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Review

Genetics and molecular basis of human peroxisome biogenesis disorders ☆

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ABSTRACT

Human peroxisome biogenesis disorders (PBDs) are a heterogeneous group of autosomal recessive disorders comprised of two clinically distinct subtypes: the Zellweger syndrome spectrum (ZSS) disorders and rhizomelic chondrodysplasia punctata (RCDP) type 1. PBDs are caused by defects in any of at least 14 different *PEX* genes, which encode proteins involved in peroxisome assembly and proliferation. Thirteen of these genes are associated with ZSS disorders. The genetic heterogeneity among PBDs and the inability to predict from the biochemical and clinical phenotype of a patient with ZSS which of the currently known 13 *PEX* genes is defective, has fostered the development of different strategies to identify the causative gene defects. These include *PEX* cDNA transfection complementation assays followed by sequencing of the thus identified *PEX* genes, and a *PEX* gene screen in which the most frequently mutated exons of the different *PEX* genes are analyzed. The benefits of DNA testing for PBDs include carrier testing of relatives, early prenatal testing or preimplantation genetic diagnosis in families with a recurrence risk for ZSS disorders, and insight in genotype–phenotype correlations, which may eventually assist to improve patient management. In this review we describe the current status of genetic analysis and the molecular basis of PBDs. This article is part of a Special Issue entitled: Metabolic Functions and Biogenesis of peroxisomes in Health and Disease.

1. Introduction

Peroxisomes are single-membrane bound organelles with a protein-rich matrix, that are present in virtually all eukaryotic cells and organisms ranging from unicellular yeasts to multicellular mammals including humans. Peroxisomes are dynamic organelles that are able to adjust their number and protein content in response to metabolic needs and physiological conditions. Consequently, the number of peroxisomes in human cells may range from 100 to more than 1000 per cell and they are most abundant in liver and kidney tubular cells. The half-life of peroxisomes in cultured skin fibroblast cells is approximately two days [1].

Peroxisomes play an important role in a number of essential metabolic pathways, including, in humans, the biosynthesis of ether phospholipids and bile acids, the α- and β-oxidation of fatty acids, and the detoxification of glyoxylate and of reactive oxygen species generated in peroxisomes, i.e. hydrogen peroxide and superoxide [2,3]. It is estimated that mammalian peroxisomes harbor more than 50 different enzyme activities, some of which are tissue-specific while others are shared with other subcellular compartments including mitochondria [3].

Given the central role of peroxisomes in human metabolism, it is not surprising that defects in genes encoding peroxisomal proteins result in a variety of different, often severe disorders. In fact, the biochemical and genetic elucidation and characterization of such disorders have been instrumental for our current understanding of peroxisome biology, biochemistry and genetics [4–8]. Peroxisomal disorders can be classified into two groups, including (*a*) the single peroxisomal enzyme deficiencies and (*b*) the peroxisome biogenesis disorders (PBDs) [3,5,6]. In this review we will focus on the molecular basis of PBDs.

2. Clinical aspects of PBDs

The PBDs consist of two distinct subtypes: the Zellweger syndrome spectrum (ZSS) disorders and rhizomelic chondrodysplasia punctata (RCDP) type 1. RCDP type 1 is clinically clearly distinct from the ZSS disorders and characterized by proximal shortening of the limbs (rhizomelia), multiple punctuate epiphyseal calcification (chondrodysplasia punctata), cataracts, facial dysmorphism, microcephaly, small stature, and psychomotor retardation [9]. RCDP type 1 is indistinguishable from RCDP type 2 and RCDP type 3, which both are single peroxisomal enzyme deficiencies due to isolated defects of respectively dihydroxyacetone phosphate acyl transferase (DHAPAT/GNPAT) and alkyl-DHAP synthase, two peroxisomal matrix enzymes involved in plasmalogen biosynthesis. Alkyl-DHAP synthase is not imported into peroxisomes in RCDP type 1 cells, indicating that the clinical phenotype of RCDP type 1 is primarily a consequence of a disturbed plasmalogen synthesis [10–14].

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The Zellweger syndrome spectrum (ZSS) consists of three overlapping clinical phenotypes: the cerebrohepatorenal syndrome or Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), and infantile Refsum disease (IRD) [7,8]. Although originally described as separate clinical entities they are now considered different presentations within the same clinical and biochemical continuum with ZS being the most and IRD the least severe. Patients who based on clinical presentation belong to the severe side of the continuum (ranging from ZS to NALD) usually come to clinical attention in the newborn period, while patients with an IRD presentation are often recognized later in childhood.

Infants with the most severe ZS presentation usually die during the first year of life, often without having made any developmental progress. ZS classically presents with severe hypotonia 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. Ocular abnormalities such as cataracts, glaucoma and corneal clouding are common. In addition, patients with ZS show seizures, renal cysts and hepatic dysfunction, evident as neonatal jaundice and elevation in liver function tests. In some severe patients calcific stippling (chondrodysplasia punctata) of the patellae and other long bones may occur [7,8,15].

The clinical course of patients with the NALD and IRD presentation is variable and may include developmental delay, hypotonia, liver dysfunction, sensorineural hearing loss, retinal dystrophy and vision impairment [16]. Children with the NALD presentation may reach their teens, while patients with the IRD presentation may reach adulthood [17]. The clinical conditions are often slowly progressive in particular with respect to loss of hearing and vision.

With the availability of genetic testing for the genes involved in PBDs, several patients have been diagnosed at later ages. These patients often present with predominantly sensory deficits, including loss of hearing and vision, but may also present with isolated ataxia (see below).

Recently, the first case of a different subclass of PBDs not associated with *PEX* genes was reported affecting the division of peroxisomes. The reported female showed mild dysmorphic facial features, truncal hypotonia, absent tendon reflexes, microcephaly, optic atrophy, failure to thrive, and severe developmental delay and died one month after birth [18]. In addition to a peroxisomal fission defect the patient showed a profound defect in the fission of mitochondria the consequences of which were most probably more severe than of the peroxisomal fission defect.

3. Biochemical aspects of PBDs

Cells of patients with a PBD are characterized by the deficiency of functional peroxisomes. Cells from patients with the severe ZS presentation entirely lack intact organelles, although peroxisomal membrane remnants are often still present. Cells from milder patients may still contain a lower number of intact functional peroxisomes or show peroxisomal mosaicism., i.e. some cells still contain intact functional peroxisomes while others do not [19,20].

Because most peroxisomal enzymes are unstable in the cytosol and rapidly degraded, the absence of intact functional peroxisomes has major consequences for most of the metabolic pathways in which peroxisomes are involved. This is evident from the biochemical aberrations observed in PBD patients, which range from the accumulation of substrates normally metabolized by peroxisomes (e.g. very-long-chain fatty acids (VLCFAs), pristanic acid, phytanic acid, dihydroxycholestanoic acid (DHCA), trihydroxycholestanoic acid (THCA)) to a shortage of end products of peroxisomal metabolism (e.g. plasmalogens, cholic and chenodeoxycholic acid, docosahexaenoic acid) [3,5,6,8].

The clinical diagnosis of a PBD can be definitively confirmed by biochemical laboratory assays. Measurement of plasma VLCFA levels is commonly used as a first informative test [3,5,6]. Elevation of the VLCFA cerotic acid (C26:0) and the ratio C26:0 to docosanoic acid (C22:0) is consistent with a defect in peroxisomal fatty acid metabolism. Additional test findings that are consistent with a PBD are elevated plasma levels of phytanic acid, pristanic acid, pipecolic acid and the bile acid precursors THCA and DHCA, and lowered concentrations of C16 and C18 plasmalogen levels in erythrocytes [3,5,6].

Ideally, the analyses in plasma samples are followed by studies in primary skin fibroblasts, which involve 1) determination of the VLCFA levels; 2) evaluation of the capacity of the cells to perform peroxisomal C26:0 and pristanic acid β-oxidation; 3) evaluation of the capacity of the cells to perform peroxisomal phytanic acid α-oxidation; 4) analysis of the activity of dihydroxyacetonephosphate acyltransferase; and 5) catalase immunofluorescence (IF) microscopy to assess the absence or phenotype of peroxisomes in the cells [3,5,6].

Table 1
PEX genes and encoded peroxins associated with peroxisome biogenesis disorders.

Gene	Locus	Exons	Size (kB)	cDNA (kb)	Protein (amino acids)	Protein size (kDa)	Description and function
PEX1	7q21.2	24	41.5	4.3	1283	142.9	Cytosolic AAA protein (ATPase associated with diverse cellular activities), part of PEX1–PEX6–PEX26 export complex.
PEX2	8q21.1	4	24.8	4.4	305	34.8	Peroxisomal membrane protein, ring-finger protein, part of PEX2–PEX10–PEX12 ubiquitin ligase complex.
PEX3	6q24.2	12	39	2.0	373	42.1	Peroxisomal membrane protein, docking protein for PEX19p.
PEX5	12p13.31	16	29.9	3.2/3.3	631/654	69.8/72.3	Cytosolic receptor protein for PTS1-targeted matrix proteins. Long form mediates PTS1 and PTS2 import; short form only PTS1 import.
PEX6	6p21.1	17	15.1	3.2	980	104	Cytosolic AAA protein (ATPase associated with diverse cellular activities), part of PEX1–PEX6–PEX26 export complex.
PEX7	6q21-q22.2	10	91	1.5	323	35.9	Cytosolic receptor protein for PTS2-targeted matrix proteins.
PEX10	1p36.32	6	7.8	1	326/346	37/39.2	Peroxisomal membrane protein, ring-finger protein, part of PEX2–PEX10–PEX12 ubiquitin ligase complex.
PEX11β	1q21.1	4	7.4	1.6	259	28.4	Peroxisomal membrane protein, member of PEX11 protein family, involved in peroxisome division.
PEX12	17q12	3	3.8	2.6	359	40.8	Peroxisomal membrane protein, ring-finger protein, part of PEX2–PEX10–PEX12 ubiquitin ligase complex.
PEX13	2p14-p16	4	31.2	1.4	403	44.1	Peroxisomal membrane protein, part of PEX13–PEX14 docking complex.
PEX14	1p36.22	9	155.8	1.9	377	41.2	Peroxisomal membrane protein, farnesylated, part of PEX13–PEX14 docking complex.
PEX16	11p11.2	11	8.4	1.7	336/346	38.6/39.3	Peroxisomal membrane protein, required for peroxisome membrane protein import.
PEX19	1q22	8	8.3	0.9	299/209	32.8/23.3	Cytosolic farnesylated receptor protein for peroxisomal membrane proteins.
PEX26	22q11.21	6	11	0.9	305	33.9	Peroxisomal membrane protein, part of PEX1–PEX6–PEX26 export complex.

4. Molecular basis of PBDs

The PBDs are autosomal recessive disorders and can be caused by a defect in any of at least 14 different *PEX* genes (Table 1). These *PEX* genes encode proteins named peroxins (denoted here as PEX) that are involved in various stages of peroxisomal protein import and/or the biogenesis of peroxisomes.

Patients affected with RCDP type 1 have mutations in the *PEX7* gene, which encodes a cytosolic receptor protein that recognizes newly synthesized peroxisomal matrix enzyme precursors containing a peroxisomal targeting signal type 2 and directs these to peroxisomes [5,10,21–23]. Mutations in *PEX7* result in defective peroxisomal import of only a small subset of peroxisomal enzymes, which causes a biochemical, cellular, and clinical phenotype that is clearly distinct from the ZSS disorders.

The remaining 13 *PEX* genes each result clinically in a ZSS disorder when defective. Already prior to the actual identification of the first human *PEX* genes, it had become clear from functional complementation studies with patient cells that PBDs are genetically heterogeneous. These complementation studies involved polyethylene glycol (PEG)-induced cell fusion of cultured fibroblasts from different PBD patients [6,24–26], followed by microscopical assessment of the peroxisomal presence using antibodies against the peroxisomal matrix enzyme catalase (Fig. 1). When both patient cells have a defect in the same *PEX* gene, peroxisomes are not formed and catalase will remain in the cytosol. When the patient cells have defects in different *PEX* genes, however, peroxisomes are formed resulting in a punctated fluorescent pattern. These complementation studies revealed 12 different genetic complementation groups indicating that peroxisome biogenesis in humans requires at least 12 different genes in addition to the *PEX7* gene defective in RCDP type 1. Most of the corresponding human *PEX* genes were subsequently identified in silico on the basis of sequence similarity with yeast *PEX* genes identified by functional complementation of peroxisome-deficient yeast mutants [9]. Two genes, *PEX2* and *PEX26*, were identified by complementation of peroxisome-deficient CHO cells [27,28].

At present more than 30 different *PEX* genes have been identified in different yeast species. The peroxins encoded by these *PEX* genes are involved in different peroxisomal processes, such as the formation of peroxisomal membranes, peroxisomal growth, fission and proliferation, and import of matrix proteins [29]. Of these yeast peroxins, 19 have been implicated in the process of peroxisome protein import and organelle assembly, whereas in humans, so far only 13 peroxins have been identified that are required for these processes. A number of additional proteins have been identified that are involved in the proliferation and division of peroxisomes, including PEX11β which very recently was found defective in a patient presenting with a mild ZSS-like phenotype [30].

There is currently no evidence that defects in additional *PEX* genes can cause ZSS disorders. In a recent study aimed at finding additional genetic complementation groups, peroxisome-deficient fibroblast cell lines from more than 600 affected ZSS patients were assigned to genetic complementation groups using a *PEX* cDNA transfection complementation assay specifically developed for this purpose and discussed below [31]. Surprisingly, all cells could be assigned to the previously defined genetic complementation groups, which suggested that only mutations in the 12 corresponding *PEX* genes would lead to peroxisome deficiency. It cannot be excluded, however, that mutations in other (*PEX*) genes do not result in peroxisome deficiency and cause milder or different biochemical and clinical phenotypes, as indeed was recently found for the *PEX11β* gene [30].

5. Peroxisome biogenesis

The biogenesis of peroxisomes involves a series of different processes including the formation of peroxisomal membranes, import of peroxisomal membrane and matrix proteins, peroxisomal growth, division and proliferation.

5.1. Peroxisome assembly and peroxisomal protein import

Peroxisomes do not contain DNA and all proteins required for the assembly and function of peroxisomes are encoded by nuclear genes and synthesized on free polyribosomes in the cytosol after which they are transported to peroxisomes. The molecular mechanisms involved in the transport and import of peroxisomal proteins are largely resolved, also because most key proteins (i.e. peroxins) that take part in these processes are encoded by one of the *PEX* genes found to be defective in cells from PBD patients. We will only briefly summarize the principles of these processes with emphasis on the role of the different peroxins therein (see also Fig. 2).

The transport of peroxisomal matrix proteins to peroxisomes is highly selective and mediated by specific import sequences known as peroxisomal targeting sequences (PTSs). The majority of peroxisomal proteins destined for the matrix contain a C-terminal peroxisomal targeting sequence PTS1, a tripeptide with consensus sequence (S/A/C)-(K/H/R)-(L/M) [32]. A small subset of matrix proteins contains an internal peroxisomal targeting sequence PTS2, an octapeptide with

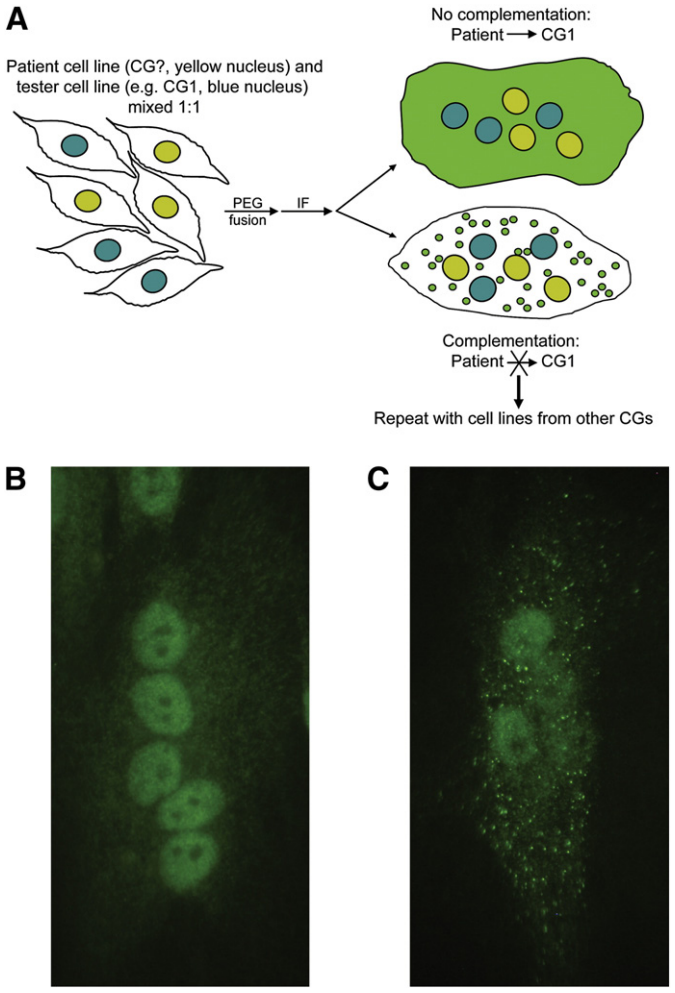

Fig. 1. Somatic cell fusion complementation assay. A. Principle of the polyethylene glycol (PEG)-induced somatic cell fusion complementation assay previously used to assign cultured cells from ZSS patients to different genetic complementation groups. B and C. Examples of multi-nuclei-containing hybrid cells processed for IF microscopy using antibodies against catalase. In panel B no genetic complementation occurs, whereas in panel C genetic complementation occurs as indicated by the punctated fluorescent staining of peroxisomes.

consensus sequence (R/K)-(L/V/I)-$(X)_5$-(H/Q)-(L/A) and localized near the N-terminus of the protein [33].

Newly synthesized matrix proteins destined for peroxisomes are recognized and bound in the cytosol by specific cytosolic receptor proteins, which subsequently direct them to the peroxisomal membrane. Newly synthesized proteins containing a PTS1 are recognized and bound by the cytosolic receptor PEX5, while proteins containing a PTS2 are recognized and bound by the cytosolic receptor PEX7. In humans, two functional isoforms of PEX5 have been identified encoded by the same *PEX5* gene and synthesized as a result of alternative splicing [34]. Both the shorter (PEX5S) and the longer form (PEX5L) can bind PTS1 proteins and direct these to the peroxisomal membrane. In addition, PEX5L contains a PEX7-binding domain required for binding and subsequent directing matrix protein-loaded PEX7 to the peroxisomal membrane [34–36]. The matrix protein-loaded receptor proteins dock onto the peroxisomal docking complex constituted by the peroxisomal membrane proteins PEX14 and PEX13. After docking, the matrix protein will be translocated across the peroxisomal membrane into the peroxisomal lumen and the receptor proteins will be released from the peroxisomal membrane for another import cycle [37,38]. These latter two steps of the cycle require the action of the peroxisomal membrane proteins PEX2, PEX10 and PEX12, which all three are zinc-binding RING finger proteins that belong to the protein family of ubiquitin ligases. PEX2, PEX10 and PEX12 catalyze either the mono- or the poly-ubiquitination of PEX5. Mono-ubiquitination will promote the recycling of PEX5 while the poly-ubiquitination makes PEX5 a substrate for proteasome-mediated degradation [37,39–41].

The cytosolic proteins PEX1 and PEX6 interact with each other, are anchored to the peroxisomal membrane via the peroxisomal membrane protein PEX26 and are involved in the release of ubiquitinated PEX5 from the peroxisomal membrane [42,43].

As follows from the proposed functions of the encoded peroxins, mutations in the *PEX1*, *PEX2*, *PEX5*, *PEX6*, *PEX10*, *PEX12*, *PEX13*, *PEX14*, *PEX26* genes typically affect only the import of peroxisomal matrix proteins. Cells from PBD patients with defects in these genes still contain peroxisomal membrane remnants containing peroxisomal membrane proteins. Cells from patients with mutations in the *PEX7* gene only display a defect in the import of PTS2-targeted peroxisomal matrix proteins, resulting in a distinct biochemical and clinical phenotype (RCDP type 1, see above).

A similar but less well characterized cycle has been proposed for the transport and membrane incorporation of peroxisomal membrane proteins (PMPs; Fig. 2). These proteins also contain specific internal targeting sequences [44] that are recognized by the cytosolic PEX19 protein, which docks onto the peroxisomal membrane protein PEX3 in the peroxisomal membrane [45,46]. PEX3 is one of the earliest membrane proteins found in peroxisomal membranes and its incorporation is independent from PEX19 [47]. The peroxisomal membrane protein PEX16 is known to interact with PEX3, but its exact role in the import of peroxisomal membrane proteins is unclear [47].

The involvement of PEX3, PEX16 and PEX19 in the transport and incorporation of peroxisomal membrane proteins also follows from the observation that severe mutations in the *PEX3*, *PEX16* and *PEX19* genes affect the import of both peroxisomal matrix and membrane proteins; cells with defects in these genes are completely devoid of any peroxisomal remnants [48–51]. It should be noted, however, that mild mutations in the *PEX16* and *PEX3* genes may result in a less severe phenotype with lower numbers of (enlarged) peroxisomes [52].

5.2. Proliferation and division of peroxisomes

Recent data indicate that new peroxisomes are predominantly formed by growth and division of pre-existing peroxisomes [1,53], but that they can also arise *de novo* from preperoxisomal vesicles that originate from specialized compartments of the endoplasmic reticulum (ER) [54]. These preperoxisomal vesicles develop into mature, metabolically active peroxisomes through the import of peroxisomal membrane proteins and subsequently matrix proteins.

Different proteins have been identified that are involved in the proliferation and division of peroxisomes. The division of peroxisomes involves three distinct sequential steps: elongation of peroxisomes, membrane constriction, and finally fission of peroxisomes [55–57]. The following proteins are involved in the fission of peroxisomes: Dynamin-Like Protein 1 (DLP1 or DRP1) [18,58], mammalian Fission 1 (hFIS1) [59,60] and mammalian Mitochondrial Fission Factor (Mff) [61]. Remarkably, these three proteins do not only play a role in the fission of peroxisomes but also in the fission of mitochondria. PEX11 proteins are involved in the elongation of peroxisomes. In mammals, including humans, three different PEX11 isoforms have been identified encoded by separate genes: *PEX11α*, *PEX11β* and *PEX11γ* [62,63]. Overexpression of PEX11α and PEX11β in mammalian cells results in an increase in peroxisome number, but PEX11γ overexpression does not have an effect on peroxisome abundance [64].

Currently, only two patients have been identified with a different defect in any of the proteins known to be involved in the proliferation and division of peroxisomes. The first reported patient was a severely affected female patient, who died one month after birth and postmortally was found to have a dominant-negative heterozygous mutation in the *DLP1* gene, which resulted in a severe fission defect of both peroxisomes and mitochondria [18].

Very recently, we identified the first patient with a complete deficiency of PEX11β due to a homozygous nonsense mutation in the encoding gene. The male patient presented with a ZSS-like disorder, including mild intellectual disability, progressive hearing loss, and sensory nerve involvement. Remarkably, although microscopical investigations of the patient's fibroblasts indicated a clear defect in peroxisome division, all standard biochemical peroxisomal parameters in plasma, erythrocytes and fibroblasts were normal [30].

6. Genetics of PBDs

When we combine published data with the partly unpublished data from our laboratory, the defective *PEX* genes of more than 1300 unrelated PBD patients have been identified by complementation testing with patient cells and/or sequence analysis (Table 2). This includes 231 patients with a defect in the *PEX7* gene, resulting in the clinically distinct RCDP type 1. For the majority of ZSS patients, the pathogenic mutations have been determined by sequencing of the implicated *PEX* genes providing a comprehensive overview of the frequency distribution of the different *PEX* gene defects and associated mutations. For a detailed listing of presently known mutations we refer to the *PEX* gene database dbPEX (http://www.dbpex.org), which provides a current update and statistics of published and unpublished mutations in the different *PEX* genes. Here we will only briefly discuss the different *PEX* genes, the *PEX* gene defect frequencies and the most common mutations found in the *PEX* genes.

6.1. PEX1

Among all diagnosed patients with a ZSS disorder almost 60% have mutations in the *PEX1* gene. *PEX1* encodes a 143-kDa cytosolic protein that belongs to the AAA ATPase protein family (ATPases Associated with various cellular Activities) and contains two highly conserved domains of 230 amino acids, which contain ATP-binding Walker A and B motifs and have ATPase activity [40,65]. At present, 90 different mutations have been identified in the *PEX1* gene, including 23 missense, 15 nonsense, 30 deletion, 11 insertion, 7 splice site, 2 indel mutations and 2 disruptions of the start codon [31]. Mutations are

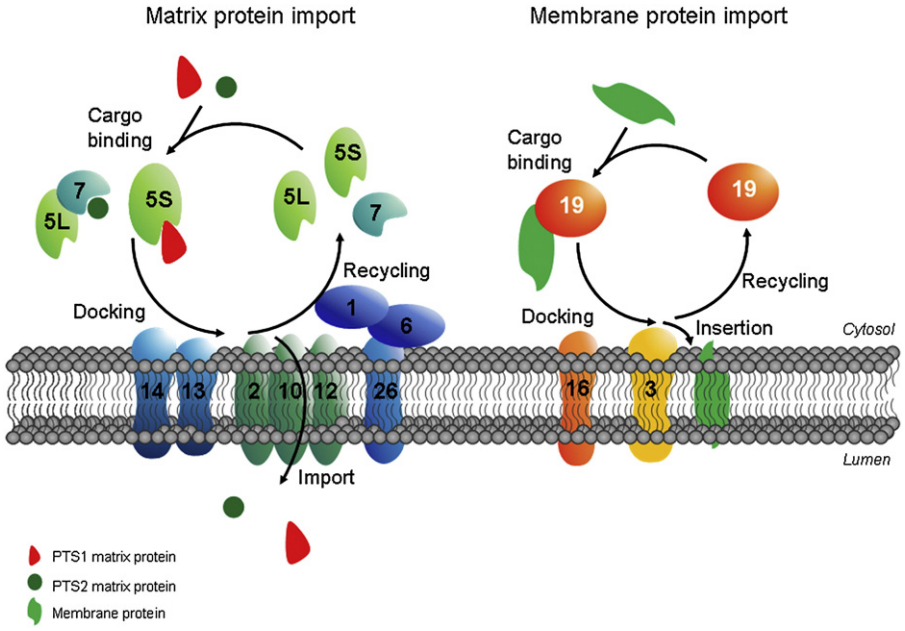

Fig. 2. Import machinery for peroxisomal protein import. Schematic presentation of the human import machinery for peroxisomal matrix and membrane proteins indicating the different roles of the 13 different peroxins. For details see main text.

distributed across the entire gene, although exons 13 and 15 harbor most mutations. By far the most common mutation in *PEX1* is the missense mutation c.2528G>A, which changes the glycine located in the second ATP-binding domain at amino acid position 843 into an aspartic acid (p.G843D). This mutation reduces the binding between PEX1 and PEX6 [66,67]. The effect of the mutation is relatively mild and cells of patients with a p.G843D mutation in the *PEX1* gene often display peroxisomal mosaicism when cultured at 37 °C. This peroxisomal mosaicism is temperature sensitive, meaning that when fibroblasts of patients homozygous for p.G843D are cultured at 30 °C, peroxisomes regain the ability to import catalase and other peroxisomal matrix proteins and the various peroxisomal metabolic functions normalize. In contrast, when cultured at higher temperatures, peroxisomes become deficient and the phenotype becomes worse [66,68]. The second most common mutation in *PEX1* is c.2097_2098insT (or c.2097dup), which results in a frame shift theoretically predicted to cause a truncated PEX1 protein [69]. Northern blot analysis indicated that this mutation results in low steady state *PEX1* mRNA levels, implying that the mRNA produced is targeted for nonsense mediated decay. Patients homozygous for this mutation are invariably affected with the severe ZS phenotype. About 80% of the patients with mutations in the *PEX1* gene are at least heterozygous for one of these two common mutations [31,67,69–72].

6.2. PEX2

The *PEX2* gene was the first identified *PEX* gene and contains four exons, although the entire coding region is included in exon 4 [28]. PEX2 is an integral peroxisomal membrane protein of 35 kDa with two transmembrane domains and a zinc-binding motif, probably involved in the poly-ubiquitination of the peroxisomal import receptor PEX5 [41]. So far 17 different mutations have been identified in exon 4 of the *PEX2* gene, including 4 missense, 5 nonsense, 6 deletion and 2 insertion mutations [31]. The most common mutation in the *PEX2* gene is c.355C>T, which changes the arginine located at amino acid position 119 into a stop codon (p.R119X). Although this mutation results in a truncated PEX2 protein lacking the zinc-binding domain, it is associated with temperature sensitivity: patient cells cultured at 30 °C show partial catalase import [73]. Also patient fibroblasts homozygous for the second common mutation c.669G>A (p.Trp223X) display a mosaic peroxisomal pattern when cultured at 37 °C [74,75].

6.3. PEX3

PEX3 is an integral peroxisomal membrane protein of 42 kDa with two putative membrane-spanning domains and its C-terminus exposed to the cytosol. The exact function of PEX3 is unclear, but it is assumed that PEX3 functions as the docking protein for the PEX19-PMP complex on the peroxisomal membrane and plays a role in the insertion of peroxisomal membrane proteins into the peroxisomal membrane [76–78]. A complete deficiency of PEX3 causes the absence of peroxisomal remnants (ghosts) in patient cell lines. In total, six different mutations in *PEX3* have been reported, including 2 nonsense, 2 deletion, 1 insertion and 1 splice site mutation [31]. The six known PEX3-defective patient cell lines

Table 2
Frequency distribution of *PEX* gene defects among 1308 patients diagnosed with a peroxisome biogenesis disorder based on published and own unpublished data.

PEX gene	Number of patients identified	Genetically analyzed patients	Frequency (%) among PBDs	Frequency (%) among ZSS disorders
PEX1	634	384	48.5	58.9
PEX2	42	42	3.2	3.9
PEX3	6	6	0.5	0.5
PEX5	18	18	1.4	1.7
PEX6	171	154	13.1	15.9
PEX7	231	231	17.7	–
PEX10	45	42	3.4	4.2
PEX11β	1	1	<0.1	<0.1
PEX12	77	66	5.9	7.1
PEX13	18	18	1.4	1.7
PEX14	3	3	0.2	0.3
PEX16	14	14	1.0	1.2
PEX19	4	4	0.3	0.4
PEX26	45	39	3.4	4.2
Total	1308	1021	100	100

all have homozygous mutations in *PEX3*, which are located in different exons [31,50,51,79,80].

6.4. PEX5

PEX5 is a 70-kDa protein with seven di-aromatic pentapeptide repeats (WxxxF/Y) in its N-terminal half and seven tetratricopeptide repeats (TPRs) in its C-terminal half [34,81]. As discussed above, in humans, two functional protein variants of PEX5, PEX5S and PEX5L are produced as a result of alternative splicing of the *PEX5* mRNA. The longest variant, PEX5L, contains an additional 111 bp encoding 37 amino acids, due to alternative splicing of exon 7 [34]. Eleven different mutations in *PEX5* have been identified, including 3 missense, 4 nonsense, 1 indel, and 2 splice site mutations [82]. Mutations are distributed across the entire gene, although exons 12 and 14 harbor most mutations [82]. The most common mutation c.1578T>G (p.N526K) results in an isolated PTS1 protein import defect [82].

6.5. PEX6

The *PEX6* gene encodes a 104-kDa protein, which is a member of the AAA ATPase family and interacts with PEX1 [65]. Mutations in the *PEX6* gene are the second most common cause for ZSS disorders (approximately 16%; Table 2). At present 104 different mutations have been reported in the *PEX6* gene, which are scattered throughout the entire *PEX6* gene, although one-third of mutations are found in exon 1 [83]. Among the 106 mutations, 37 missense, 5 nonsense, 32 deletion, 9 insertion, 1 indel and 20 splice site mutations have been identified. Although many mutations are found in exon 1, the most common mutation in *PEX6* is a small deletion, c.1314_1321delGGAGGCCT, located in exon 5.

6.6. PEX7

The *PEX7* gene encodes the cytosolic receptor protein required for the import of PTS2-targeted peroxisomal matrix proteins. The 36-kDa protein contains six series of WD40 repeats [84]. A defect in PEX7 is the cause of RCDP type 1, which is the underlying defect in approximately 18% of the patients with a PBD but clearly distinctive from the ZSS disorders. At present, 38 different mutations have been identified in the *PEX7* gene, including 14 missense, 7 nonsense, 6 deletion, 4 insertion and 7 splice site mutations. Mutations are distributed across the entire gene, although exons 1 and 9 harbor most mutations [21,22]. The nonsense mutation c.875T>A (p.L292X) is by far the most common *PEX7* mutation and is due to a founder effect [85].

6.7. PEX10

PEX10 is a 37-kDa integral peroxisomal membrane protein, which contains two transmembrane domains and a C-terminal zinc-binding motif, similar as PEX2 and PEX12. Two mRNA splice forms of *PEX10* have been identified in skin fibroblasts, resulting from the use of a different splice acceptor site at the 3′ end of intron 3. The longer form accounts for 10% of the *PEX10* mRNA in the cell and appears to be slightly less functional [86]. So far, 30 different mutations have been identified in the *PEX10* gene, including 13 missense, 6 nonsense, 5 deletion, 2 insertion, 1 splice site mutation and 3 disruptions of the start codon [31]. Mutations are distributed across the entire gene, but exon 5 harbors most mutations. The most common *PEX10* mutation is c.814_815delCT, which is found mainly in the Japanese population [87]. The second most common mutation is the c.704_705insA leading to a truncated protein missing the zinc binding motif [88].

6.8. PEX11β

PEX11β is a 28.5-kDa integral peroxisomal membrane protein, and member of the PEX11 protein family, which in mammals includes three different isoforms encoded by separate genes: *PEX11α*, *PEX11β* and *PEX11γ* [62,63]. PEX11 proteins are involved in the elongation of peroxisomes, which is part of peroxisome division. A defect in PEX11β was reported only very recently in one patient with a mild ZSS-like presentation, who was homozygous for a nonsense mutation, c.64C>T (p.Q22X), as a consequence of which no PEX11β protein is produced [30].

6.9. PEX12

Mutations in the *PEX12* gene are the third common cause of a ZSS disorder: 7% of the reported patients with a ZSS disorder have mutations in *PEX12* (Table 2). The 41-kDa PEX12 protein is, like PEX2 and PEX10, one of the RING (abbreviation for 'really interesting new gene') finger proteins; it contains two transmembrane domains and a C-terminal zinc-binding motif [89–91]. Currently 36 different mutations have been identified in the *PEX12* gene, including 7 missense, 6 nonsense, 15 deletion, 6 insertion and 2 splice site mutations [31]. Mutations are distributed across the entire gene, although exons 2 and 3 harbor most mutations. The most common *PEX12* mutation, c.959C>T (p.S320F) is located in the zinc-binding domain and predominantly found in patients from Turkish descent. Patients homozygous for this mutation present with an atypical mild biochemical phenotype in cultured fibroblasts: the biochemical peroxisomal parameters are mostly normal to slightly abnormal. The mutation causes a temperature-sensitive phenotype in cells: when cultured at 37 °C, 70% of the cells contain catalase-positive peroxisomes, when cultured at 30 °C, all cells contain catalase import competent peroxisomes [92]. The c.126+1G>T is the second common *PEX12* mutation and causes aberrant splicing of *PEX12*.

6.10. PEX13

PEX13 is a 44-kDa peroxisomal transmembrane protein, which contains a transmembrane domain as well as an Src homology 3 (SH3) domain [93] and interacts with PEX14. Currently 10 different mutations have been reported in the *PEX13* gene, including 3 missense, 3 nonsense, 3 deletions, and 1 splice site mutation [31]. Most of the identified mutations are located in exon 2 or 4, but no common mutations have been found [94–97].

6.11. PEX14

The *PEX14* gene encodes a 41-kDa peroxisomal membrane protein. PEX14 interacts with PEX13 and forms the peroxisomal docking site for the PTS1 receptor PEX5 [98]. A mutation in *PEX14* is one of the least common causes of a ZZS disorder; so far only 3 different mutations have been identified, including 1 missense, 1 deletion and 1 splice site mutation [31,99,100].

6.12. PEX16

The *PEX16* gene encodes a 39-kDa integral peroxisomal membrane protein with two putative membrane-spanning domains and its C- and N-terminal domains exposed to the cytosol. Two different mRNA variants of *PEX16* are produced as a result of alternative splicing, each with an alternate exon 11 (exon 11a and exon 11b). Both transcription variants are expressed in human fibroblasts, but the variant containing exon 11a is most abundant. Two groups of PEX16-defective patients have been reported; patients with a severe clinical presentation of whom the fibroblasts displayed a defect in the import of both peroxisomal matrix and membrane proteins,

resulting in a total absence of peroxisomal remnants [48,101,102], and, more recently, several patients with a relatively mild clinical phenotype of whom the fibroblasts showed enlarged, import-competent peroxisomes [52]. Currently, 10 different mutations have been identified in the *PEX16* gene, including 2 missense, 1 nonsense, 3 deletions, 1 insertions and 1 splice site mutation [31]. Mutations are localized across the entire gene. The mutations found in the milder patients are located in the C-terminus of the protein.

6.13. PEX19

The *PEX19* gene encodes a mainly cytosolic 30-kDa protein that binds many peroxisomal membrane proteins, strongly suggesting that PEX19 functions as the receptor for peroxisomal membrane proteins [46,103]. Patients with a defect in PEX19 presented with a severe ZS phenotype and the fibroblasts displayed a total absence of peroxisomal remnants [49]. 3 different mutations have been identified in the *PEX19* gene, including 1 nonsense, 1 deletion and 1 insertion mutation [31]. The mutations are localized in exons 3 and 6. A defect in PEX19 is one the least common causes of a ZSS disorder (Table 2).

6.14. PEX26

The *PEX26* gene encodes a 34-kDa peroxisomal membrane protein with one putative membrane-spanning domain [104]. PEX26 interacts with PEX1 and PEX6. Human PEX26 is the orthologue of yeast PEX15. Twenty three different mutations have been identified in the *PEX26* gene, including 6 missense, 5 nonsense, 5 deletion, 3 insertion, 3 splice site mutations and 1 disruption of the start codon [31]. Mutations are distributed across the entire gene, although exons 2 and 3 harbor most mutations. The most common mutation is c.292C>T (p.R98W), which results in a temperature-sensitive biochemical phenotype [27,104].

7. Genetic diagnosis of PBDs

Because it is not possible to predict from the biochemical and clinical phenotype of a patient with ZSS which of the currently known 13 *PEX* genes is defective, different approaches have been developed to rapidly identify the affected gene, including *PEX* cDNA transfection complementation assays followed by sequencing of the implicated *PEX* gene, and a genetic *PEX* gene screen algorithm in which the most frequently mutated exons of the different *PEX* genes are analyzed.

The identification of the defective *PEX* gene and the mutations causing a PBD enables carrier testing of relatives and allows early prenatal testing or preimplantation genetic diagnosis in families with a recurrence risk for ZSS disorders. In addition, the knowledge can be used to determine genotype–phenotype correlations which may delineate the effect of certain mutations and could eventually assist to improve patient management.

7.1. PEX cDNA transfection complementation assay

The *PEX* cDNA transfection complementation assay developed in our laboratory (Fig. 3) takes advantage of the known identities of the 12 most common human *PEX* genes that can cause a ZSS disorder (*PEX11β* not included) [31] and has replaced the above discussed complementation analysis involving somatic cell hybridization. A similar approach with some variations has been developed and reported by Krause et al. [105]. In the assay developed and also used for diagnostic purpose in our laboratory [31], peroxisome-deficient ZSS primary fibroblast cells are co-transfected with a mammalian expression vector containing one of the 12 different *PEX* cDNAs and a second vector that expresses Green Fluorescent Protein (eGFP) tagged with the peroxisomal targeting signal-SKL, which allows microscopical detection of peroxisomes when they are present. Two to three days after transfection, cells are examined by means of fluorescence microscopy to determine the subcellular localization of the peroxisomal reporter protein eGFP-SKL. A punctuate fluorescent pattern indicates the reappearance of peroxisomes and thus genetic complementation, whereas a diffuse, cytosolic fluorescent staining indicates non-complementation. To identify the defective *PEX* gene, the transfections of cell lines are performed in three subsequent series based on the currently known frequency of the different *PEX* gene defects (Table 2). In a first series, cells are transfected separately with *PEX1*, *PEX6* and *PEX12*. Cells that are not functionally complemented are then transfected separately with *PEX2*, *PEX10* and *PEX26* and finally with *PEX5*, *PEX13* and *PEX14*. Transfection with *PEX3*, *PEX16* and *PEX19* is performed when patient fibroblasts show no peroxisomal membrane remnants based on IF microscopy using antibodies against a peroxisomal membrane protein, such as PMP70 or ALDP.

After the defective *PEX* gene is identified by complementation testing, all exons plus flanking intronic sequences of the *PEX* genes are sequenced following amplification of these by PCR from genomic DNA isolated from the patient's cells [31].

Although the *PEX* cDNA transfection complementation assay has turned out to be very efficient, reliable and reproducible in the identification of the defective *PEX* gene in ZSS patients it has a few drawbacks. For example, it requires the availability of cultured fibroblasts from the patients. Moreover, the assay works only well for cells that in majority display a complete peroxisome deficiency and for cell lines displaying peroxisomal mosaicism that is sensitive to culturing at 40 °C, which exacerbates the defect in peroxisome biogenesis [19]. This implies that primarily cells from (more) severely affected patients can be used. When patient fibroblasts are not available or do not display peroxisome deficiency after culturing at 40 °C, different approaches will be required, such as sequential sequencing of the 13 known *PEX* genes in an order based on the currently known frequency of the different *PEX* gene defects.

7.2. PEX gene screen algorithm

As an alternative to sequential sequencing of the entire *PEX* genes, a *PEX* gene screen algorithm has been proposed, which involves systematic screening of selected exons of the six *PEX* genes that are found most commonly defective in ZSS patients. Sequential sequence analysis of exons 13, 15, and 18 of the *PEX1* gene, exon 4 of the *PEX2* gene, exon 1 of the *PEX6* gene, exons 2 and 3 of the *PEX12* gene, exons 3–5 of the *PEX10* gene, and finally exons 2 and 3 of the *PEX26* gene in a cohort of 91 patients identified at least one pathogenic mutation in 72 cases, i.e. 79% [106]. Steinberg and colleagues proposed to use this *PEX* gene screen algorithm as the first in a three-step strategy. If the *PEX* gene screen does not identify mutations in any of the analyzed exons, they recommend subsequently analyzing first the entire *PEX1* and *PEX6* genes, which in their cohort of 91 patients increased the sensitivity to 92%. Eventually, patients remaining undefined would then be candidates for mutations in *PEX3*, *PEX5*, *PEX11β*, *PEX13*, *PEX14*, *PEX16*, and *PEX19* or in a *PEX* gene not yet associated with human disease [106,107].

The *PEX* gene screen algorithm could be helpful in screening DNA from patients for which no fibroblasts are available, or whose cells do not show a distinct peroxisome-deficient phenotype and thus would not be suitable for complementation testing. In addition, the approach could be helpful by screening parental DNAs in those cases where no DNA is available from the index patient.

It is expected that in the near future the defective *PEX* genes in patients will be identified by whole exome sequencing or other next generation sequencing technologies that are rapidly evolving and becoming cost-effective.

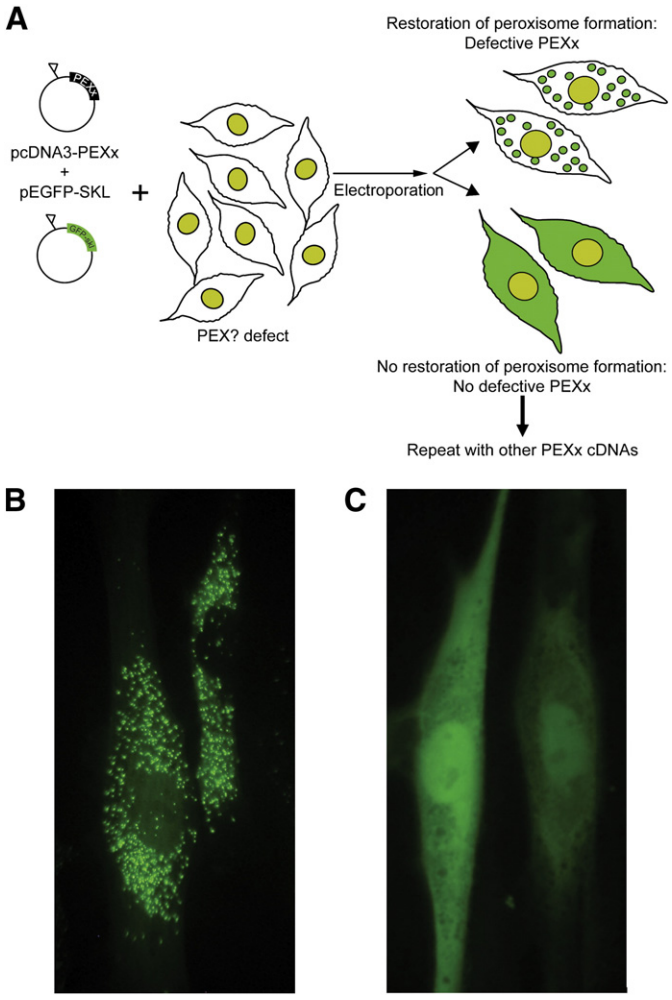

Fig. 3. *PEX* cDNA transfection complementation assay. A. Principle of *PEX* cDNA transfection complementation assay used to identify the defective *PEX* gene in cells from ZSS patients. B and C. Examples of cells cotransfected with a PEXx-containing expression plasmid and a plasmid expressing the peroxisomal reporter protein GFP-SKL. In panel B genetic complementation occurs as indicated by the punctated fluorescent staining of peroxisomes, whereas in panel C no genetic complementation occurs, and the reporter protein is localized in the cytosol.

7.3. Carrier testing and prenatal diagnosis

The identification of the defective *PEX* gene and pathogenic mutations causing a PBD makes carrier testing possible in families of affected patients as well as more distant relatives. To this end, relatives are first analyzed for the presence of the familial mutation in the known *PEX* gene. If that is the case, the entire *PEX* gene of the partner can be sequenced to determine or exclude the presence of any potential pathogenic mutation. In the vast majority of cases no mutation will be identified in the partner's *PEX* gene, which does not per se exclude but significantly lowers the chance of carriership and, accordingly, the chance on diseased progeny. It should be noted that carriers for PBD cannot be identified by biochemical testing.

The poor outcome and often early death associated with PBDs makes prenatal and preimplantation diagnosis an important service for families with previously affected children. Prenatal diagnosis for a PBD can be performed by biochemical as well as genetic testing and is carried out using chorionic villus samples, cultured chorionic villus cells, cultured amniotic fluid cells or DNA isolated thereof. Genetic testing is currently the preferred method as it is fast, accurate and allows one to exclude maternal contamination of the fetal DNA. To be eligible for prenatal or preimplantation genetic testing, however, the identity of the defective *PEX* gene and disease-causing mutations in the index patient have to be known and preferably confirmed in the corresponding parental genes. When the defective *PEX* gene and disease-causing mutations are unknown at the time of prenatal testing, biochemical prenatal testing is the method of choice. Biochemical prenatal testing for a PBD mostly involves measurement of the activity of DHAP-AT in chorionic villus samples, cultured chorionic villus cells or cultured amniotic fluid cells, immunoblot analysis of peroxisomal thiolase (is processed after import in peroxisomes) and determination of VLCFA concentrations in cultured chorionic villous or amniotic fluid cells. In addition, IF microscopy using antibodies against catalase can be used in cultured chorionic villous or amniotic fluid cells to assess the presence of peroxisomes. Biochemical prenatal testing for a PBD is reliable and conclusive when the index patient has a clear biochemical phenotype. For milder defects, however, biochemical testing can be problematic and not always conclusive and thus genetic testing is preferred.

7.4. Genotype–phenotype correlations

ZSS disorders are autosomal recessive disorders that display considerable clinical and genetic heterogeneity. Dependent on their type and coding effect, mutations in any of the known *PEX* genes can result in a severe early-lethal ZS clinical presentation, a relatively mild IRD clinical presentation or an intermediate phenotype. Indeed, for most *PEX* genes mutations have been identified that are associated with severe as well as other mutations that are associated with less severe presentations. Although from the clinical, biochemical, or cellular phenotype it is often not possible to predict the defective *PEX* gene, there is in general a good correlation between on the one hand the defective *PEX* gene and the type of mutation and on the other hand the impact on peroxisome assembly and function, and the clinical severity. Homozygosity or compound heterozygosity for two severe mutations in any of the 12 *PEX* genes most commonly associated with a ZSS disorder will invariably result in a severe biochemical and clinical phenotype. Two mild mutations generally result in a milder biochemical and clinical phenotype, although the outcome for such patients can be variable due to secondary complications such as respiratory problems and infections that may severe the disease and can have fatal consequences [108]. In addition, the course of disease in milder patients often shows a progressive trend. Compound heterozygosity for one mild and one severe mutation will result in an intermediate biochemical and clinical phenotype with patients even more susceptible for the negative consequences of secondary complications.

8. Special cases

The availability of genetic testing for *PEX* genes and other peroxisome-related genes in combination with improved laboratory diagnostic tests including in particular microscopical assessment of the peroxisomal phenotype in patient cells has resulted in the diagnosis of several patients at later ages, who previously had not been recognized. These patients are often relatively mildly affected and/or show only a few of the symptoms that are typically associated with ZSS disorders.

8.1. "Isolated" cerebellar ataxia

Two independent patients with cerebellar ataxia, axonal motor neuropathy, and posterior column dysfunction but without mental retardation [109] and a third patient with a similar phenotype, but with cerebral white matter changes [110] were found by sequential

sequencing of *PEX* genes to have mutations in the *PEX10* gene. Biochemically, their absolute plasma levels of VLCFA were normal with a mild increased ratio of C26:0/C22:0. Plasma phytanic and pristanic acid levels were elevated, as were the plasma levels of the bile acid intermediates DHCA and THCA and pipecolic acid, which suggested involvement of multiple peroxisomal pathways. Biochemical studies in cultured fibroblasts of these patients revealed no abnormalities, and the peroxisome appearance analyzed by catalase IF microscopy was either normal or slightly mosaic. Culturing of the fibroblasts at 40 °C did not exacerbate the defect in peroxisome biogenesis.

A similar approach was used to identify the genetic defect in two brothers with isolated progressive cerebellar ataxia [111]. Brain MRI showed atrophy of the vermis and lateral hemispheres of the cerebellum, but there were no signs of demyelination or neuronal migration defects in forebrain and cerebellum in both patients. Biochemical analysis in plasma revealed a moderate increase of the bile acid intermediates DHCA and THCA, and of phytanic and pristanic acid, but normal levels of VLCFAs and pipecolic acid. Biochemical studies in fibroblasts showed only a slight decrease of the oxidation of phytanic acid. Sequence analysis of the candidate genes *PEX2*, *PEX10* and *PEX12* revealed a homozygous nonsense mutation in the *PEX2* gene as cause for the isolated progressive cerebellar ataxia in these two brothers.

These cases indicate that a diagnosis of a PBD cannot always be made or excluded based solely on the analysis of plasma VLCFA levels, but that additional analyses are required, including the measurement of the bile acid intermediates DHCA and THCA, which are probably the best diagnostic predictors for a PBD.

8.2. Clinical overlap with other syndromes

Several patients have been reported, who in fact showed clear peroxisomal biochemical aberrations, but because they initially were diagnosed with a different disease based on their clinical presentation, they had not been examined for peroxisomal defects. For example, Michelakakis et al. described a patient, who initially was diagnosed with Leber congenital amaurosis (LCA) at the age of 6 months, but was neurologically examined at the age of 2 years because of myoclonic seizures [112]. Based on the development of mild psychomotor retardation with cerebellar tract involvement, mild facial dysmorphism and mild demyelinating sensorimotor neuropathy, the initial diagnosis was questioned and metabolic studies were initiated, which revealed a peroxisome biogenesis disorder due to homozygosity of the p.G843D mutation in the *PEX1* gene.

Very recently, a similar case was reported involving a 28-year cognitively normal patient who was diagnosed with isolated LCA, although the patient also showed sensorineural hearing loss and Arnold-Chiari malformation [113]. After eliminating 500 common mutations in 15 LCA genes, the patient's DNA was subjected to whole exome sequencing which again identified the homozygous p.G843D mutation in the *PEX1* gene. Peroxisomal biochemical studies subsequently confirmed a ZSS disorder in the patient. Subsequent screening of a cohort of LCA patients, revealed another 9 months old baby with the same homozygous mutation, who at the age of 20 months was hospitalized for developmental delay, hypotonia and seizures, and a diagnosis of ZSS was confirmed.

These three cases consolidate the hypothesis of Ek and coworkers already made in 1986 that some patients with LCA may turn out to have a ZSS disorder [114].

Another example of a syndrome with clinical overlap with ZSS disorders is Usher syndrome, characterized by deafness and gradual vision loss. Two unrelated patients, diagnosed with Usher syndrome on the basis of sensorineural deafness and retinitis pigmentosa, turned out to have mutations in the *PEX6* gene, which in cells caused a relatively mild peroxisomal biochemical dysfunction and peroxisomal mosaicism [115].

8.3. Unusual peroxisomal phenotypes

An important aid in recognizing the possibility of a peroxisomal disorder in patients, who only show minor or sometimes even normal peroxisomal biochemical parameters, is the microscopical observation of the peroxisomal phenotype in cultured skin fibroblasts of the patients. As discussed above this also resulted in the identification of a novel class of peroxisomal defects affecting the division of peroxisomes. The two patients currently known with a defect in peroxisome fission due to mutations in DLP1, also affecting mitochondrial fission [18], and PEX11β [30] both only showed an aberrant peroxisomal phenotype while peroxisomal biochemical parameters were essentially normal.

Another example of an unexpected peroxisomal phenotype was recently reported in several patients with mutations in the *PEX16* gene [52]. Before the identification of these patients, mutations in *PEX16* were associated with a severe, lethal clinical presentation of a ZSS disorder and fibroblasts of patients displayed a defect in the import of both peroxisomal matrix and membrane proteins. The reported PEX16-defective patients, however, were moderately affected and the biochemical parameters in fibroblasts were only mildly abnormal or even within the normal range. Catalase IF microscopy revealed the presence of import-competent peroxisomes, which were increased in size but reduced in number [52].

8.4. Dominant mutations, digenic and oligogenic inheritance

So far, haplo insufficiency, dominant negative mutations or digenic/oligogenic inheritance have not been reported for any of the 14 *PEX* genes associated with PBDs. The occurrence of haplo insufficiency, i.e. a single functional copy of a gene is insufficient to maintain normal function, seems unlikely because parents of diagnosed patients, who are obligate carriers of one of the two mutations of the patient, never show any clinical signs that could be related to peroxisomal dysfunction.

Given the fact that most of the peroxins encoded by the *PEX* genes have interactions with each other, it may be anticipated that examples of dominant negative mutations and/or digenic/oligogenic inheritance will be found in future. In the case of dominant-negative mutations one would expect to find only one heterozygous pathogenic mutation in one of the *PEX* genes from a patient, while this mutation is absent in the corresponding parental genes. In the case of digenic or oligogenic inheritance, one may expect to find heterozygous pathogenic mutations in two (digenic) or more (oligogenic) *PEX* genes that encode peroxins which interact with each other.

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