochondria (463). An increased localization of vMIA to peroxisomes was observed throughout infection, culminating in the modulation of peroxisome morphology late in infection through the activation of PEX11β (464). The interdependency between HCMV and peroxisomes is a great example of the dynamic interplay between viruses and host cells: HCMV has evolved a dual strategy to take advantage of the multifunctional nature of peroxisomes, initially blocking peroxisome-dependent antiviral signaling and later promoting peroxisome biogenesis and metabolism to benefit the formation of new virus particles.

Infection by the herpes simplex virus 1 (HSV-1), was also shown to increase peroxisome numbers and alter the organelle's morphology, mainly at late stages of infection (Figure 12) (457). HSV-1 is also able to restrict peroxisomal MAVS signaling via its protein VP16, although the specific mechanism is still to be determined (Figure 11) (465).

An increase in peroxisome numbers and metabolism was also reported upon latent infection by the Kaposi's sarcoma-associated herpesvirus (KSHV) (Figures 12 and 13) (466). The authors showed that docosahexaenoic acid (DHA) and its precursors are upregulated during KSHV latency (466). Furthermore, ACOX1 and ABCD3 (PMP70) were found to be important for the survival of endothelial cells latently infected by KSHV (466). Finally, it was shown that peroxisomal MAVS, through the induction of TRAF-mediated post-translational modifications, stabilizes the viral FLICE-inhibitory protein (vFLIP), which has been suggested to interact with PEX19 and localize at peroxisomes (467). Thus, KSHV seems to rely on peroxisomal MAVS and modulate peroxisome biogenesis and metabolism to maintain infection and establish latency in infected cells.

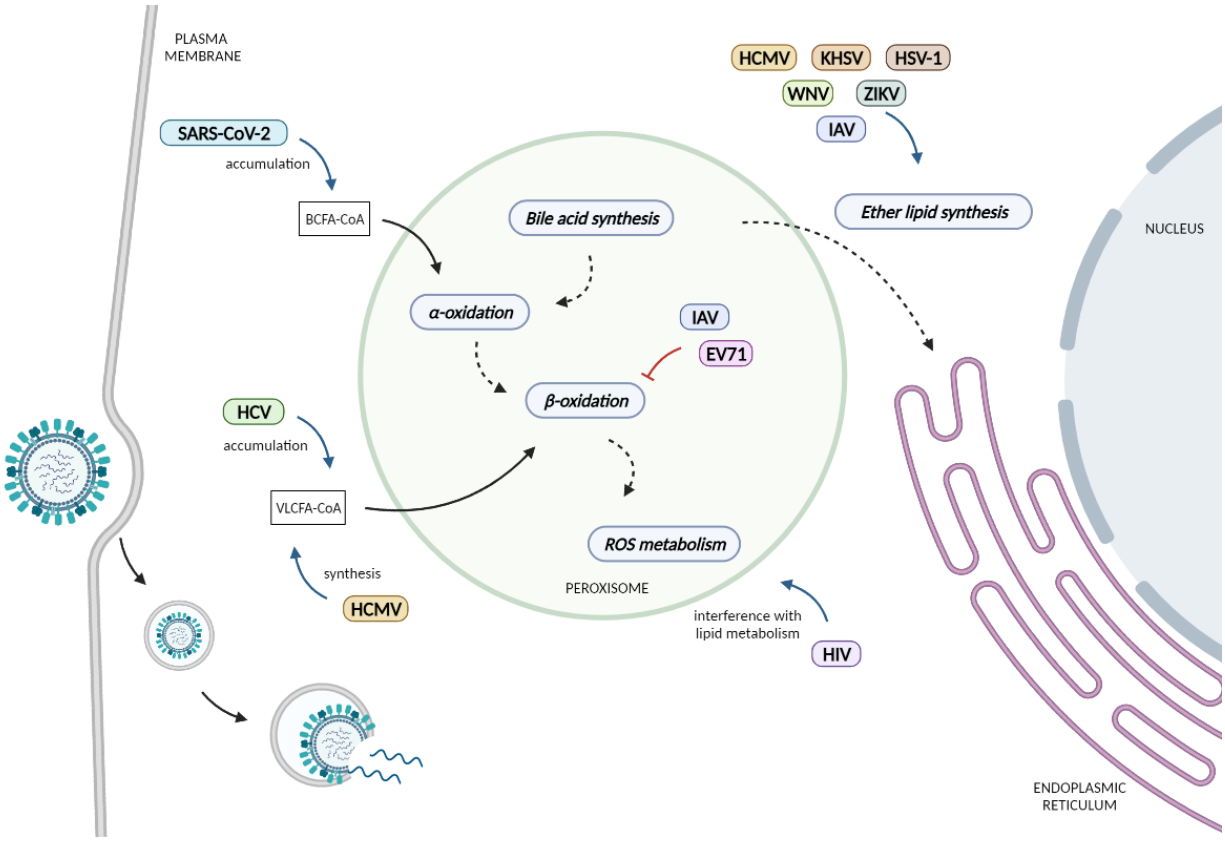


Figure 13. Schematic representation of the modulation of peroxisome metabolism by different viruses. *Different viruses modulate peroxisome metabolism in order to sustain or improve the formation of the infectious viral particles. Influenza A virus (IAV), Zika virus (ZIKV), West Nile virus (WNV), human cytomegalovirus (HCMV), Kaposi's sarcoma-associated herpesvirus (KSHV), and herpes simplex virus 1 (HSV-1) have been shown to induce the synthesis of ether lipids during infection. Enterovirus 71 (EV71) and IAV have also been shown to downregulate peroxisome β-oxidation. Hepatitis C virus (HCV) infection induces the accumulation of very long chain fatty acids (VLCFAs) through the reduction of the expression of peroxisomal genes. Infection by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been shown to induce the accumulation of branched-chain fatty acids (BCFAs). Black arrows represent host-related processes; blue and red arrows represent virus-related processes; red arrows indicate inhibition mechanisms. Figure prepared using BioRender.com.*

6.2. Hepatitis C virus

The hepatitis C virus (HCV) is an RNA enveloped virus, member of the *Flaviviridae* family. Although not much is known concerninmg the interplay between peroxisomes and HCV, it has been shown that HCV infection leads to an impairment of peroxisome metabolism and the intracellular accumulation of VLCFA, consistent with the formation of intrahepatic lipid droplets, which are essential for virus particle assembly (468). Another study has shown that VLCFA synthesis is important for infection and pathogenesis (469) (Figure 13). Although further studies are needed to confirm this, it is tempting to hypothesize that the stimulation of peroxisomal β-oxidation of VLCFA or inhibition of VLCFA synthesis may negatively influence HCV infection.

HCV is also able to counteract peroxisomal MAVS signaling in order to evade the cellular immune response (Figure 11). The viral serine protease complex NS3-4A, which cleaves MAVS at the outer-mitochondrial membrane and MAM (450, 454)**,** also were shown to cleave peroxisomal MAVS (Figure 11), and thereby impair antiviral signaling originating from this organelle, with similar kinetics as observed for mitochondria (470).

Peroxisome metabolism has recently been implicated in the progression of HCV-induced hepatocellular carcinoma (HCC), although the results from different studies are somewhat contradictory. The susceptibility to develop HCC upon HCV infection was shown to increase in patients carrying polymorphisms that upregulate the levels of the peroxisomal enzyme catalase, especially when combined with polymorphisms in the gene coding for the mitochondrial antioxidant enzyme glutathione peroxidase 1 (GPX1) (471). However, another study has found peroxisomal impairment in tumors and tissues adjacent to HCC (468) and ROS accumulation has been shown to suppress HCV replication in HCC cells (472). Further studies are required to allow a better understanding of the molecular mechanisms involved and the importance of peroxisome metabolism for HCV-induced HCC.

6.3. Dengue, West Nile and Zika viruses

Dengue virus (DENV), West Nile virus (WNV) and Zika virus (ZIKV) are arthropod-borne RNA viruses belonging to the *Flaviviridae* family. Different studies have addressed the interplay between these viruses and peroxisomes.

DENV and WNV have been suggested to evade the peroxisomal antiviral response by disrupting peroxisome biogenesis. Both viral infections induce a reduction in the number of peroxisomes and the inhibition of type III IFNs production (473) (Figures 11 and 12). Infection by ZIKV also induces a reduction of peroxisome numbers as well as of the expression of certain peroxisomal proteins**,** such as PEX19 (474) (Figure 12). ZIKV viral protease complex NS2A has been shown to localize at peroxisomes and interact with both PEX19 and PEX3 (475). The capsid proteins of DENV, WNV and ZIKV were found to form a stable complex with PEX19 (473, 476), and overexpression of the ZIKV capsid protein alone was shown to decrease PEX19 protein levels and peroxisome number. It has been proposed that these viruses evade the peroxisomal antiviral signaling by capsid protein-mediated sequestration and/or degradation of PEX19 (473). However, overexpression of the individual capsid proteins (473) had little effect on poly(IC)-induced production of type III IFNs, and knockdown of PEX19 did not result in increased viral replication (473). Further studies are needed to clarify the exact mechanism of inhibition of peroxisomal signaling by these viruses, taking into account the specific contribution of peroxisome biogenesis and

metabolism to the different phases of the viruses' infection cycles. Although not much data exists in this regard, a lipidomics study has identified increased levels of plasmalogens in the serum of ZIKV-infected patients (474). Furthermore, induction of peroxisome proliferation by overexpression of PEX11β has been shown to strongly impair ZIKV infection (474). Similarly, WNV infection has been shown to increase the levels of glycerophospholipids, including plasmalogens, and sphingolipids, which are essential building blocks for virus particle assembly (Figure 13) (477). Thus, it is likely that both WNV and ZIKV modulate peroxisome lipid metabolism throughout their infection cycle to virus particle formation and viral propagation.

6.5. Pestivirus

Pestivirus, another member of the *Flaviviridae* family, was also shown to interfere with peroxisomes. The viral protein N^{pro} was found to redistribute, together with IRF3, to peroxisomes and mitochondria (478). Although further studies are needed to confirm this, it has been speculated that, together with mitochondria, peroxisomes may represent novel sites for IRF3 regulation by pestivirus (478).

6.6. Coronaviruses

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), an RNA virus from the *Coronaviridae* family, has recently been shown to induce a profound remodeling of several organelles, including peroxisomes, which are recruited to viral replication centers at perinuclear regions (479). An increase in peroxisome numbers and the accumulation of PMP70 were also observed upon SARS-Cov-2 infection (Figure 12) (479). Although the molecular mechanisms and biological significance of this peroxisomal relocalization have not yet been explored, it may be related to the prevention of oxidative damage to viral RNA, provision/degradation of specific lipids or even to the cellular antiviral response. These results are in contrast with results of another study, which reports a loss of peroxisome integrity and function during SARS-CoV-2 infection (480). This study showed that the viral protein ORF9c (also named ORF14) interacts with PEX14, and thereby impairs the import of peroxisomal matrix proteins, which ultimately results in less abundant and dysfunctional organelles (Figure 12) (480). Although this study suggests that the reduction in the number of peroxisomes may have an impact on the peroxisome-mediated antiviral response, further studies should be performed to substantiate this conclusion. In fact, another report, in which the SARS-Cov-2 protein ORF9b was found to be an interactor of MAVS and an inhibitor of type I and type III IFNs production, has shown that ORF9c overexpression had no effect on type I and type III IFNs expression (481). One should therefore consider

the possibility that the effect of ORF9c on peroxisomes may not directly be related to the role of this organelle on antiviral signaling but to its contribution to the virus life-cycle and particle formation. The opposing data concerning peroxisome numbers and integrity upon SARS-Cov-2 infection may result from the distinct time points upon infection and cell lines that were analyzed in the two studies. Further analyses at several different time points during a single infectious cycle, to avoid the presence of multiple cells displaying different stages of infection, are required to conclude whether or not these differences reflect temporal variations of the role of peroxisomes throughout SARS-CoV-2 infection. Furthermore, ORF9b's role on the manipulation of RLR/MAVS signaling should also be further studied in the specific context of the peroxisome-dependent antiviral response.

Other coronaviruses have been shown to interfere with peroxisome biogenesis, metabolism and peroxisome-dependent antiviral signaling. Infection by the porcine epidemic diarrhea virus (PEDV) or the porcine deltacoronavirus (PDCov) induce a reduction in the number of peroxisomes and the inhibitions of the IRF1-mediated type III IFNs response in intestinal epithelial cells (482, 483) (Figure 11 and 12). While the specific mechanisms remain to be further elucidated, PEDV nsp1 appears to be mainly responsible for the modulation of peroxisome morphology and type III IFNs expression (482).

6.7. Influenza A virus

The influenza A virus (IAV) is an RNA virus that belongs to the *Orthomyxoviridae* family. IAV particle formation has been linked specifically to peroxisomal lipid metabolism. A recent lipidomics study analyzed the lipid profile of IAV virus particles and revealed the enrichment of peroxisome-derived ether-linked phosphatidylcholines relative to ester-linked phosphatidylcholines (484). The combined analysis of these results with previous genomics and proteomics data, led the authors to propose that IAV infection induces the downregulation of peroxisomal β-oxidation and the upregulation of ether lipids and sphingolipids synthesis (Figure 13) (484). Although the biological meaning remains to be explored, the IAV protein NS1 was found to interact with HSD17B4 (also known as DBP), an important peroxisomal enzyme catalyzing the second and third step of peroxisomal β-oxidation as described above, in different human and avian influenza virus species (485). Although further *in vivo* and *in vitro* studies must be performed to substantiate this, these results indicate that IAV may interfere with peroxisome lipid metabolism throughout its life cycle, for efficient formation of infectious virus particles.

6.8. Human immunodeficiency virus

The human immunodeficiency virus (HIV), an RNA retrovirus belonging to the *Retroviridae* family, has

also developed specific mechanisms to interfere with peroxisome biogenesis and metabolism at different stages in the virus' life cycle and with clear implications for disease progression. Upon infection, HIV has been shown to induce, via its protein Vpu, a reduction in the number of peroxisomes in the brain as well as in primary macrophages through the upregulation of specific microRNAs targeting peroxisome biogenesis factors (PEX2, PEX7, PEX11β and PEX13) (Figure 12) (486, 487). This impairment of peroxisome biogenesis may contribute to the evasion of the peroxisome-dependent antiviral signaling or even to the development of HIV-associated neurocognitive deterioration, as pathogenic variants in peroxisome biogenesis proteins have been associated with the impairment of neuronal functions in various autosomal recessive disorders (488).

HIV has also been shown to interfere with peroxisome metabolism (Figure 13). The viral protein Nef has been shown to localize at peroxisomes and interact with ACOT8 (also known as human Thiosterase II). This interaction was linked to the downregulation of CD4 and MHC class I expression at the surface of infected cells (489), and has been suggested to be related to ACOT8's capacity to induce the palmitoylation of membrane proteins. This interaction may also interfere with peroxisomal lipid metabolism, as ACOT8 is also involved in the regulation of peroxisomal β-oxidation by means of its capacity to cleave acyl-CoAs into free fatty acids and coenzyme A (489).

The immunodeficiency syndrome/AIDS caused by HIV infection is characterized by progressive cell death of CD4+ T lymphocytes, the majority of which are uninfected bystander cells. Peroxisomes have been implicated in the development of this immunodeficiency, as the HIV Env protein has been shown to induce a reduction in the number of peroxisomes by pexophagy and the accumulation of ROS in uninfected bystander CD4+ T lymphocytes, leading to their death by apoptosis (490).

6.9. Enterovirus

The enterovirus 71 (EV71), an RNA virus belonging to the *Picornaviridae* family and causing hand-foot-and-mouth disease, induces apoptosis and autophagy in order to release the newly formed virus particles from infected neuronal cells. EV71 infection has been shown to decrease peroxisome numbers and downregulate the expression of ACOX1 and PEX19 in neuronal cells, and at the same time induce the accumulation of ROS and subsequent activation of autophagy and apoptosis (491) (Figure 12 and 13). In accordance with these results, a knockdown of ACOX1 was shown to favor EV71 propagation, while overexpression of ACOX1 had a negative impact on viral replication (491). Antioxidant treatment induced the downregulation of EV71 replication (491), emphasizing the importance of further studies to determine whether these peroxisome-dependent mechanisms could be explored as possible targets for

antiviral therapy.

6.10. Rotavirus

Rotavirus, an RNA virus from the family *Reoviridae* and the etiologic agent for infantile gastroenteritis, was the first human virus to be directly associated with peroxisomes. Its protein Vp4 was found to localize at peroxisomes due to the presence of a PTS1 domain at its C terminus (492). Although further studies should be performed, it has been suggested that the interplay between the rotavirus and peroxisomes may reflect a strategy developed by the virus to control lipid metabolism and posttranslational protein modification (492).

6.11. Human papillomavirus

The human papillomavirus (HPV) is a DNA virus from the *Papillomaviridae* family and the main cause of cervical and anogenital cancers. A recent study, using a genome-wide CRISPR screen, has identified PMP34 as an important protein for the HPV life cycle, although the underlying mechanisms have not been resolved (493).

7. CONCLUDING SUMMARY

In recent decades much has been learned about the role of peroxisomes in human health and disease as comprehensively reviewed in this review. At the same time it is clear that the current state of knowledge about peroxisomes especially in humans is still immature for various reasons. First, only very few different human cell types have been studied so far due to the restricted access to different human cell types other than fibroblasts and blood cells. This also implies that the proteome of human peroxisomes has remained ill-defined with so far only one single proteomics study performed in human liver peroxisomes. Recent technological developments which allow the generation of different human cell types from induced pluripotent stem cells derived from primary cells like cultured fibroblasts and blood cells, combined with the new, much more sensitive methods for proteome analysis, will surely lead to the identification of new peroxisomal proteins and hitherto unknown functions of peroxisomes. It will be especially exciting to use these newly acquired cells albeit hepatocytes, astrocytes, or whatever cell type of interest, for future metabolomics analyses in which the metabolome of control cells is compared with that of mutant cells with a selective defect either in peroxisome metabolism or in peroxisome biogenesis. This is all the more relevant since the field of metabolomics analysis has progressed tremendously in the last few years. The knowledge gained from these studies will also be of

key importance to understand the role of peroxisomes in different tissues. Indeed, although we have learned much about the pathophysiology of peroxisomes from the different mouse models as reviewed here, there are still many gaps in our knowledge not in the least because there are major differences between mice and humans in terms of their physiology.

The same is true for the role of peroxisomes in viral infections. Although different viruses from different families and origins were shown to have developed specific strategies to interfere with peroxisomes and/or peroxisomal metabolism, research in this field is still relatively immature and only specific mechanisms and infection stages have been analyzed for most viruses. Studies of complete infection cycles may reveal that viruses down-regulate peroxisome biogenesis and metabolism to interfere with the cellular antiviral respons at early stages, but induce their upregulation at later stages to favor the assembly of virus particles. Although much work remains to be done to resolve all intricate details, current data have already provided important evidence that may lead to the development of novel peroxisome-related therapies. The common manipulation of peroxisomal pathways by different viruses further support the notion that peroxisome modulation may lead to the development of innovative broad-spectrum antiviral strategies. Taken together, the field of peroxisome research is rapidly moving forward and will surely produce exciting new information including new peroxisomal functions relevant to human health and disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHORS CONTRIBUTIONS

R.W, M.B, D.R, S.F. and H.W. drafted the manuscript and prepared figures. R.W and H.W. edited and revised the manuscript; all authors approved the final version of the manuscript.

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