Original Article

Journal of Inborn Errors of Metabolism & Screening
2018, Volume 6: 1–16

DOI: 10.1177/2326409818810285
journals.sagepub.com/home/iem
SAGE

Laboratory Diagnosis of Peroxisomal Disorders in the -Omics Era and the Continued Importance of Biomarkers and Biochemical Studies

Ronald J. A. Wanders, PhD[1], Frédéric M. Vaz, PhD[1], Sacha Ferdinandusse, PhD[1], Stephan Kemp, PhD[1], Merel S. Ebberink, PhD[1], and Hans R. Waterham, PhD[1]

Abstract

The clinical as well as biochemical and genetic spectrum of peroxisomal diseases has markedly increased over the last few years, thanks to the revolutionary advances in the field of genome analysis and several -omics technologies. This has led to the recognition of novel disease phenotypes linked to mutations in previously identified peroxisomal genes as well as several hitherto unidentified peroxisomal disorders. Correct interpretation of the wealth of data especially coming from genome analysis requires functional studies at the level of metabolites (peroxisomal metabolite biomarkers), enzymes, and the metabolic pathway(s) involved. This strategy is not only required to identify the true defect in each individual patient but also to determine the extent of the deficiency as described in detail in this article.

Keywords

peroxisome metabolism, peroxisomes, omics, biomarkers, Zellweger syndrome

Introduction

Peroxisomes are subcellular organelles that play an essential role in a variety of different catabolic and anabolic pathways, which include the α- and β-oxidation of different fatty acids (FAs); the synthesis of ether phospholipids (EPLs), bile acids, and docosahexaenoic acid (C22:6omega3); and the detoxification of glyoxylate as well as other metabolic functions. The importance of peroxisomes for humans is stressed by the existence of a still expanding group of inherited diseases caused by mutations in genes coding for proteins required for the proper functioning of peroxisomes. The group of peroxisomal disorders (PDs) is generally divided into 2 subgroups: (1) the disorders of peroxisome biogenesis (PBD) and (2) the single peroxisomal enzyme deficiencies (PED).

The introduction of whole-exome sequencing (WES) and whole-genome sequencing (WGS) methods and other technological advances have led to the recognition of novel disease phenotypes linked to mutations in previously identified peroxisomal genes as well as several new PDs. In this review, we provide an update about the current state of knowledge about PDs, with particular emphasis on the continued importance of biomarker analysis and biochemical studies, especially now that WES/WGS has conquered such a dominant position in the diagnostic process.

Peroxisome Biogenesis: An Update

For many years, peroxisomes were believed to originate from preexisting peroxisomes through growth and division and were thus marked as *autonomous* organelles. In more recent years, however, the original growth and division model has been challenged, and current evidence holds that peroxisomes are in fact *semiautonomous* organelles that can also form de novo from a specific subdomain of the endoplasmic reticulum

[1] Laboratory Genetic Metabolic Diseases, Departments of Clinical Chemistry and Pediatrics, EmmaChildren's Hospital, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

Received September 09, 2018. Accepted for publication September 27, 2018.

Corresponding Author:
Ronald J. A. Wanders, PhD, Laboratory Genetic Metabolic Diseases, Academic Medical Center, University of Amsterdam, Meibergdreef 9, Room F0-514, 1105 AZ Amsterdam, the Netherlands.
Email: r.j.wanders@amc.uva.nl

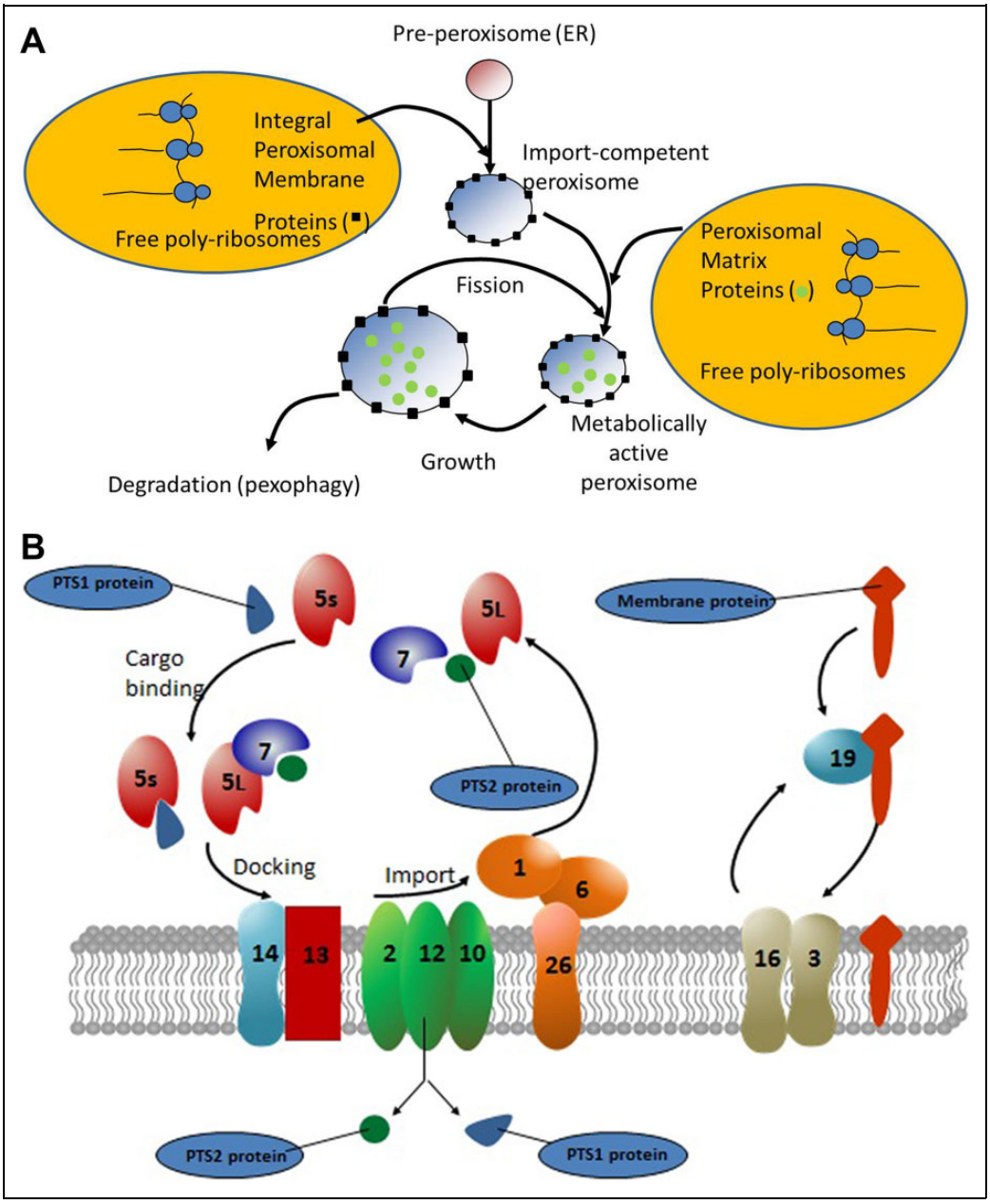

Figure 1. A, The current model of peroxisome biogenesis. Peroxisomes were long thought to be autonomous organelles originating from preexisting peroxisomes through growth and division but have now been identified as semiautonomous organelles that can also form de novo from a specific subdomain of the endoplasmic reticulum (ER) as depicted in the figure. B, The synthesis of peroxisomal membrane and matrix proteins on free polyribosomes and the subsequent targeting of these proteins to peroxisomes is mediated by different peroxins (PEX). See text for details.

(ER; Figure 1A). Once assembled and released from the ER, the preperoxisome acquires additional membrane proteins including those constituting the peroxisomal protein import machinery. As soon as the multiprotein import machinery, composed of various peroxins (PEX proteins), has been put together, the import of matrix proteins begins (see Figure 1A). It is important to mention that virtually all peroxisomal membrane proteins (PMPs), except those involved in the formation of the preperoxisome at the ER, are synthesized on free polyribosomes and are posttranslationally inserted into the peroxisomal membrane via a cycling mechanism that involves PEX19 as cycling receptor. PEX19 is able to bind PMPs in the cytosol and delivers these proteins at the peroxisomal membrane through interaction with PEX3 (see Figure 1B).

The majority of the PMPs identified to date play a central role in the transport of peroxisomal matrix proteins across the peroxisomal membrane (Figure 1B). This includes the PEX13–PEX14 docking complex, the PEX2–PEX10–PEX12 ring finger complex, and the PEX26–PEX1–PEX6–recycling complex as main representatives. Other PMPs include the different half-ATP-binding cassette family D (ABCD) ABC transporters, ABCD1, ABCD2 and ABCD3, which catalyze the import of

different acyl-CoA esters and also the tail-anchored protein ACBD5 identified by Gronemeyer et al in 2013 in human peroxisomes following proteomic analyses.[1] Peroxisomal matrix proteins are targeted to peroxisomes via 1 of 2 different targeting signals, peroxisomal targeting signal (PTS) 1 and PTS2. The canonical PTS1 sequence is the C-terminal tripeptide serine–lysine–leucine (SKL), which was found to be necessary and sufficient to target peroxisomal proteins to peroxisomes.[2] Many variations of this SKL sequence have been identified which also promote import of peroxisomal proteins. This has resulted in the following consensus PTS1 sequence: (SAC)-(KRH)-(LM), as reviewed by Kim and Hettema.[3] The PTS1 sequence is recognized in the cytosol by the cycling receptor PEX5, which contains a C-terminal tetratricopeptide repeat domain that interacts with the PTS1.[4,5] PEX5 occurs in 2 forms, a short form (PEX5S) and a long form (PEX5L). PEX5S is involved in the import of PTS1 proteins in contrast to PEX5L, which plays a key role in the import of PTS2 proteins (see Figure 1B). A subset of peroxisomal matrix proteins is targeted to peroxisomes via a different PTS (PTS2) located at the amino terminus. The different PTS2 sequences identified thus far fit the following consensus sequence: -R-(LIVQ)-X-X-(LIVQH)-(LSGA)-X-(HQ)-(LA).[6] While PEX5S is sufficient for the targeting of PTS1-containing proteins to the peroxisomal docking complex followed by import into the peroxisomal matrix, PEX7 requires different coreceptors to be functionally active which differ depending upon the species involved. In humans, PEX5L is required for PEX7-mediated protein import (see Figure 1B). Not all peroxisomal matrix proteins contain a PTS1 or PTS2. At least some of these non-PTS1/non-PTS2 containing proteins are imported into peroxisomes via a so-called piggyback mechanism by which the protein is cotransported together with a protein that does have a PTS1- or a PTS2 sequence (see Kim and Hettema[3] for details).

After docking of the 2 different cargo-loaded receptors, the PTS1 and PTS2 proteins must be translocated across the membrane to be released inside the peroxisomes. The PEX2–PEX10–PEX12 complex is involved in the release of the cargo from the receptors, thereby preparing the receptors for the next import cycle. The unloaded PEX5 and PEX7 proteins are recycled back into the cytosol by means of the PEX26–PEX1–PEX6 complex (see Figure 1B). Importantly, mechanisms are in place to recycle not-needed, damaged, and/or aged proteins as well as to degrade the entire organelle via a specific mechanism called pexophagy.[7]

Peroxisome Metabolism in Humans

Human peroxisomes contain a large variety of enzyme activities that allow peroxisomes to exert their functions in metabolism which include: the α- and β-oxidation of fatty acids (FAs); the biosynthesis of bile acids, docosahexaenoic acid, and etherphopholipids (EPLs); and the detoxification of glycolate and glyoxylate as briefly discussed below (see Figure 2):

(1) Peroxisomal FA β-oxidation: Peroxisomes in human cells catalyze the β-oxidation of a large variety of FAs of which some can be oxidized in both mitochondria and peroxisomes, whereas others are solely oxidized in peroxisomes or in mitochondria. Very long-chain acyl-CoAs such as C24:0-CoA and C26:0-CoA are unique substrates for the peroxisomal β-oxidation system. Peroxisomes are able to oxidize these straight-chain fatty-acyl CoAs all the way to short-chain acyl-CoAs such as hexanoyl-CoA (C6:0-CoA), but the bulk of short-, medium- and long-chain FAs is oxidized in the mitochondria. This is supported by the finding of normal short-, medium-, and long-chain acylcarnitines in plasma from patients with Zellweger syndrome (ZS) who lack functional peroxisomeas, whereas very long-chain acylcarnitines are elevated.[8] Other FAs and FA derivatives unique to peroxisomal β-oxidation include pristanic acid (2,4,6,10-tetramethylpentadecanoic acid), long-chain dicarboxylic acids, tetracosahexaenoic acid (C24:6omega3), and the bile acid intermediates di- and trihydroxycholestanoic acid (DHCA/THCA) as well as several prostaglandins, thromboxanes, and other isoprenoids.[9,10]

The first step in the oxidation of FAs in peroxisomes involves their transport across the peroxisomal membrane. Peroxisomes do not contain a carnitine cycle like in mitochondria. Most FAs are activated to the corresponding acyl-CoAs outside peroxisomes. Current evidence holds that they are transported across the peroxisomal membrane as acyl-CoA esters rather than as free FAs or acylcarnitines. Peroxisomes contain 3 different half-ABC transporters that predominantly form homodimers including ABCD1, ABCD2, and ABCD3 which all catalyze the transport of acyl-CoAs. ABCD1 transports very long-chain acyl-CoAs,[11,12] whereas ABCD3 (PMP70) catalyzes the transport of the branched-chain acyl-CoAs pristanoyl-CoA and phytanoyl-CoA as well as the CoA-esters of the bile acid intermediates DHCA and THCA.[13] Until recently, it was believed that human peroxisomes contain 2 different acyl-CoA oxidases, 2 bifunctional proteins with enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities, and 2 thiolases. Recently, however, we identified a third acyl-CoA oxidase, catalyzing the oxidation of branched-chain acyl-CoAs (see the study by Ferdinandusse et al[14]).

(2) Peroxisomal FA α-oxidation: Fatty acids with a methyl group at the 3-position, including phytanic acid, cannot be handled by the peroxisomal β-oxidation system and first need to undergo oxidative decarboxylation also called α-oxidation, which solely occurs in peroxisomes. The enzymology of the phytanic acid α-oxidation pathway has been delineated to a great extent and involves the enzymes phytanoyl-CoA 2-hydroxylase, 2-hydroxyphytanoyl-CoA lyase, and pristanal dehydrogenase, which results in the chain shortening of 3-methyl FAs by 1 carbon atom to produce 2-methyl FAs

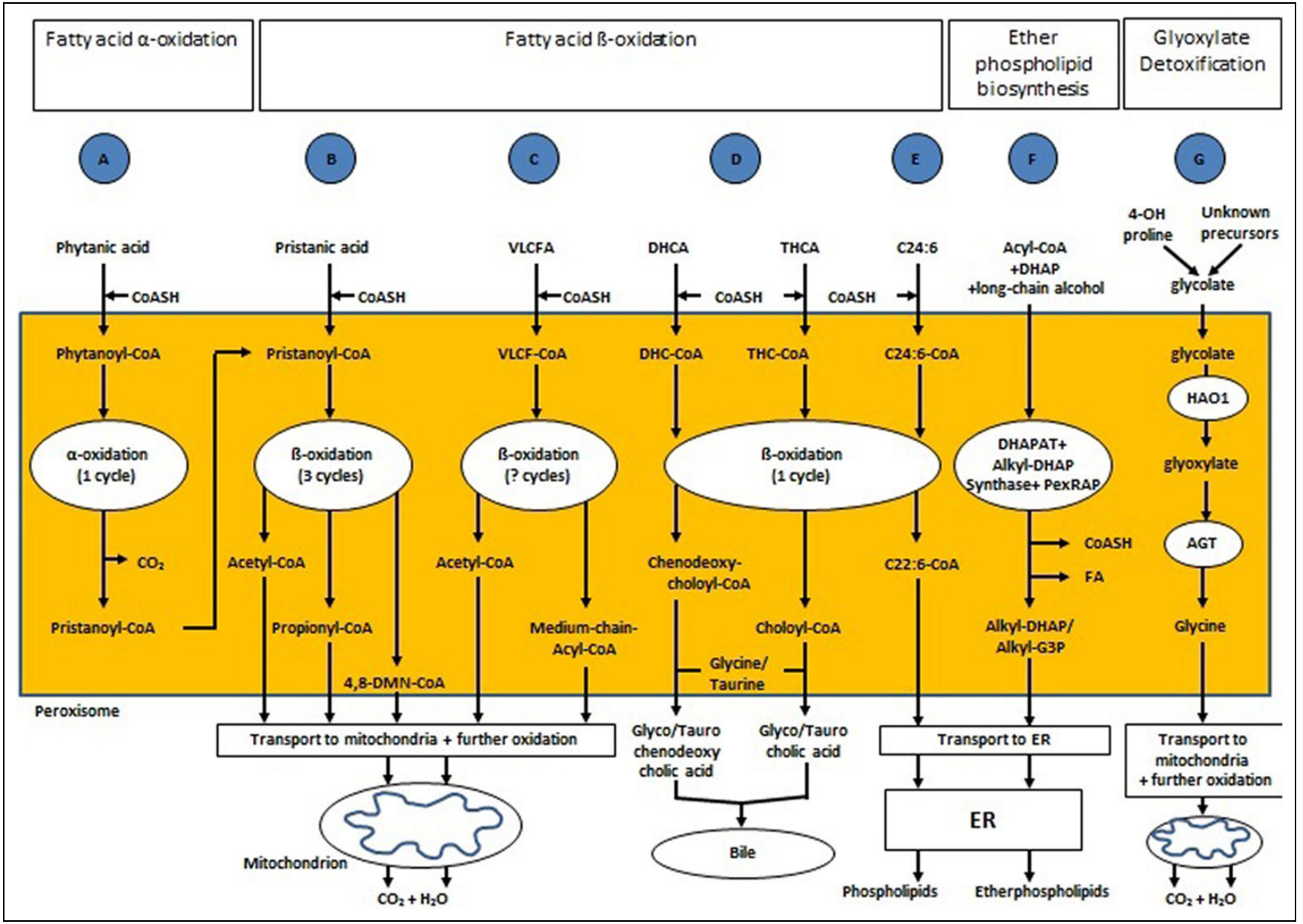

Figure 2. Schematic diagram showing the different metabolic functions of peroxisomes and their interaction with other subcellular organelles. See text for details.

which can then be degraded by peroxisomal β-oxidation. Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is the major substrate for α-oxidation in humans and generates pristanic acid (for review see [9,15,16])

(3) Ether phospholipid (EPL) biosynthesis: Ether phospholipids are a specific class of phospholipids, characterized by an ether bond at the sn-1 position. In humans, most EPLs occur in their plasmalogen form characterized by an unsaturated 1-O-alkenyl rather than 1-O-alkyl group at sn-1. Peroxisomes play a key role in EPL synthesis, since the first 2 steps in EPL biosynthesis are catalyzed by peroxisomal enzymes that include: (1) glycerone-3-phosphate O-acyltransferase (GNPAT) and (2) alkylglycerone-3-phosphate synthase (AGPS), both localized within peroxisomes. The peroxisomal tail-anchored protein FAR1 with acyl-CoA reductase activity generates the long-chain alcohol required in the AGPS reaction (see the study by Wanders et al[16]).

(4) Peroxisomal glyoxylate detoxification: Glyoxylate is a very toxic substance in itself and also because it undergoes rapid conversion into oxalate if not detoxified immediately. Although much remains to be learned about the exact sources of glyoxylate in humans, 4-hydroxyproline is definitely an important source of glyoxylate. Degradation of 4-hydroxyproline solely occurs in mitochondria, and the glyoxylate produced in the 4-hydroxy-2-oxoglutarate aldolase (HOGA) reaction is first converted into glycolate which is then transported out of the mitochondrion to peroxisomes, where glycolate is converted into glyoxylate via the peroxisomal enzyme glycolate oxidase. Glyoxylate is then converted into glycine by the enzyme alanine glyoxylate aminotransferase (AGXT) followed by the retrograde transport of glycine back to mitochondria for oxidation to CO_2 and H_2O (see Figure 2).

(5) Bile acid synthesis: Peroxisomes play an indispensable role in the biosynthesis of the primary bile acids, cholic acid and chenodeoxycholic acid. Indeed, while all steps from cholesterol to the bile acid intermediates DHCA and THCA take place outside peroxisomes, the subsequent oxidation of the side chains of di- and trihydroxycholestanoyl-CoA takes place in peroxisomes. To this end, they are first transported across the peroxisomal membrane by ABCD3 (PMP70) followed by chain shortening of the 2 cholestanoyl-CoAs via 1 cycle of β-oxidation and, subsequently, the conjugation of the products choloyl-CoA and chenodeoxycholoyl-CoA with taurine and/or glycine, after which the taurine and/or glycine esters are exported out of the peroxisome to be excreted into bile by the bile salt exchange pump (ABCA11).[17]

The Expanding Clinical and Biochemical Spectrum of PDs

The PDs identified to date are usually classified into 2 groups: (1) the PBDs and (2) single PEDs. Thanks to the increased awareness about PDs and the application of improved technologies, including WES and WGS, the phenotypic spectrum of patients affected by a PD has markedly widened over the years which makes the clinical recognition of patients affected by a PD much more difficult. The laboratory diagnosis of PD can also be very complicated, since the various peroxisomal biomarkers may be entirely normal in some patients.

Zellweger Spectrum Disorders

The prototypic Zellweger spectrum disorder (ZSD) is ZS that is a classic malformation syndrome originally described as cerebro-hepato-renal syndrome (see[18–20] for review). Patients typically present with severe hypotonia, seizures, cranial facial dysmorphia with a high forehead, large anterior fontanelles, hypertelorism, epicanthal folds, a high arched palate, and micrognatia. Microgyria, pachygyria, and heterotopia are seen upon brain magnetic resonance imaging (MRI). Ocular abnormalities are frequent and include cataract, glaucoma, and corneal clouding. Cortical renal cysts are usually identifiable on ultrasound. Chondrodysplasia punctata especially in the knees and hips has been observed on skeletal X-rays.[18] Cardiovascular malformations and pulmonary hypoplasia have also been documented. Infants with ZS generally do not survive beyond the first year of life. The discovery of the first peroxisomal biomarkers in patients with ZS in the early 1980s, including elevated very long chain fatty acid (VLCFA) levels in the plasma[21] and decreased plasmalogen levels in erythrocytes,[22] followed by the finding of other metabolic abnormalities (see Figure 3), has led to the discovery of the different metabolic functions of peroxisomes in humans as shown in Figure 3. Furthermore, recognition of this peroxisomal biomarker panel as a good readout of in vivo peroxisome functioning followed by its introduction in metabolic laboratories for the sake of patient's diagnostics has prompted the identification of many new PDs as well as the assignment of earlier described disorders to the group of PDs, including neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD). Figure 4 shows the PDs identified so far as classified into the 2 different groups including the group of PBDs and the group of peroxisomal function disorders. Figure 4 also provides up-to-date information on the abbreviations used in literature, the gene(s) involved with the different PDs as well as information on the peroxisomal biomarker(s) for each individual PD.

Taken together, it has become clear over the years that classification of patients in the ZS, NALD, or IRD categories has become obsolete because of the identification of patients showing clinical signs and symptoms different from ZS, NALD, and IRD as well as the description of patients with an incomplete phenotype. This has prompted introduction of the name ZSD, which gives credit to the fact that the phenotypic variability is large and involves a disease spectrum rather than separate disease entities. Despite the extensive phenotypic variability, patients with ZSD can roughly be divided into 3 groups which include: (1) a neonatal–infantile form, (2) a childhood form, and (3) an adolescent–adult form as discussed in detail by Klouwer et al[20] (Figure 5).

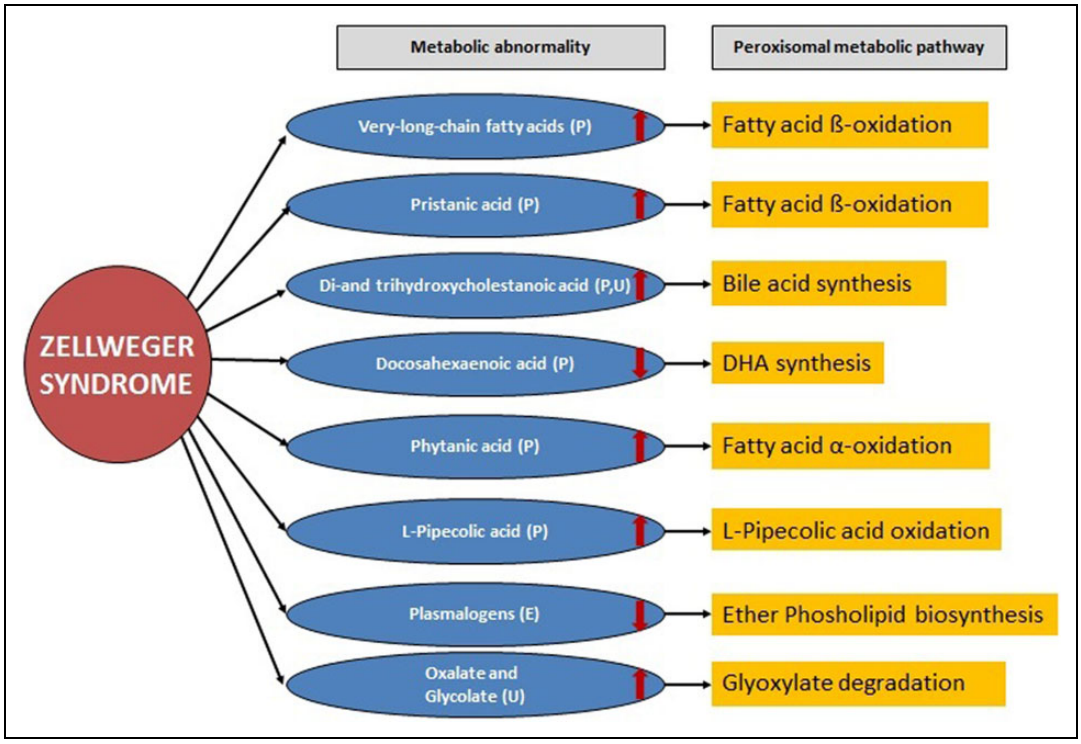

Figure 3. Zellweger syndrome and its important role in the identification of the different metabolic functions of peroxisomes in humans as deduced from the metabolic abnormalities discovered over the years.

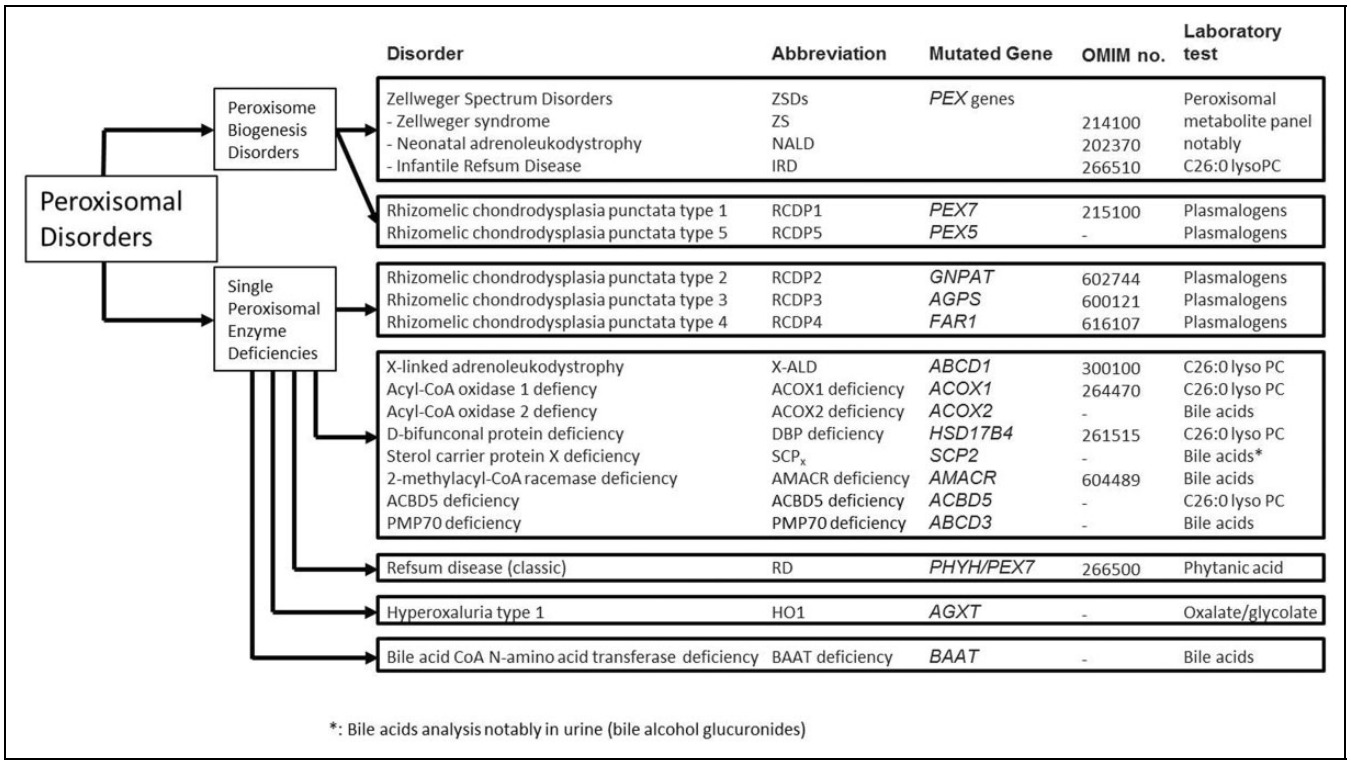

Figure 4. The list of peroxisomal disorders as identified up to now with information about the abbreviations used in literature, OMIM number(s), the gene(s), and the different peroxisomal biomarkers involved with each individual peroxisomal disorder (PD).

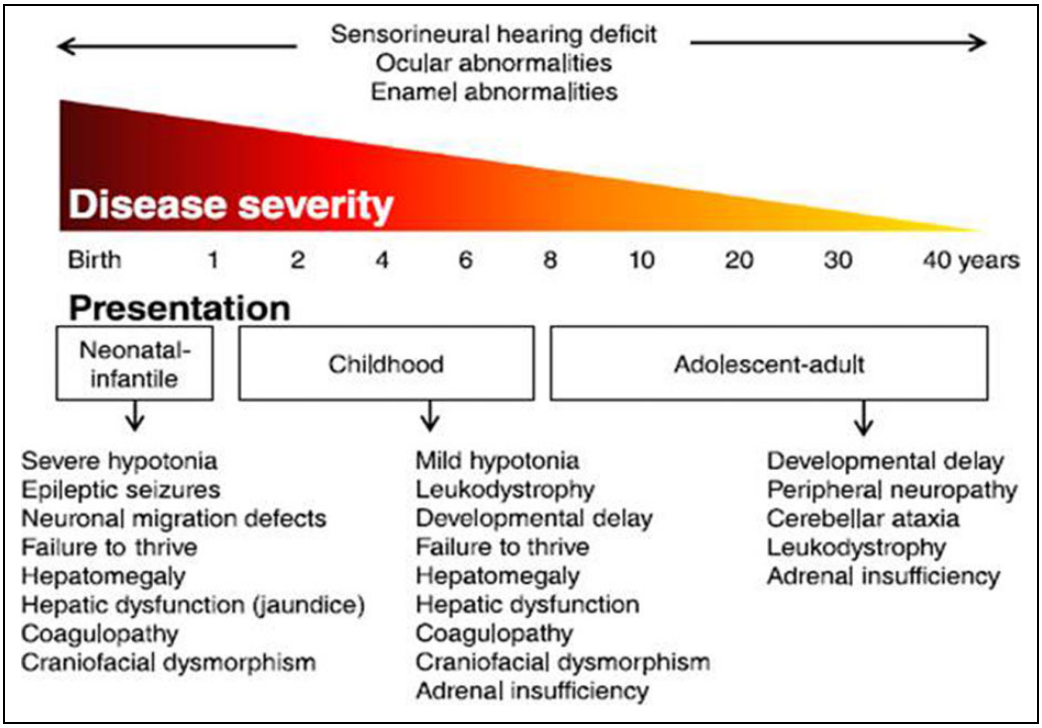

Figure 5. Schematic diagram showing the wide clinical spectrum of patients affected by a peroxisome biogenesis disorder as modified from figure 1 in the article by Klouwer et al.[20]

Neonatal–infantile presentation. ZSD patients with the neonatal–infantile presentation usually show a severe phenotype that closely resembles the originally described classic ZS and is characterized by multiple congenital defects. Patients typically present after birth with severe hypotonia, seizures, typical dysmorphic features, hepatic dysfunction, and other abnormalities as detailed in Figure 5. Most patients do not reach any developmental milestones and usually die in the first year of life.

Childhood presentation. Although partially overlapping with the neonatal–infantile presentation, the disease spectrum of the childhood form is more variable with the onset usually within the first to second year of life with patients coming to clinical attention because of delayed developmental milestones. Usually, there is progressive bilateral visual and sensory neural hearing impairment. Ocular abnormalities include retinitis pigmentosa, cataract, optic nerve atrophy, glaucoma, and Brushfield spots. Facial dysmorphia is usually much less pronounced compared to that observed in patients with the classic neonatal–infantile form. Prognosis is variable. Patients usually die later in childhood.

Adolescent–adult presentation. Patients with the adolescent–adult presentation are even more difficult to diagnose. Sensory neural hearing loss and ocular features are important clues, whereas additional signs and symptoms may be absent or occur later in life. Patients at the mildest end of the ZSD spectrum usually have mental retardation plus visual and hearing impairments and nonspecific symptoms, including teeth and nail abnormalities as in Heimler syndrome.[23] Cranial facial dysmorphia is usually absent or very subtle. Adrenal insufficiency is common although asymptomatic in more than 50% of the patients (see the study by Klouwer et al[24]).

Biochemistry and Molecular Basis of the ZSDs

A defect in peroxisome biogenesis as in ZSD patients affects the global formation of peroxisomes and is thus associated with the loss of basically all peroxisomal functions. This includes FA α- and β-oxidation; the synthesis of bile acids, EPLs, and DHA; the detoxification of glyoxylate as well as the degradation of L-pipecolic acid. The different metabolic functions of peroxisomes can be assessed in fibroblasts, although bile acid synthesis, glyoxylate detoxification, and pipecolic acid oxidation are liver specific and cannot be analyzed in fibroblasts.[25] Fortunately enough, there are good readouts for each of the metabolic functions of peroxisomes in humans which can be determined in a single blood sample. These include (1) VLCFA (plasma) and C26:0-lysoPC (bloodspot); (2) pristanic acid (plasma); (3) phytanic acid (plasma); (4) DHCA and THCA (plasma/urine); (5) pipecolic acid (plasma); and (6) plasmalogens (erythrocytes; see Figure 3). It should be noted that it is only very rare to find all these abnormalities in a single patient. This is first due to the fact that some parameters such as pristanic acid and phytanic acid are only derived from dietary sources and thus accumulate in an age- and diet-dependent manner. Second, for reasons that have remained poorly understood, one or more peroxisomal biomarkers may be completely

normal despite the fact that there is a marked deficiency of all peroxisomal functions when studied in fibroblasts.[24,26] In the literature, several patients have been described who turned out to have a PBD, whereas all peroxisomal biomarkers in a blood sample were found to be normal. In addition, milder affected PBD patients who may show abnormalities in one or more peroxisomal biomarkers early in life tend to normalize with increasing age.[24,26] This has important implications for the laboratory diagnosis of patients as discussed further on.

The ZSD group is not only clinically and biochemically very heterogeneous, but the molecular basis of the ZSDs is also very diverse. At present bi-allelic mutations in *PEX1*, *PEX2*, *PEX3*, *PEX5*, *PEX6*, *PEX10*, *PEX12*, *PEX13*, *PEX14*, *PEX16*, *PEX19*, and *PEX26* have been identified in PBD patients.[27] More recently, genetic defects in peroxisome division have been reported due to mutations in *DLP1*, *MFF*, *GDAP1*, and *PEX11*-β (see[28] for review).

Rhizomelic Chondrodysplasia Type 1 (PEX7 Deficiency) and Type 5 (PEX5L Deficiency)

Although clinically much different from the ZSDs, peroxisome biogenesis is also defective in rhizomelic chondrodysplasia punctata type 1 (RCDP1), albeit only partially, due to mutations in the *PEX7* gene that causes a deficiency of only 1 of the 2 import pathways for peroxisomal matrix proteins, that is, the PTS2 pathway (see Figure 1B). As a consequence, all peroxisomal enzymes equipped with a PTS2 signal which include AGPS and phytanoyl-CoA 2-hydroxylase (PHAX) are not imported into peroxisomes which results in a deficiency of plasmalogens (AGPS-deficiency) and the accumulation of phytanic acid (PHAX-deficiency). In its classic form, RCDP is characterized by a disproportionally short stature primarily affecting the proximal parts of the extremities, typical facial appearance including a broad nasal bridge, epicanthus, high arched palate, dysplastic external ears and micrognathia, congenital contractures, characteristic ocular involvement, dwarfism, and severe mental retardation with spasticities. X-ray studies usually show a series of abnormalities, including symmetrical shortening of femur and humerus with irregular and broad metaphyses, calcific stippling mainly of the epiphyses, absent capital femur epiphysis, coronal clefts of vertebrae, increased intravertebral disc spaces, cupping of dorsal ribs, and a barrel-formed thorax. Neurological impairments include truncal hypertonia, spastic tetraplegia, and epilepsy. Patients usually show profound mental retardation and a disproportionate growth failure. In contrast to earlier reports, patients usually survive beyond one year of age. A subset of patients with RCDP have been described with a milder clinical phenotype with less severe neurological impairments including moderate to severe mental retardation with motor problems but nearly normal skeletal findings with bone dysplasia only.[29] Recently, a variant form of RCDP was described in which the PTS2 pathway was affected as in RCDP type 1 but not because of mutations in *PEX7*. Instead, a homozygous frameshift mutation was identified in the PEX5L-specific exon 9 which results in the loss of PEX5L but not of PEX5S which explains why the PTS1 pathway has remained intact in this patient.[30] Apart from the two forms of RCDP described earlier caused by a defect in the import of PTS2 proteins into peroxisomes, there are three additional peroxisomal forms of RCDP caused by mutations in the genes coding for the peroxisomal EPL enzymes GNPAT, AGPS, and FAR1 (see[24] and[18] for review).

Biochemistry and Molecular Basis of RCDP

In all 5 forms of RCDP discussed earlier, there is a deficiency of plasmalogens in all tissues including erythrocytes, although in milder affected patients plasmalogen levels may only be mildly decreased. Furthermore, in type 1 and type 5, plasma phytanic acid levels may be increased but only in an age- and diet-dependent manner. The gene affected in type 1 is the *PEX7* gene which codes for the PTS2 receptor (see Figure 1B). In the patient with PEX5L deficiency, the PTS2 pathway is dysfunctional as in PEX7 deficiency (see Figure 1B) as a consequence of a homozygous frameshift mutation in exon 9 in *PEX5* (see[30]). In RCDP, type 2, 3, and 4 bi-allelic mutations have been identified in *GNPAT*, *AGPS*, and *FAR1*.[18,27,28]

X-Linked Adrenoleukodystrophy

The clinical expression of X-linked adrenoleukodystrophy (X-ALD) varies widely with different phenotypic variants described.[31] The classification of X-ALD is somewhat arbitrary and based on the age of onset and the organs principally involved. The 2 most frequent phenotypes are childhood cerebral ALD (CCALD) and adrenomyeloneuropathy (AMN). Age of onset of CCALD is usually between 3 and 10 years of age, with progressive behavioral, cognitive, and neurological deterioration often leading to total disability within 3 years.[32] The cerebral phenotype is not only observed in childhood but may also present later in life in adolescence or adulthood. There is a marked difference between cerebral ALD on the one hand and AMN on the other hand, since cerebral ALD shows an inflammatory reaction in the cerebral white matter which resembles but can be distinguished from what is observed in multiple sclerosis. In contrast to cerebral ALD, the inflammatory response is absent or mild in AMN which has a much later age of onset (>20 years of age) and a much slower rate of progression. It is important to mention that 85% of women heterozygous for X-ALD develop AMN-like symptoms in middle age or later.[33] X-ALD is one of the few PDs for which an effective therapy is available. Indeed, allogeneic hematopoietic cell transplantation (HCT) is an effective therapy provided it is performed at an early stage of brain disease.[34] The benefit of HCT in cerebral ALD is thought to be mediated by donor-derived replacement of myeloid-derived cells, possibly including microglial cells. Nevertheless, there are important limitations to HCT including the risk of graft failure and graft versus host disease. To this end, gene therapy with autologous hematopoietic stem cells has been investigated as an alternative to allogeneic HCT. In the initial proof-of-principle study, 4 boys

with cerebral ALD received autologous CD34+ hematopoietic stem cells transduced ex vivo with a lentiviral vector that contained human *ABCD1* complementary DNA (cDNA).[35] Results for 2 of these patients have been reported with both patients showing functional expression of adrenoleukodystrophyprotein (ALDP) and disease stabilization. Unfortunately, hematopoietic cell transplantation does not prevent the onset of AMN in adulthood.[36] Recently, Eichler et al[37] reported the results of 17 boys who received autologous hematopoietic stem cell gene therapy using a lentiviral vector. The authors concluded that Lenti-D gene therapy is a safe and effective alternative to allogeneic stem cell transplantation in boys with early-stage CCALD.[37]

Biochemistry and Molecular Basis of X-ALD

The product of *ABCD1* is ALDP which is a homodimeric half-ABC transporter localized in the peroxisomal membrane, taking care of the transport of very long-chain acyl-CoAs across the peroxisomal membrane.[31] A deficiency of ALDP leads to the accumulation of very long-chain acyl-CoAs in the cytosol which are subsequently incorporated into different lipid species including cholesterol esters. Plasma VLCFA analysis has been the method of choice for the identification of patients with X-ALD with all other peroxisomal biomarkers being normal. Work especially done by Moser et al[38] has shown that VLCFA analysis is a very robust method with very few false negatives. In recent years, C26:0-lysoPC (1-hexacosanoyl-2-lyso-sn-3-glycerolphosphorylcholine) has been proposed as an even better analyte to be determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS).[39] Recent work from our own center[40] has shown that C26:0-lysoPC in bloodspot is indeed a better and more accurate biomarker for X-ALD than plasma VLCFA levels. Approximately 15% of women with X-ALD have normal levels of VLCFA in plasma. Interestingly, these women all had elevated C26:0-lysoPC levels which suggests that heterozygosity for X-ALD can now be assessed realibly via a simple blood test. These observations clearly indicate that C26:0-lysoPC should be added to the routine biochemical array of diagnostic tests for X-ALD and other PDs as discussed in more detail later in this review.

Acyl-CoA Oxidase 1 Deficiency

Acyl-CoA oxidase 1 (ACOX1) deficiency first reported in 1988 by Poll-Thé et al[41] has so far been described in about 30 patients in the literature. The clinical, biochemical, and mutational spectrum of a cohort of 22 patients has been published.[42] All patients identified had psychomotor retardation but did acquire limited skills. In most (83%) patients, however, there was a loss of motor achievements. Brain imaging (MRI and/or CT scan) revealed cerebral and/or cerebellar white matter abnormalities in all patients investigated (12/12). Three of these patients showed neocortical dysplasia. Other abnormalities included hypotonia (97%), seizures (91%), visual system failure (78%), impaired hearing (77%), facial dysmorphia (50%), hepatomegaly (50%), and failure to thrive (37%). Interestingly, a late-onset form of ACOX1 deficiency has been described in 2 adult patients with normal early developmental milestones in whom the disease was progressive.[43]

Biochemistry and Molecular Basis of ACOX1 Deficiency

Acyl-CoA oxidase 1 plays a key role in the oxidation of VLCFAs but not other metabolites β-oxidized in peroxisomes, including pristanic acid and DHCA and THCA, which are degraded via 1 of the 2 other acyl-CoA oxidases.[14] This explains why there is only accumulation of VLCFAs in ACOX1 deficiency. Remarkably, in the literature patients have been described in whom there was no accumulation of VLCFAs in plasma (as discussed in more detail later in this review).[44] The molecular basis of ACOX1 has been described in detail[42] in the review of 22 patients. In all patients, bi-allelic mutations were identified in *ACOX1*.

D-Bifunctional Protein Deficiency

The clinical, biochemical, and mutational spectrum of D-bifunctional protein (DBP) deficiency has been described in 126 patients.[45] The clinical presentation of DBP deficiency is dominated by neonatal hypotonia (98%) and seizures (93%) within the first months of life. Failure to thrive was observed in 43% of the patients. Visual system failure, including nystagmus, strabismus, or failure to fixate objects, was reported in 39 (54%) patients. In addition, a progressive loss of vision and hearing was noted in 35% and 46% of the patients, respectively. Virtually none of the patients acquired any psychomotor development. External dysmorphia was present in 52 (58%) patients and resembled that of patients with ZS. Frequently described features include a high forehead, high arched palate, a large fontanel, long philtrum, epicanthal folds, hypertelorism, macrocephaly, shallow supraorbital ridges, retrognathia, and low-set ears. Recently, several adult patients have been identified through WES who presented with a slowly progressive, juvenile-onset phenotype that included cerebellar atrophy and ataxia, hearing loss, hypogonadism, hyperreflexia, and a demyelinating sensorimotor neuropathy.[46,47] Moreover, DBP deficiency has also been described in patients with Perrault syndrome, which is a genetically heterogeneous recessive disease characterized by ovarian dysgenesis in females and sensorineural deafness in both males and females.[48]

Biochemistry and Molecular Basis of DBP Deficiency

DBP is not only involved in the β-oxidation of VLCFAs but also of pristanic acid and the bile acid intermediates DHCA and THCA. This explains why in patients with classical DBP deficiency all these metabolites accumulate. Nevertheless, several patients have been described in the literature with an incomplete profile of peroxisomal biomarker abnormalities and in some patients with even a fully normal panel of metabolites.[45] With respect to the molecular basis of DBP deficiency, a large number of private mutations has been identified in the

HSD17B4 gene encoding DBP, with only 1 mutation being more frequent (c.46G→A; allele frequency: 24%). The latter mutation causes a glycine to serine amino acid substitution, which effectively prevents binding of nicotinamide adenine dinucleotide (NAD) because of a so-called steric clash.

α-Methylacyl-CoA Racemase Deficiency

α-Methylacyl-CoA Racemase (AMACR) deficiency has so far been described in only a few patients with different phenotypes. The first one involves a relatively mild form of the disease mimicking Refsum disease,[49] whereas the other is dominated by early-onset severe liver abnormalities.[50] In recent years yet other variants have been described, including tremor and deep white matter changes,[51] relapsing encephalopathy,[52] relapsing rhabdomyolysis,[53] and two patients with an adult-onset phenotype with peripheral neuropathy, epilepsy and relapsing encephalopathy, bilateral thalamic lesions, cataract, pigmentary retinopathy, and tremor.[54]

Biochemisty and Molecular Basis of AMACR Deficiency

The enzyme AMACR has 2 isoforms, one localized in peroxisomes and the other in mitochondria. The peroxisomal form of AMACR is not only involved in the racemization of (25*R*)-DHCA and THCA into the corresponding (25*S*)-compounds but also of (2*R*)-pristanic acid into the (2*S*)-form. This explains why there is accumulation of the peroxisomal biomarkers DHCA, THCA, and pristanic acid in patients with AMACR deficieny.[17] All patients have bi-allelic mutations in *AMACR*.

Refsum Disease (phytanoyl-CoA 2-Hydroxylase Deficiency)

Refsum disease is the only disorder known to be caused by a deficiency of FA α-oxidation. The disease is caused by a defect in phytanoyl-CoA 2-hydroxylase.[55] Clinical features of Refsum disease include retinitis pigmentosa as the most frequent abnormality, which usually culminates into blindness, followed by anosmia, cerebellar ataxia, peripheral neuropathy, and a number of other abnormalities that are less frequent.[56,57]

Biochemistry and Molecular Basis of Refsum Disease

In all patients with bona fide Refsum disease due to phytanoyl-CoA 2-hydroxylase deficiency, plasma phytanic acid levels are always abnormal, although the levels may vary considerably among patients.[58] In most cases, Refsum disease is caused by bi-allelic mutations in the PHYH gene encoding phytanoyl-CoA 2-hydroxylase. In rare variant cases, the phytanoyl-CoA 2-hydroxylase deficiency is secondary to a PTS2 protein import defect caused by mutations in the *PEX7* gene.[59]

Primary Hyperoxaluria Type 1

Primary hyperoxaluria type 1 (PH-1) belongs to the primary hyperoxalurias, which is a collective term encompassing an indeterminate number of genetically inherited conditions of which only PH-1, PH-2, and PH-3 have been characterized. PH-1 is clinically variable in terms of symptoms, age of onset, and mode of progression. Approximately one-fourth of patients have an infantile form with early nephrocalcinosis of whom 50% developed renal failure by 3 years of age, whereas 30% of patients have recurrent urolithiasis and renal failure in adolescence or early adulthood, and 21% have late-onset urolithiasis diagnosed in adulthood.[60] It is not unusual for the disease to go unrecognized and be first diagnosed after kidney transplantation. The PH-1 is the only type of primary hyperoxaluria caused by a deficiency of a peroxisomal enzyme AGXT. In contrast, PH-2 and PH-3 are due to deficiencies of the cytosolic enzyme glyoxylate reductase and the mitochondrial enzyme HOGA, respectively.[60]

Biochemistry and Molecular Basis of PH-1 Deficiency

The most common method of investigating PH-1 is by measuring urinary oxalate excretion. Markedly elevated urinary oxalate levels usually are indicative of either PH-1, PH-2, or PH-3. For differential diagnosis, urinary glycolate (elevated in PH-1), L-glycerate (elevated in PH-2), and 2,4-dihydroxyglutarate (elevated in PH-3) must be measured. It should be noted, however, that a significant number of patients with PH-1 with proven AGT deficiency have hyperoxaluria but no hyperglycolic aciduria. The PH-1 is caused by mutations in the *AGXT* gene coding for AGT rendering alanine glyoxylate aminotransferase inactive. However, in a substantial number of patients, the enzyme AGXT is enzymatically active, at least in homogenates, but physiologically inactive due to a peculiar peroxisome–to–mitochondria mistargeting phenotype (see[60] for review).

Bile Acid CoA: Amino Acid N-Acyltransferase Deficiency

The enzyme Amino acid N-acyltransferase BAAT is localized in peroxisomes and catalyzes the formation of tauro- and glycocholate as well as tauro- and glycochenodeoxycholate. The first patients with a defect in bile acid conjugation were identified by Setchell and coworkers.[61] Subsequently, Carlton and coworkers[62] described a series of patients from the Amish community with bonafide mutations in the *BAAT* gene. Patients were homozygous for a C.226A>G mutation (M76 V) and showed increased bile acids that were virtually fully unconjugated. Clinical features of these patients included fat malabsorption, failure to thrive, coagulopathy, pleuritis, and chronic upper respiratory infections. They did not have jaundice and had normal serum *gamma*-glutamyltransferase (γ GT) levels. In 2007, Heubi et al[63] reported 6 additional patients with mutations in the *BAAT* gene. The clinical phenotype included growth delay (3/6), neonatal cholestasis (3/6) including one with fulminant liver failure, and 5/6 with fat soluble vitamin deficiency, that is, vitamin A, E, and/or D. At diagnosis (5 months to 19 years), all but 1 had normal bilirubin and normal or minimally elevated transaminases.

Biochemistry and Molecular Basis of BAAT Deficiency

Laboratory diagnosis of BAAT deficiency requires bile acid analysis, which can best be performed by means of tandem mass spectrometry. In patients with BAAT deficiency, the glycine and taurine bile acid conjugates are virtually completely deficient in all body fluids, with the unconjugated form of cholic acid predominating.

Rare PDs

PMP70 deficiency[13] and sterol carrier protein X (SCPx) deficiency[64] have so far been described in single patients only and will not be discussed in detail here. Sufficient to say that SCPx-deficiency is an isolated PED affecting the degradation of 2-methyl branched-chain FAs by virtue of the fact that SCPx is the peroxisomal thiolase specifically involved in the peroxisomal β-oxidation of the 2-methyl branched-chain FAs pristanic acid and DHCA and THCA. Interestingly, in SCPx deficiency only trace amounts of DHCA and THCA are present in plasma, whereas urine bile acid analysis revealed a range of abnormal bile acids, including several bile-alcohol glucuronides (m/z: 611, 613, 627, 629, 643, and 645).[64] In PMP70 deficiency, there is accumulation of DHCA and THCA in the plasma.[13]

Newly Identified PDs

Acyl-CoA oxidase 2 (ACOX2) deficiency is a recently identified peroxisomal disorder of bile acid biosynthesis. All patients had intermittently or persistently elevated transaminases.[14,65,66] In one patient, this was combined with liver fibrosis, mild ataxia, and cognitive impairment, whereas in another patient, severe liver damage occurred after exposure to non-steroidal anti-inflammatory drugs (NSAIDs). The identification of patients with ACOX2 deficiency led to reinvestigation of the human peroxisomal acyl-CoA oxidases and the recognition that human peroxisomes harbor not 2 but 3 ACOX enzymes.[14] ACOX1 is responsible for the oxidation of the very long-chain fatty acyl-CoA's, ACOX2 and ACOX3 can both handle the branched-chain acyl-CoAs, whereas ACOX2 is the only enzyme involved in bile acid biosynthesis. This explains why in patients with ACOX2 deficieny VLCFA and pristanic acid levels are normal, but the bile acid intermediates DHCA and THCA accumulate while the primary bile acid levels are low.

ACBD5 deficiency is a recently described disorder of VLCFA metabolism identified in patients with progressive white matter disease and retinal dystrophy.[67] ACBD5 is a tail-anchored PMP with a cytosolic acyl-CoA binding domain and interacts with the ER proteins VAPA/B, thereby tethering peroxisomes to the ER. Very long chain fatty acids are synthesized by chain-elongation of dietary long-chain FAs at the ER, and for degradation they need to be transported to the peroxisome. This process is facilitated by ACBD5 through tethering of peroxisomes to the ER and its acyl-CoA-binding capacity.[68,69] As a consequence, in case of a deficiency of ACBD5, VLCFA metabolism is disturbed resulting in an accumulation and increased levels of C26lysoPC.

Laboratory Diagnosis of PDs

The laboratory diagnosis of PDs has long been based on the analysis of the relevant biomarkers in blood and/or urine in candidate patients with clinical signs and symptoms indicative of a PD. Figure 4 lists the various biomarkers for each of (groups of) PDs. This classical approach starting from the clinical signs and symptoms to biochemical analyses in blood and urine followed by enzymatic and molecular studies has been extremely rewarding and continues to do so. However, through the years, it has become clear that this approach also has its limitations, especially since biomarkers may be minimally abnormal to completely normal in a subset of patients. Indeed, in patients with the classical neonatal–infantile form of ZSD, the full spectrum of metabolic abnormalities may be observed which includes elevated plasma levels of VLCFAs, the bile acid intermediates DHCA and THCA, phytanic acid, pristanic acid, pipecolic acid, and a deficiency of plasmalogens in erythrocytes, but there are also patients in whom biomarker analysis only revealed minimal to no abnormalities. Moreover, it is well established by now that the peroxisomal biomarkers may well be abnormal in ZSD patients early in life but tend to normalize later in life.[26] The underlying basis for this peculiar finding has not yet been established but must involve an increase in the number of functional peroxisomes, notably in the liver of patients, especially since peroxisomal biomarkers such as the bile acid intermediates and L-pipecolic acid are solely degraded in the liver. Work done in our own laboratory by Klouwer et al[24] has shown that the classical C26:0–C22:0 ratio was only abnormal in 29 of the 37 ZSD patients of the Amsterdam ZSD-cohort. Importantly, the finding of normal peroxisomal biomarkers is not confined to ZSD patients but is also true for other PDs such as ACOX1 deficiency, DBP deficiency, and X-ALD.

The finding that biomarker analysis can lead to false-negative results is a real diagnostic problem and has prompted the question whether this diagnostic dilemma can be resolved. There are at least 3 possibilities to resolve this issue, including (1) search for improved biomarkers; (2) biochemical studies, notably in fibroblasts; and/or (3) genome sequencing.

(1) *Search for improved biomarkers*. Many of the biomarkers known today in the field of inborn errors of metabolism (IEM) have not been identified based on a thorough investigation of the complete metabolome which implies that it may well be that for many IEMs there may actually be better biomarkers compared to those that are currently being used. This has already been demonstrated for the C26:0–C22:0 ratio used as a biomarker for some of the PDs. Work done by Hubberd et al[39] had suggested that C26:0-lysoPC as measured by LC-MS/MS may well be a better biomarker, at least for X-ALD, and our own recent work is in full

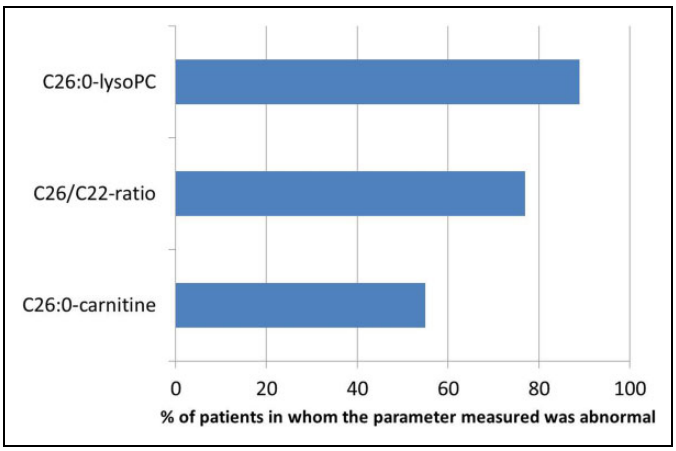

Figure 6. Discriminative power of 3 peroxisomal biomarkers in a series of 37 patients belonging to the Amsterdam ZSD cohort.

support of this notion.[40] First, Klouwer et al[70] showed that analysis of C26:0-lysoPC has a much better predictive value for ZSD diagnostics than the classical C26:0–C22:0 ratio, since C26:0-lysoPC was found to be abnormal in 33 (89%) of the 37 patients of the Amsterdam ZSD cohort, whereas the C26:0–C22:0 ratio in these patients was only abnormal in 29 (77%) of 37 patients (see Figure 6). Second, work done by Huffnagel and coworkers in our laboratory has shown that C26:0-lysoPC is also the preferred biomarker for X-ALD and is superior to other biomarkers, including the C26:0–C22:0 ratio and C26:0-acylcarnitine.[40] Indeed, the discriminative power of C26:0-lysoPC was not only found to be much better for males with ALD but also for females with ALD. In fact, C26:0-lysoPC analysis gave a 100% score in a series of 46 obligate heterozygotes from the Amsterdam X-ALD cohort, whereas the classical C26:0–C22:0 ratio was only abnormal in 40 of 46 ALD females (sensitivity: 87%). Despite these improvements, there is certainly still room for improved biomarker discovery also in the field of PDs, and the rapid technological developments in the field of metabolite analysis have opened up the way to achieve this goal. Thanks to the introduction of high-resolution tandem MS, the metabolome can now be analyzed in much more detail than ever before which has led to the development of new platforms covering lipids (*lipidomics*), glycans (*glycomics*), and a much wider spectrum of other metabolites using both targeted and nontargeted methods.

We have already begun to use lipidomics for the identification of improved biomarkers which would not only allow patients to be identified with greater precision but also reflect the clinical status of individual patients and guide the way toward the best treatment and prediction of disease severity in case of early diagnosis. Work by Herzog et al[71] has resulted in the identification of disease-specific phospholipid ratios which, at least in fibroblasts, discriminate between patients affected by a mild or severe form of ZSD. Lipidomic analysis in plasma from patient affected by different PDs has also identified specific abnormalities.[72]

(2) *Biochemical studies*. As described in greater detail by Ferdinandusse et al,[25] biochemical studies remain of utmost importance for the proper diagnosis of patients. First, since biomarker analysis may reveal no abnormalities as discussed earlier, biochemical studies notably in fibroblasts are the way to go, especially if the clinical signs and symptoms are indicative for a peroxisomal disorder. Many examples of the success of this approach have been described in the literature (see the study by Ferdinandusse et al[25]). One example is the case published by Rosewich et al[44] of a patient who presented with dysmorphic features (high forehead, broad nasal bridge, high-arched palate, syndactyly of the second and third toes on both feet), muscular hypotonia, and clonic seizures at 2 months of life. Retinopathy and psychomotor retardation became evident later. The MRI analysis revealed a typical pattern, showing pachygyria, perisylvian polymicrogyria, and cerebral and cerebellar white matter abnormalities. Repetitive analysis of plasma VLCFAs and other peroxisomal metabolites revealed no abnormalities. Because of the suggestive MRI findings, biochemical studies in fibroblasts were performed which revealed a deficiency of acyl-CoA oxidase 1 due to biallelic mutations in ACOX1. Apart from the importance of biochemical studies in fibroblasts for the primary diagnosis of PDs, biochemical studies may also be of utmost importance following WES/WGS studies as exemplified. In addition, biochemical and metabolite studies in fibroblasts can be valuable for the prediction of the clinical severity which is of great importance in determining treatment stratagies.

(3) *Genome sequencing (WES/WGS)*. The amazing technological achievements in the field of DNA-sequencing now allow sequencing of the complete exome and genome with increasing accuracy and fidelity. As a consequence, WES/WGS is now an essential element in the repertoire of methods used to identify the underlying defect in IEM patients. In some centers, WES/WGS has taken over from metabolite screening as first-line test, and metabolic screening may not be performed at all, at least in some centers. In our own center in Amsterdam, we take a different approach based on 2 different scenarios as described (see Figure 7).

Scenario 1: In case of patients with clinical signs and symptoms suggestive for a certain IEM which includes PDs, metabolic studies should be done first at least when a relatively reliable biomarker is available. For instance, in case of a patient with clinical signs and symptoms suggestive for a ZSD, we strongly advocate

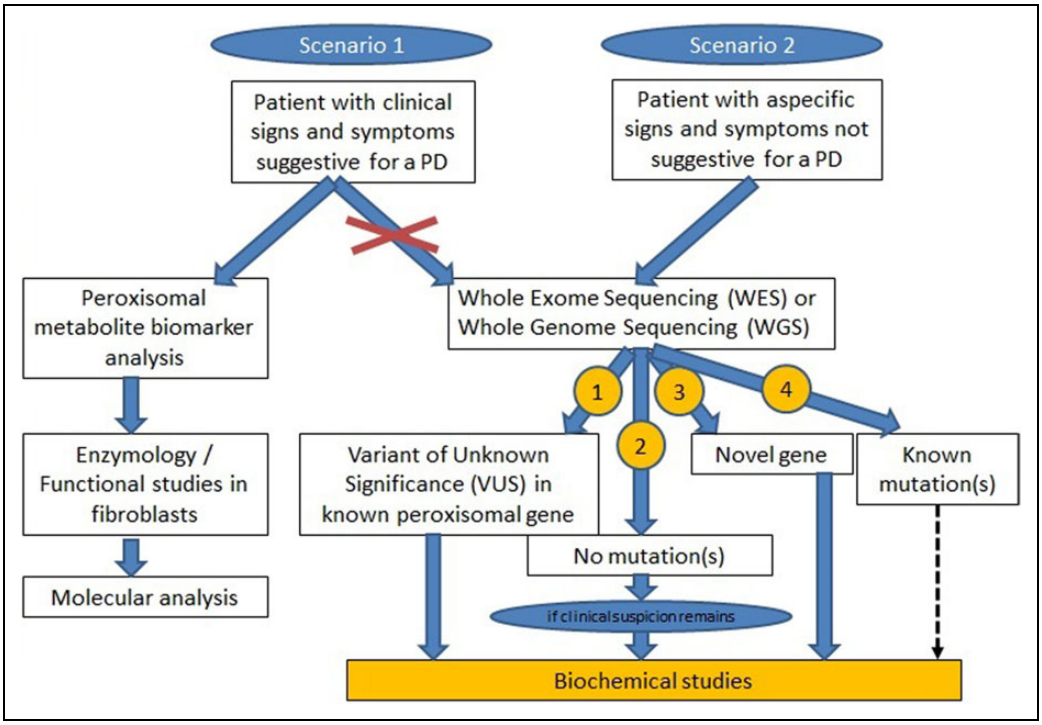

Figure 7. Flowchart for the laboratory diagnosis of patients showing clinical signs and symptoms suggestive for a peroxisomal disorder (PD; scenario 1) or not (scenario 2). See text for detailed information.

to do C26:0-lysoPC analysis first ideally combined with the analysis of other peroxisomal biomarkers. If abnormal, the patient is almost certainly affected by some PD, and subsequent work in fibroblasts has to be done to resolve whether the patient is affected by a PBD or a defect in one of the metabolic pathways in peroxisomes which should then be followed up by molecular studies, either using Sanger sequencing of individual genes or panel sequencing.[28]

Scenario 2: If a patient is presenting with aspecific signs and symptoms and there is no a priori basis to suspect a peroxisomal disorder or, in fact, a certain IEM, WES/WGS is the way to go, although we always advocate to perform metabolite studies in parallel for various reasons as detailed below, which include:

(1) Metabolite studies in patients selected for WES/WGS may well be very helpful in interpreting the results obtained by WES/WGS. Indeed, since WES/WGS analysis usually generates a list of candidate genes rather than a single one, it is extremely helpful if biochemical analyses have already identified 1 or more biochemical abnormalities. Again, there are many examples in the literature supporting this notion. One example is that of NANS deficiency in which Trio WES had identified 19 candidate genes with pathogenic variants. The finding of N-acetylmannosamine upon next-generation metabolic screening paved the way to the identification of the affected gene out of the series of 19 candidate genes.[73] Another example is that of NADK2 deficiency, again with a long list of candidate genes that could rapidly be reduced to a single candidate gene because of the accumulation of a peculiar acylcarnitine (dodecadienoyl carnitine; C10:2-acylcarnitine).[74]

(2) Metabolic studies are of key importance to allow interpretation of variants of unknown significance. Indeed, interpretation of sequence variants produced by WES/WGS is often not straightforward because many of the variants may not have been reported in the literature and/or may not have been tested experimentally. This is true for new exonic variants and even more so for intronic variants. Moreover, prediction programs like POLYPHEN and SIFT are suboptimal, and prediction rates are far removed from100% accuracy. In clinical molecular genetics, 5 different classes of sequence variants are being discriminated of which only class 1 ("clearly not pathogenic") and class 5 ("clearly pathogenic") are useful in terms of correct molecular diagnosis in patients. It is clear that the identification of a class 2, 3, or 4 variant should definitely be followed up by biochemical studies (see Figure 7). Again, there are many examples to be found in the literature emphasizing this point. An example from our own work is that of Schackmann et al which started with a female patient having clinical features suggestive for ALD and an otherwise uninformative family.[75] Plasma VLCFA analysis was done in the patient which revealed no abnormalities. However, since this did not rule out heterozygosity for X-ALD because of the fact

that VLCFAs are only abnormal in 85% of obligate heterozygotes, molecular analysis of *ABCD1* was performed which revealed a c.50 G>A-variant (p.Arg17His) suggested to be pathogenic. Using a novel method based on the use of SV-40 immortalized fibroblasts from the patient which were then clonally selected and biochemically analyzed, it became clear that the variant was in fact not pathogenic.

(3) Mono-allelic mutations rather than bi-allelic mutations: We recently discovered an apparently dominant form of ZS which is in principle an autosomal recessive disorder, with important consequences for the interpretation of WES and WGS data in clinical diagnostic laboratories.[76] It all started with a patient with signs and symptoms suggestive for a PD. Peroxisomal biomarker analysis revealed clearly abnormal VLCFAs, and biochemical studies in fibroblasts from the patient showed a severe import defect pointing to a PBD. Subsequently, complementation analysis was performed which identified *PEX6* as the gene involved. Molecular analysis, however, only revealed a single heterozygous variant (c.2578C>T) which turns a higly conserved arginine into tryptophane, whereas no mutations were found on the other *PEX6* allele. The disease mechanism was only resolved when cDNA analysis was done in fibroblasts from the patient and his mother which revealed that there was allelic expression imbalance promoting the mutant *PEX6* allele in the patient but not in his mother. Collaborative efforts led to the identification of a series of patients with a similar genotype. The importance of this finding lies in the fact that allelic expression levels are not functionally verified in general unless messenger RNA analysis is done specifically and variants inherited from asymptomatic parents are usually filtered out and considered nonpathogenic.[76]

In summary, the clinical as well as biochemical and genetic spectrum of PDs and in fact IEMs in general has widened tremendously in the last few years, thanks to the revolutionairy developments in the area of genome sequencing and other -omics technologies. Correct interpretation of the data gathered requires functional studies including (improved) biomarker analysis and biochemical studies whether a patient presents with suggestive clinical signs and symptoms (scenario 1) or not (scenario 2; see Figure 7). Only if the mutations identified by WES/WGS have been functionally characterized and described in the literature before, one may refrain from biochemical studies although in our hands subsequent work in fibroblasts remains important, also in these cases, since this allows one to establish the extent of the deficiency in greater detail.

Acknowledgments

The authors gratefully acknowledge the expert help of Mrs Maddy Festen for preparing the manuscript and Mr Jos Ruiter, MSc, for drawing of the figures.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

References

1. Gronemeyer T, Wiese S, Ofman R, et al. The proteome of human liver peroxisomes: identification of five new peroxisomal constituents by a label-free quantitative proteomics survey. *PLoS One*. 2013;8(2):1-12.
2. Gould SG, Keller GA, Subramani S. Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase. *J Cell Biol*. 1987;105(6 Pt 2):2923-2931.
3. Kim PK, Hettema EH. Multiple pathways for protein transport to peroxisomes. *J Mol Biol*. 2015;427(6 Pt A):1176-1190.
4. Gatto GJ Jr, Geisbrecht BV, Gould SJ, Berg JM. Peroxisomal targeting signal-1 recognition by the TPR domains of human PEX5. *Nat Struct Biol*. 2000;7:1091-1095.
5. Stanley WA, Filipp FV, Kursula P, et al. Recognition of a functional peroxisome type 1 target by the dynamic import receptor pex5p. *Mol Cell*. 2006;24(5):653-663.
6. Petriv OI, Tang L, Titorenko VI, Rachubinski RA. A new definition for the consensus sequence of the peroxisome targeting signal type 2. *J Mol Biol*. 2004;341(1):119-134.
7. Zientara-Rytter K, Subramani S. Autophagic degradation of peroxisomes in mammals. *Biochem Soc Trans*. 2016;44:431-440.
8. Rizzo C, Boenzi S, Wanders RJ, Duran M, Caruso U, Dionisi-Vici C. Characteristic acylcarnitine profiles in inherited defects of peroxisome biogenesis: a novel tool for screening diagnosis using tandem-MS. *Pediatr Res*. 2003;53(6):1-6.
9. Van Veldhoven PP. Biochemistry and genetics of inherited disorders of peroxisomal fatty acid metabolism, *J Lipid Res*. 2010; 51(10):2863-2895.
10. Wanders RJA, Waterham HR. Biochemistry of mamalian peroxisomes revisited. *Annu Rev Biochem*. 2006;75:295-332.
11. van Roermund CW, Visser WF, Ijlst L, et al. The human peroxisomal ABC half transporter ALDP functions as a homodimer and accepts acyl-CoA esters. *FASEB J*. 2008;22:4201-4208.
12. Wiesinger C, Kunze M, Regelsberger G, Forss-Petter S, Berger J. Impaired very long-chain Acyl-CoA beta-oxidation in human X-ALD fibroblasts is a direct consequence of ABCD1 transporter dysfunction. *J Biol Chem*. 2013;288(26):19269-19279.
13. Ferdinandusse S, Jimenez-Sanchez G, Koster J, et al. A novel bile acid biosynthesis defect due to a deficiency of peroxisomal ABCD3. *Hum Mol Genet*. 2015;24(2):361-370.
14. Ferdinandusse S, Denis S, van Roermund CWT, et al. A novel case of ACOX2 deficiency leads to recognition of a third human peroxisomal acyl-CoA oxidase. *Biochim Biophys Acta*. 2018;1864(3):952-958.
15. Wanders RJA, Komen JC, Ferdinandusse S. Phytanic acid metabolism in health and disease. *Biochim Biophys Acta*. 2011;1811(9):498-507.
16. Wanders RJ, Waterham HR, Ferdinandusse S. Metabolic interplay between peroxisomes and other subcellular organelles including mitochondria and the endoplasmic reticulum. *Front Cell Dev Biol*. 2015;3:83.

17. Vaz FM, Ferdinandusse S. Bile acid analysis in human disorders of bile acid biosynthesis, *Mol Aspects Med*. 2017;56:10-24.
18. Argyriou C, D'Agostino MD, Braverman N. Peroxisome biogenesis disorders. *Transl Sci Rare Dis*. 2016;1(2):111-144.
19. Braverman NE, D'Agostino MD, Maclean GE. Peroxisome biogenesis disorders: biological, clinical and pathophysiological perspectives. *Dev Disabil Res Rev*. 2013:17(3):187-196.
20. Klouwer FC, Berendse K, Ferdinandusse S, Wanders RJ, Engelen M, Poll-The BT. Zellweger spectrum disorders: clinical overview and management approach. *Orphanet J Rare Dis*. 2015;10:151.
21. Brown FR, McAdams AJ, Cummins JW, et al. Cerebro-hepatorenal (Zellweger) syndrome and neonatal adrenoleukodystrophy: similarities in phenotype and accumulation of very long chain fatty acids. *Johns Hopkins Med. J*. 1982;151(6):344-351.
22. Heymans HS, Schutgens RB, Tan R, van den Bosch H, Borst P. Severe plasmalogen deficiency in tissues of infants without peroxisomes (Zellweger syndrome). *Nature*. 1983;306(5938):69-70.
23. Ratbi I, Falkenberg KD, Sommen M, et al. Heimler syndrome is caused by hypomorphic mutations in the peroxisome-biogenesis genes PEX1 and PEX6. *Am J Hum Genet*. 2015;97(4):535-545.
24. Klouwer FC, Huffnagel IC, Ferdinandusse S, et al. Clinical and biochemical pitfalls in the diagnosis of peroxisomal disorders. *Neuropediatrics*. 2016;47(4):205-220.
25. Ferdinandusse S, Ebberink MS, Vaz FM, Waterham HR, Wanders RJ. The important role of biochemical and functional studies in the diagnostics of peroxisomal disorders. *J Inheri Metab Dis*. 2016;39(4):531-543.
26. Berendse K, Engelen M, Ferdinandusse S, et al. Zellweger spectrum disorders: clinical manifestations in patients surviving into adulthood. *J Inherit Metab Dis*. 2016;39(1):93-106.
27. Waterham HR, Ebberink MS. Genetics and molecular basis of human peroxisome biogenesis disorders. *Biochim Biophys Acta*. 2012;1822(9):1430-1441.
28. Waterham HR, Ferdinandusse S, Wanders RJ. Human disorders of peroxisome metabolism and biogenesis. *Biochim Biophys Acta*. 2016;1863(5):922-933.
29. Smeitink JA, Beemer FA, Espeel M, et al. Bone dysplasia associated with phytanic acid accumulation and deficient plasmalogen synthesis: a peroxisomal entity amenable to plasmapheresis. *J Inherit Metab Dis*. 1992;15(3):377-380.
30. Barøy T, Koster J, Strømme P, et al. A novel type of rhizomelic chondrodysplasia punctata, RCDP5, is caused by loss of the PEX5 long isoform. *Hum Mol Genet*. 2015;24(20):5845-5854.
31. Kemp S, Huffnagel IC, Linthorst GE, Wanders RJ, Engelen M. Adrenoleukodystrophy - neuroendocrine pathogenesis and redefinition of natural history. *Nat Rev Endocrinol*. 2016;12(10):606-615.
32. Engelen M, Kemp S, de Visser M, et al. X-linked adrenoleukodystrophy (X-ALD): clinical presentation and guidelines for diagnosis, follow-up and management. *Orphanet J Rare Dis*. 2012;7:51.
33. Engelen M, Barbier M, Dijkstra IM, et al. X-linked adrenoleukodystrophy in women: a cross-sectional cohort study. *Brain*. 2014; 137(Pt 3):693-706.
34. Cartier N, Hacein-Bey-Abina S, Bartholomae CC, et al. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science*. 2009;326(5954):818-823.
35. Flavigny E1, Sanhaj A, Aubourg P, Cartier N. Retroviral-mediated adrenoleukodystrophy-related gene transfer corrects very long chain fatty acid metabolism in adrenoleukodystrophy fibroblasts: implications for therapy. *FEBS Lett*. 1999;448(2-3):261-264.
36. van Geel BM, Poll-The BT, Verrips A, Boelens JJ, Kemp S, Engelen M. Hematopoietic cell transplantation does not prevent myelopathy in X-linked adrenoleukodystrophy: a retrospective study. *J Inherit Metab Dis*. 2015;38(2):359-361.
37. Eichler F, Duncan C, Musolino PL, et al. Hematopoietic stem-cell gene therapy for cerebral adrenoleukodystrophy. *N Engl J Med*. 2017;377(17):1630-1638.
38. Moser AB, Kreiter N, Bezman L, et al. Plasma very long chain fatty acids in 3,000 peroxisome disease patients and 29,000 controls. *Ann Neurol*. 1999;45(1):100-110.
39. Hubbard WC, Moser AB, Liu AC, et al. Newborn screening for X-linked adrenoleukodystrophy (X-ALD): validation of a combined liquid chromatography-tandem mass spectrometric (LC-MS/MS) method, *Mol Genet Metab*. 2009;97(3):212-220.
40. Huffnagel IC, van de Beek MC, Showers AL, et al. Comparison of C26:0-carnitine and C26:0-lysophosphatidylcholine as diagnostic markers in dried blood spots from newborns and patients with adrenoleukodystrophy. *Mol Genet Metab*. 2017;122(4):209-215.
41. Poll-The BT, Roels F, Ogier H, et al. A new peroxisomal disorder with enlarged peroxisomes and a specific deficiency of acyl-CoA oxidase (pseudo-neonatal adrenoleukodystrophy). *Am J Hum Genet*. 1988;42(3):422-434.
42. Ferdinandusse S, Denis S, Hogenhout EM, et al. Clinical, biochemical, and mutational spectrum of peroxisomal acyl-coenzyme A oxidase deficiency. *Hum Mutat*. 2007;28(9):904-912.
43. Ferdinandusse S, Barker S, Lachlan K, et al. Adult peroxisomal acyl-coenzyme A oxidase deficiency with cerebellar and brainstem atrophy. *J Neurol Neurosurg Psychiatry*. 2010;81(3): 310-312.
44. Rosewich H, Waterham HR, Wanders RJ, et al. Pitfall in metabolic screening in a patient with fatal peroxisomal beta-oxidation defect. *Neuropediatrics*. 2006;37(2):95-98.
45. Ferdinandusse S, Denis S, Mooyer PA, et al. Clinical and biochemical spectrum of D-bifunctional protein deficiency. *Ann Neurol*. 2006;59(1):92-104.
46. Lines MA, Jobling R, Brady L, et al. Peroxisomal D-bifunctional protein deficiency: three adults diagnosed by whole-exome sequencing. *Neurology*. 2014;82(11):963-968.
47. Lieber DS, Hershman SG, Slate NG, et al. Next generation sequencing with copy number variant detection expands the phenotypic spectrum of HSD17B4-deficiency. *BMC Med Genet*. 2014;15:30.
48. Pierce SB, Walsh T, Chisholm KM, et al. Mutations in the DBP-deficiency protein HSD17B4 cause ovarian dysgenesis, hearing loss, and ataxia of Perrault Syndrome. *Am J Hum Genet*. 2010; 87(2):282-288.
49. Ferdinandusse S, Denis S, Clayton PT, et al. Mutations in the gene encoding peroxisomal alpha-methylacyl-CoA racemase cause adult-onset sensory motor neuropathy. *Nat Genet*. 2000;24(2): 188-191.
50. Setchel KD, Heubi JE, Bove KE, et al. Liver disease caused by failure to racemize trihydroxycholestanoic acid: gene mutation

and effect of bile acid therapy. *Gastroenterology*. 2003;124(1): 217-232.
51. Clarke CE, Alger S, Preece MA, et al. Tremor and deep white matter changes in alpha-methylacyl-CoA racemase deficiency. *Neurology*. 2004;63(1):188-189.
52. Thompson SA, Calvin J, Hogg S, Ferdinandusse S, Wanders RJ, Barker RA. Relapsing encephalopathy in a patient with alpha-methylacyl-CoA racemase deficiency. *J Neurol Neurosurg Psychiatry*. 2008;79(4):448-450.
53. Kapina V, Sedel F, Truffert A, et al. Relapsing rhabdomyolysis due to peroxisomal alpha-methylacyl-CoA racemase deficiency. *Neurology*. 2010;75(14):1300-1302.
54. Haugarvoll K, Johansson S, Tzoulis C, et al. MRI characterisation of adult onset alpha-methylacyl-coA racemase deficiency diagnosed by exome sequencing. *Orphanet J Rare Dis*. 2013;8:1-10.
55. Jansen GA, Wanders RJA, Watkins PA, Mihalik S. J. Phytanoyl-Coenzyme A hydroxylase deficiency - the enzyme defect in Refsum's disease. *N Engl J Med*. 1997;337(2), 133-134.
56. Wierzbicki AS., Lloyd MD., Schofield CJ, Feher MD, Gibberd F. B. Refsum's disease: a peroxisomal disorder affecting phytanic acid alpha-oxidation. *J. Neurochem*. 2002;80(5):727-735.
57. Wanders RJA, Jakobs C, Skjeldal OH. Refsum disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic & Molecular Bases of Inherited Disease*. 8th ed. New York, NY: Mc Graw-Hill; 2001:3303-3321.
58. Wanders RJA, Waterham HR, Leroy B. Refsum Disease. *GeneReviews*. 2006. www.genetests.org.
59. van den Brink DM, Brites P, Haasjes J, et al. Identification of PEX7 as the Second Gene Involved in Refsum Disease. *Am J Hum Genet*. 2003;72(2):471-477.
60. Salido E, Pey AL, Rodriguez R, Lorenzo V. Primary hyperoxalurias: disorders of glyoxylate detoxification. *Biochim Biophys Acta*. 2012;1822(9):1453-1464.
61. Bove KE, Daugherty CC, Tyson W, et al. Bile acid synthetic defects and liver disease. *Pediatr Dev Pathol*. 2000;3(1):1-16.
62. Carlton VE, Harris BZ, Puffenberger EG, et al. Complex inheritance of familial hypercholanemia with associated mutations in TJP2 and BAAT. *Nat Genet*. 2003;34(1):91-96.
63. Heubi JE, Setchell KD, Bove KE. Inborn errors of bile acid metabolism. *Semin Liver Dis*. 2007;27(3):282-294.
64. Ferdinandusse S, Kostopoulos P, Denis S, et al. Mutations in the gene encoding peroxisomal sterol carrier protein X (SCPx) cause leukencephalopathy with dystonia and motor neuropathy. *Am J Hum Genet*. 2006;78(6):1046-1052.
65. Vilarinho S, Sari S, Mazzacuva F, et al. ACOX2 deficiency: A disorder of bile acid synthesis with transaminase elevation, liver fibrosis, ataxia, and cognitive impairment. *Proc Natl Acad Sci U. S. A*. 2016;113(40):11289-11293.
66. Monte MJ, Alonso-Peña M, Briz O, et al. ACOX2 deficiency: an inborn error of bile acid synthesis identified in an adolescent with persistent hypertransaminasemia. *J Hepatol*. 2017;66(3):581-588.
67. Ferdinandusse S, Falkenberg KD, Koster J, et al. ACBD5 deficiency causes a defect in peroxisomal very long-chain fatty acid metabolism. *J Med Genet*. 2017;54(5):330-337.
68. Costello JL, Castro IG, Hacker C, et al. ACBD5 and VAPB mediate membrane associations between peroxisomes and the ER. *J Cell Biol*. 2017;216(2):331-342.
69. Hua R, Cheng D, Coyaud É, et al. VAPs and ACBD5 tether peroxisomes to the ER for peroxisome maintenance and lipid homeostasi. *J Cell Biol*. 2017;216(2):367-377.
70. Klouwer FCC, Ferdinandusse S, van Lenthe H, et al. Evaluation of C26:0-lysophosphatidylcholine and C26:0-carnitine as diagnostic markers for Zellweger spectrum disorders. *J Inherit Metab Dis*. 2017;40(6):875-881.
71. Herzog K, Pras-Raves ML, Vervaart MA, et al. Lipidomic analysis of fibroblasts from Zellweger spectrum disorder patients identifies disease-specific phospholipid ratios. *J Lipid Res*. 2016;57(8):1447-1454.
72. Herzog K, Pras-Raves ML, Ferdinandusse S, et al. Functional characterisation of peroxisomal beta-oxidation disorders in fibroblasts using lipidomics. *J Inherit Metab Dis*. 2018;41(3):479-487.
73. van Karnebeek CD, Bonafé L, Wen XY, et al. NANS-mediated synthesis of sialic acid is required for brain and skeletal development. *Nat Genet*. 2016;48(7):777-784.
74. Houten SM, Denis S, Te Brinke H, et al. Mitochondrial NADP(H) deficiency due to a mutation in NADK2 causes dienoyl-CoA reductase deficiency with hyperlysinemia. *Hum Mol Genet*. 2014;23(18):5009-5016.
75. Schackmann MJ, Ofman R, van Geel BM, et al. Pathogenicity of novel ABCD1 variants: the need for biochemical testing in the era of advanced genetics. *Mol Genet Metab*. 2016;118(2):123-127.
76. Falkenberg KD, Braverman NE, Moser AB, et al. Allelic expression imbalance promoting a mutant PEX6 allele causes zellweger spectrum disorder. *Am J Hum Genet*. 2017;101(6):965-976.