Molecular, biochemical and clinical aspects of peroxisomes biogenesis disorders

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Chapter 1

General introduction
Introduction

The disorder currently known as Zellweger syndrome (ZS, MIM# 214100) was first described in 1964 in two sib pairs as a familial syndrome of multiple congenital defects. In the following years, two more reports on similar patients were published in which the term cerebro-hepato-renal syndrome was introduced. In 1969, Opitz et al. suggested the name Zellweger syndrome. A major hallmark in the research on Zellweger syndrome was the discovery by Goldfischer et al. that peroxisomes where absent in the liver and kidney tubules of Zellweger patients. Later, peroxisomes were also found to be deficient in the two other disorders neonatal adrenoleukodystrophy (NALD, MIM# 202370) and infantile Refsum disease (IRD, MIM# 266510). ZS, NALD and IRD together form a spectrum of disease severity with ZS being the most, and IRD the least severe disorder. The disorders are collectively called peroxisome biogenesis disorders (PBDs). They have an incidence of approximately 1 per 50,000 births.

Peroxisomes

Peroxisomes are the last discovered true subcellular organelles. In the 1950s, Rhodin discovered these organelles in kidney cells of mice and named them microbodies. In 1966, de Duve et al. found hydrogen peroxide producing as well as degrading enzymes in these microbodies and therefore named them peroxisomes. The significance of peroxisomes remained unclear for a long time, until the observation of their absence in Zellweger syndrome. Peroxisomes are single-membrane bounded organelles with a diameter of 0.1-1.0 μm. They are found in virtually all eukaryotes, ranging from microorganisms (except archaea) to plants and animals. In humans, peroxisomes are present in every cell, except for red blood cells. Human cells typically contain several hundred peroxisomes, and they are most abundant in liver and kidney. They are usually round or oval vesicles, although they sometimes appear to form elongated, tubular structures, or reticula. Peroxisomes have a very high matrix-protein concentration, sometimes leading to crystalline inclusions.

Peroxisome biogenesis

Peroxisome biogenesis disorders are due to a defect in peroxisome formation. Cell fusion complementation studies using patient fibroblasts (described later) revealed the existence of 12 distinct genetic groups (complementation groups, CG), representing defects in different genes. Currently all of the corresponding PEX genes have been identified. These PEX genes encode proteins involved in the biogenesis of peroxisomes, called peroxins (PEX). Currently, 29 peroxins have been identified in various species, of which 14 orthologs have been described in humans.

Early studies of PBD cell lines demonstrated a deficiency in peroxisomal matrix protein import, whereas peroxisomal membrane assembly was relatively normal. In these cells, empty peroxisomal membrane remnants that still contain some peroxisomal membrane proteins (PMPs), but lack most of their internal content, were present, which are called ghosts. Later studies also reported patient cell lines in which these peroxisomal ghosts
were absent,\textsuperscript{15,16} which implied a segregation of peroxisomal matrix protein import and peroxisomal membrane biogenesis.

\textbf{Peroxisomal membrane biogenesis}

There has been much controversy on the question how peroxisomes are formed. According to the earliest model, formation of new peroxisomes occurred by vesicle budding from the ER. This hypothesis was based on the electron microscopy observations, showing that peroxisomes were found in close proximity to the ER.\textsuperscript{17} Later studies provided evidence against this postulate, and abundant evidence suggested that peroxisomes multiply by fission of pre-existing peroxisomes (reviewed in Lazarow and Fujiki\textsuperscript{18}). This model proposes that newly synthesized peroxisomal membrane and matrix proteins are incorporated in pre-existing peroxisomes. When a certain size is obtained, a new peroxisome buds off, and the process starts over again (figure 1).

\textbf{Figure 1} Overview of the different hypothetical models of peroxisome biogenesis. In the model of Lazarow and Fujiki\textsuperscript{18} new peroxisomes are formed by budding from pre-existing peroxisomes. In the model of South and Gould\textsuperscript{20} for the re-introduction of peroxisomes in ghost-less PEX16 cell lines, PEX16, peroxisomal membrane proteins (PMPs) and matrix proteins (MP) are inserted subsequently into pre-peroxisomes (pp) to form mature peroxisomes. The model of Titorenko\textsuperscript{22} involves pre-peroxisomal vesicles (P1 and P2) derived from the ER, which fuse into P3 vesicles that develop via P4 and P5 into mature peroxisomes.

Waterham et al. found that peroxisomes in temperature-sensitive mutants of \textit{H. polymorpha}, in which peroxisomes were absent when grown at 43°C, but present at 35°C, re-emerged after a shift from restrictive to permissive temperature.\textsuperscript{19} Furthermore, South and Gould described the re-introduction of peroxisomes into cells of ghost-less PEX16 deficient human cell lines.\textsuperscript{20} These results suggest that peroxisomes are not necessarily derived from pre-existing peroxisomes. South and Gould proposed a model involving the existence of (ER independent) prereroxosomal structures into which PEX16 is inserted after complementation/transfection (figure 1). After the import of other PMPs this model converges with the model by Lazarow and Fujiki. However, recent experiments in the yeast \textit{Y. lipolytica} by Titorenko et al. indicated a role for the ER in peroxisome biogenesis. They provided experimental evidence indicating that peroxisomes develop in a multistep process that starts with the formation of pre-peroxisomal vesicles, thought to arise from a subdomain of the ER, containing components of coat protein II vesicles (figure 1).\textsuperscript{21,22} Inhibition with COPI and COPII inhibitors, however, was found to have no effect on peroxisome formation.\textsuperscript{23,24} Electron microscopy, immunocytochemistry and three-dimensional image reconstruction of peroxisomes and associated compartments in mouse dendritic cells also support the involvement of the ER.\textsuperscript{25} Future studies will show if these results are species-specific, or if both models can converge into one hybrid model.
Regardless of the way peroxisomes are formed, both models require that PMPs, which are synthesized on free polyribosomes, are imported into the membrane, probably using a peroxisomal targeting sequence for membrane proteins (mPTS) to facilitate this. mPTTs have been identified in several integral peroxins and other PMPs in different species (see references in Jones et al.\textsuperscript{26}). Available evidence indicates that there is no apparent consensus sequence. A hydrophilic peptide containing a group of positively charged amino acids adjacent to at least one hydrophobic patch or transmembrane domain was observed several times, although there was no common amino acid sequence among them, and any individual amino acid could be changed.

The mPTTs need to be recognized in the cytosol, after which the PMPs are inserted into the peroxisomal membrane. Based on the absence of peroxisomal ghosts in cell lines from patients with defects in \textit{PEX3}, \textit{PEX16} and \textit{PEX19},\textsuperscript{15,16,27} it is assumed that the peroxins encoded by these genes play a role in these processes.

\textit{PEX19} is a farnesylated protein that is approximately 95% cytosolic and 5% peroxisomally associated.\textsuperscript{28,29} It binds to an array of PMPs of diverse functions,\textsuperscript{28,29,30,31,32,33} including peroxins (\textit{PEX3, 10, 11,12,13,14,16} and 17) and other PMPs. These results suggest a role for \textit{PEX19} as a soluble mPTS receptor, which is supported by the finding of an overlap between the regions of PMPs with which \textit{PEX19} binds and the mPTTs.\textsuperscript{28} However, others reported cases in which there was no overlap, and suggested a chaperone-like role for \textit{PEX19}.\textsuperscript{31,34}

The \textit{PEX3} gene encodes a 42- to 52-kDa membrane protein with its C-terminus facing the cytosol. Opinions differ on whether the N-terminus is also cytosolic\textsuperscript{35} or intraperoxisomal.\textsuperscript{33} The exact function of \textit{PEX3} is unclear. It interacts with \textit{PEX19} via its C-terminal domain, not with its mPTS,\textsuperscript{28,33,35,36} and many authors have suggested that \textit{PEX3} is required for other membrane-bound peroxins to assemble in the peroxisome membrane (reviewed in Purdue and Lazarow\textsuperscript{37}) although there is no direct evidence.

\textit{PEX16} has two putative membrane-spanning domains and exposes its C- and N-terminal domains to the cytosol.\textsuperscript{20} Its function is unknown, but it is likely to function upstream of \textit{PEX3}.\textsuperscript{38} In contrast to \textit{PEX3} and \textit{PEX19}, there is no \textit{S. cerevisiae} \textit{PEX16} ortholog. Besides humans, the only other organisms in which it has been reported, thus far, are \textit{Y. lipolytica} and \textit{Arabidopsis thaliana}. In \textit{Y. lipolytica}, \textit{PEX16} has different properties than human ortholog, and plays no role in membrane assembly.\textsuperscript{39} In \textit{A. thaliana}, \textit{PEX16} has a role in protein and oil body biogenesis.\textsuperscript{40}

In conclusion, there are still many uncertainties with respect to peroxisomal membrane biogenesis. \textit{PEX19} is likely to serve as PMP receptor or chaperone, the functions of \textit{PEX3} and \textit{PEX16} are less clear.

\textit{Import of peroxisomal matrix enzymes}

Like PMPs, the peroxisomal matrix proteins are synthesized on free polyribosomes in the cytosol, and posttranslationally imported into the peroxisome. The peroxisomal import machinery accepts folded proteins, oligomerized proteins and even gold particles fused to import signals as substrates.\textsuperscript{41,42} To reach their correct cellular location, the peroxisomal matrix proteins contain specific peroxisomal targeting signals (PTS). More than 90% of all matrix proteins contain a PTS1, a C-terminal tripeptide with a (S/A/C)-(K/R/H)-L consensus sequence.\textsuperscript{43} Later studies found extended sequence lengths as well as species-dependent ranges of possible conservative exchanges of the residues.\textsuperscript{44-47} A few matrix
proteins are targeted by a PTS2, which is located near the N-terminus and has an (R/K)-(L/V/I)-(X)5-(H/Q)-(L/A) consensus sequence. Recently a third PTS was identified in yeast: PTS3. No human PTS3 proteins have been identified so far.

The PTSs are bound by receptor proteins in the cytosol which target them to the peroxisome (figure 2). PEX5 is the receptor for proteins containing a PTS1 or PTS3 and PEX7 is the receptor for proteins containing a PTS2. PEX5 has been described in a wide range of species. Mutations in PEX5 have been found as a cause of disease in PBD patients belonging to CG2. PEX5 contains six tetraticopeptide repeats (TPRs) which together constitute the binding site for the PTS1 of the cargo proteins, while highly conserved N-terminal pentapeptide repeats were shown to be essential for the interaction with the members of the docking complex. The PTS2 receptor PEX7 contains six WD repeats, which are each approximately 40 amino acids long and contain a central tryptophan (W)-aspartate (D) motif. To carry out its receptor function, PEX7 requires help from different species-specific auxiliary proteins: PEX18 or PEX21 in S. cerevisiae, PEX20 in Y. lipolytica and Neurospora crassa, or the longer of two splice isoforms of PEX5 (PEX5L) in mammals. These non-orthologous proteins possess a conserved sequence region that probably represents a common PEX7-binding site, suggesting the evolutionary conservation of a functional module rather than an entire protein. PEX7 mutations are the cause of disease in patients belonging to CG11. These patients are affected with rhizomelic chondrodysplasia punctata (RCDP) type 1, a phenotype different from the Zellweger-spectrum. These patients are only deficient in the few enzymes imported by the

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**Figure 2** Peroxisomal matrix protein import. PEX proteins (peroxins) are depicted as hexagons. Filled hexagons represent human PEX proteins, open hexagons represent PEX proteins that have not been identified in humans yet. Dashed arrows depict the extended shuttle model proposed by Damai and Subramani. The PTS2 protein/PEX7/PEX5L complex is imported comparable to the PTS1 protein/PEX5 complex. See text for details.
PTS2 pathway, and present with proximal shortening of the limbs, periarticular calcifications, microcephaly, coronal vertebral clefting, dwarfinng, congenital cataract, ichthyosis and severe mental retardation.\textsuperscript{6}

After binding their ligands, PEX5 and PEX7 bind to the components of the docking machinery (figure 2). The transmembrane proteins PEX13 and PEX14, have been established as members of the docking complex (reviewed in Holroyd and Erdmann\textsuperscript{30}). PEX13 and PEX14 both provide binding sites for PEX5 and PEX7. It is believed that PEX14 represents the initial docking site for both receptor proteins, because PEX14 has a higher affinity for cargo-loaded PEX5, whereas PEX13 has a higher affinity for PEX5 alone.\textsuperscript{62} Moreover, PEX13 and PEX14 form a complex with cargo-loaded PEX5, but dissociate in the presence of unloaded PEX5.\textsuperscript{73} Mutations in PEX13 have been shown to cause the disease in CG13 patients.\textsuperscript{65} PEX17 has been identified as a peripheral protein in \textit{Y. lipolytica}\textsuperscript{64} and as an integral membrane protein in \textit{P. pastoris}.\textsuperscript{32} Although there has been some controversy about PEX17 being involved in PMP import,\textsuperscript{32} most evidence suggests PEX17 to be involved in matrix import only. The protein interacts with PEX14,\textsuperscript{65} but its exact function is unknown. No human PEX17 has been identified thus far.

After docking of the receptor-cargo complexes, the matrix proteins need to be translocated over the peroxisomal membrane (figure 2). The three peroxins PEX2, PEX10 and PEX12 have been implicated in this process. Mutations in these genes are causing the disease in CG10,\textsuperscript{66} CG7\textsuperscript{67,68} and CG3,\textsuperscript{69} respectively. All three belong to the family of RING zinc finger proteins, and have been identified in different yeast species and mammals. They are all integral peroxisomal membrane proteins and have a cytosolic carboxy-terminal zinc-binding domain, which is thought to mediate protein-protein interactions. PEX10 and PEX12 interact with each other, and both proteins can also directly bind the PTS1 receptor PEX5.\textsuperscript{70,72} Moreover, PEX2, PEX5, PEX12 and PEX14 were found in a complex in rat liver peroxisomes.\textsuperscript{73} PEX13 is also present in these complexes, but in non-stochiometric amounts. Cells lacking one of these zinc-binding proteins accumulate PEX5 at the peroxisomal membrane, which suggests these proteins to act downstream of receptor docking. How the translocation occurs mechanistically, and how the machinery can accommodate folded and oligomerized proteins is unknown. PEX8 is an intra-peroxisomal peroxin, first identified in \textit{H. polymorpha}\textsuperscript{74} and so far cloned in several yeast species only. It behaves like a peripheral peroxisomal membrane protein in \textit{P. pastoris},\textsuperscript{75} \textit{Y. lipolytica}\textsuperscript{64} and \textit{S. cerevisiae},\textsuperscript{76} whereas it is localized in the peroxisomal matrix in \textit{H. polymorpha}.\textsuperscript{74} All orthologs contain a PTS1 at the carboxy-terminus; \textit{H. polymorpha} PEX8 also contains a PTS2. PEX8 directly interacts with PEX5, independent of its PTS1,\textsuperscript{76} although it has not been shown if this interaction takes place inside the peroxisome. Since PEX8 contains a PTS1 and in \textit{H. polymorpha} also a PTS2, it has been suggested to be involved in the dissociation of the cargo from the PTS receptors or in the subsequent recycling of the receptors,\textsuperscript{76} although others have implicated PEX8 in the association of the docking complex with the translocation complex.\textsuperscript{77}

Initially, the general model for peroxisomal matrix protein import proposed that the PTS receptors recycle right back into the cytosol after dropping their cargo at the docking site.\textsuperscript{79} However, recent experiments indicate that PEX5 enters human peroxisomes during the course of its function, and then re-emerges into the cytosol to carry out another round of import: the extended shuttle model (figure 2).\textsuperscript{79} Dammai and Subramani elegantly demonstrated the proteolytic cleavage of a PEX5 fusion protein within peroxisomes and
detected the processed PEX5 in the cytosol. These experiments, however, cannot exclude that the fusion protein has only been inserted into the membrane with its amino-terminal site accessible to the protease, without actual transport across the membrane. Moreover, the complete translocation of PEX5 would imply the presence of a, yet unknown, PEX5 export machinery.

After translocation of the cargo proteins, the PTS receptors PEX5 and PEX7 need to recycle back to the cytosol for another cycle of protein import (figure 2). PEX1 and PEX6 have been implicated in this recycling, and are both members of the AAA protein family (ATPases associated with a various cellular activities). These proteins contain highly conserved AAA domains of 230 amino acids, which contain Walker ATP binding sequences and have ATP activity. Mutations in the genes encoding these proteins are disease-causing in CG1[80,81] and CG4[82] respectively. Both proteins were found to interact with each other.[83] Localization studies have shown remarkable differences between various organisms. The proteins have been found peroxisomally associated and/or cytosolic, and also present in vesicles distinct from mature peroxisomes.[22,83-87] Cell lines defective in PEX1 and PEX6 have a defect in the import of matrix proteins.[88] They are both important for the stability of PEX5,[78,84] and they function in the terminal steps of the matrix proteins import pathway.[89] However, because other members of the AAA family are involved in membrane fusion processes[90] and in some organisms PEX1 and PEX6 were found present in vesicles distinct from mature peroxisomes, they were also suggested to be involved in the fusion of these small vesicles, leading to the maturation of peroxisomes.[22,83] Very recently, two proteins interacting with PEX1 and PEX6 have been reported. In S. cerevisiae, PEX15 was described as an integral membrane protein that binds PEX6 in an ATP-dependent manner.[91] In humans, the transmembrane protein PEX26 was discovered to cause the disease in PBD patients of CG8.[92] This protein was shown to anchor PEX6 and PEX1 to the peroxisomal membrane, in a PEX6-dependent manner. Later studies showed that in human cell lines defective of PEX26, catalase and PTS2 protein import was disturbed but PTS1 protein was normal,[93] which makes the role of PEX26 and the moment of action of PEX1 and PEX6 unclear.

PEX4 is a peripherally associated peroxisomal membrane protein, located at the cytosolic face of the peroxisome. The protein is kept at the peroxisomal membrane via interaction with PEX22, an integral peroxisomal membrane protein.[94] In cell lines defective in these proteins, PEX5 protein level was severely decreased.[89] Both PEX4 and PEX22 are involved in the import of peroxisomal matrix proteins,[94,95] and act in the final step, after PEX1 and PEX6,[89] suggesting a role in receptor recycling. Their exact functions, however, are still unclear. No human PEX4 and PEX22 have been identified so far.

**Peroxisome proliferation**

PEX11 has been postulated to play a regulatory role in peroxisome proliferation, based on the finding that cells lacking PEX11 contain few giant peroxisomes and appear to be unable to segregate the giant peroxisomes to daughter cells.[96] When PEX11 is overexpressed, hyperproliferation occurs.[97,98] However, PEX11 was also found to play a metabolic role in peroxisomal β-oxidation in S. cerevisiae.[99] These authors suggested the role of PEX11 in peroxisome division to be secondary due to ongoing β-oxidation. Recently, both the dynamin-like protein Vsp1p (DLP1 in humans)[100,101] and the newly identified PEX25 and PEX27[102-104] in S. cerevisiae were shown to be involved in peroxisome
Chapter 1

proliferation. Cells lacking these proteins had fewer and larger peroxisomes. The C-termini of PEX25 and PEX27 are similar to the entire PEX11. Some of the cells lacking PEX25 had no peroxisomes detectable with immunofluorescence with antibodies against PTS1- or PTS2-containing matrix proteins. Yeast two-hybrid analyses showed that PEX27 interacts with PEX25 and itself, PEX25 interacts with PEX27 and itself, and PEX11 interacts only with itself. Furthermore, in cells deleted for two other peroxins in S. cerevisiae, PEX28 and PEX29, peroxisomes are increased in number, exhibit extensive clustering, are smaller in area than peroxisomes of wild-type cells, and often exhibit membrane thickening between adjacent peroxisomes in a cluster. This implies a role for these two peroxins in peroxisome dynamics. Thus, PEX11, PEX25, PEX27, PEX28, PEX29 and Vps1p/DLP1 are candidates for a role in the regulation of peroxisome number, size, and distribution. The underlying mechanisms, however, are still unclear.

Peroxisomal functions

Peroxisomes are involved in a number of essential metabolic functions in humans. The major functions are:

**β-oxidation of fatty acids and fatty acid derivatives**
The peroxisome handles the β-oxidation of many substrates. These include very-long chain fatty acids (VLCFAs), notably C26:0, the branched chain fatty acids, such as pristanic acid, the bile acid intermediates di- and trihydroxycholestanolic acid (DHCA and THCA), some long chain fatty acids and long chain dicarboxylic acids (products of ω-oxidation), and the side chains of eicosanoids. Furthermore, it has become clear that the peroxisomal β-oxidation system is also involved in the last step of the biosynthesis of the poly-unsaturated fatty acid docosahexaenoic acid (DHA).

**Phytanic acid α-oxidation**
Phytanic acid and other 3-methyl-branched fatty acids are taken up from the diet with dairy products, meat and fish being the most important sources. These compounds cannot be degraded by the normal peroxisomal β-oxidation pathway, because of the methyl group on the C3 position. Therefore, they are broken down by the peroxisomal α-oxidation system. Phytanic acid is thereby broken down to pristanic acid, which can be degraded by β-oxidation. The deficiency in the second enzyme of phytanic acid α-oxidation: phytanoyl-CoA hydroxylase, leads to Refsum disease [MIM# 266500].

**Ether-phospholipids biosynthesis**
The first two steps in the ether-phospholipid biosynthesis, catalyzed by the enzymes dihydroxyacetonephosphatase acyltransferase (DHAPAT) and alkyl-dihydroxyacetonephosphatase-synthase (alkyl-DHAP-synthase), take place in the peroxisomes. Deficiencies in these two enzymes in humans lead to RCDP type 2 [MIM# 222765] and RCDP type 3 [MIM# 600121], respectively. In mammals, the main end-products of the ether-phospholipid biosynthetic pathway are the plasmalogens, which are characterized by the presence of an alkylenyl group at the sn-1 position of the glycerol backbone. Plasmalogens are components of cellular membranes, make up approximately half of the heart's phospholipids, but are most abundant in nervous tissue. They make up...
approximately 80 to 90% of the ethanolamine phospholipid class in myelin. Furthermore, plasmalogens are decreased in the affected areas of the brain of Alzheimer’s patients.\textsuperscript{109} However, the functional significance of plasmalogens is unknown.

Other peroxisomal functions

Other metabolic pathways that take place inside peroxisomes, at least partly, are L-pippecolic acid oxidation, glyoxylate metabolism, D-amino acid metabolism, hydrogen peroxide metabolism, purine metabolism and fatty acid chain elongation. Although the involvement of peroxisomes in the biosynthesis of isoprenoid/cholesterol biosynthesis was generally accepted for many years,\textsuperscript{110} recent data strongly suggest that peroxisomes are not involved in human isoprenoid/cholesterol biosynthesis.\textsuperscript{111,112}

PBD Phenotypes

As described in the introduction, the peroxisome biogenesis disorders form a disease spectrum comprising of the three disorders Zellweger syndrome, neonatal adrenoleukodystrophy and infantile Refsum disease.

Zellweger syndrome

The cerebro-hepato-renal syndrome of Zellweger is the most severe PBD, and is characterized by the complete absence of functional peroxisomes and peroxisomal functions. Patients display a characteristic facial appearance with a large anterior fontanel, high forehead, hypoplastic supraorbital ridges, epicanthal folds and a broad nasal bridge. Ocular abnormalities include cataracts, glaucoma, corneal clouding, Brushfield spots, pigmentary retinopathy and optic nerve dysplasia. Sensorineural deafness is often present and patients are mentally retarded. Additionally, there is severe hypotonia, weakness and neonatal seizures. The development of internal organs (brain, liver, kidney) and the skeleton is disturbed. Radiologic examination reveals abnormal punctuate calcifications in the patella and epiphyses of the long bones. Renal cysts are common. Most patients die within the first year of life.\textsuperscript{6}

Neonatal adrenoleukodystrophy

NALD was first described by Ulrich et al. as connatal ALD in a boy who presented at birth with hypotonia, convulsions, absent grasp reflex, slight Moro response, and little spontaneous movements.\textsuperscript{113} He showed all signs diagnostic of ALD, including the characteristic demyelination of the central nervous system white matter, atrophy of the adrenal cortex, ballooned adrenocortical cells and splinter-like lamellar elements composed of electron-dense leaflets separated by a clear space. NALD should not be confused with X-linked adrenoleukodystrophy (X-ALD, MIM# 300100), which is not a peroxisome biogenesis disorder, but a defect in the \textit{ABCD1} gene resulting in a deficiency in the peroxisomal β-oxidation and accumulation of VLCFAs. To distinguish between ZS and NALD, Kelley et al. defined criteria to distinguish between the two disorders.\textsuperscript{114} Although many cases could be classified by these criteria, a number of patients remained with symptoms of both disorders. However, since it is now firmly established that the PBDs form a disease continuum, NALD is now defined as a less severe form of ZS,
involving progressive white matter disease, with patients surviving from several months of life up until their mid-teens.

**Infantile Refsum disease**
IRD is the mildest phenotype of the PBDs. It was first described by Scotto and co-workers in three cases of young children presenting with several neurological abnormalities and hepatomegaly. Initially, IRD was thought to be a variant of Refsum disease, a single enzyme defect, in which phytic acid accumulates. However, later studies revealed the presence of a general peroxisomal dysfunction, which was confirmed by the absence of peroxisomes in liver. Patients with IRD have external features reminiscent of ZS, but no neuronal migration disorder, and no progressive white matter disease. Their cognitive and motor development varies between severe global handicap and moderate learning disorder with deafness and visual impairment due to retinopathy. Their survival is variable. Most patients with IRD reach childhood and some even reach adulthood.

**Diagnosis of PBDs**

**Biochemical analysis**
The laboratory diagnosis of a PBD starts with the analysis of various parameters in plasma (concentrations of VLCFAs, pristanic acid, phytanic acid, bile acid intermediates, polyunsaturated fatty acids and L-pipeolic acid) and erythrocytes (plasmalogens and polyunsaturated fatty acids). When a PBD is suspected, this is followed by the measurement of various parameters in cultured skin fibroblasts (VLCFA concentration, C26:0 and pristanic acid β-oxidation, phytanic acid α-oxidation, DHAPAT activity and immunoblot analysis to assess the peroxisomal processing of the β-oxidation enzymes straight chain acyl-CoA oxidase and 3-ketoacyl-CoA thiolase). The diagnosis is completed by immunofluorescence microscopy with antibodies against the peroxisomal enzyme catalase, to confirm the absence of peroxisomes, and with antibodies against the peroxisomal membrane protein ALDP to reveal the presence or absence of peroxisomal ghosts.

![Complementation analysis](image)

**Figure 3** Complementation analysis. Cells from a new patient are fused with cells from a patient belonging to a known complementation group (CG). When no complementation occurs, the defective genes in both patients are the same. Otherwise, the procedure is repeated with other CGs.

**Complementation analysis**
When the diagnosis of a PBD is established, cell fusion complementation analysis is performed, to identify the defective PEX gene. In this technique, fibroblasts from a new
patient are fused with cells from a patient belonging to a known complementation group, thereby combining the genetic information of both patients (figure 3). When the cells do not complement each other and there is no restoration of peroxisome formation, the defective genes in both patients are the same. If peroxisomes are formed, the defective gene in both patients is different. Peroxisome formation is assayed by catalase immunofluorescence microscopy.

In our laboratory, so far, 246 PBD patients have been assigned to the 12 different complementation groups (table 1). The percentages in this table are comparable to the data in literature. Most complementation groups are associated with more than one clinical phenotype. The large majority of the patients belong to CG1, caused by mutations in the PEX1 gene. The second most common CG is CG4, in which PEX6 is defective. Together, defects in these two AAA-ATPases account for more than 70% of the PBD patients.

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* Kennedy Krieger institute, Baltimore, USA, Gifu university, Gifu, Japan. % of cell lines complemented with this CG, CG11 was excluded, n.i. not included

Mutations in the PEX genes
Mutation analysis has been performed in all of the complementation groups. Only for two mutations in CG1 (PEX1) a high allele frequency has been found. The first mutation is a point mutation 2528G>A causing the missense mutation G843D. This mutation attenuates the activity of the protein and is associated with the mild IRD phenotype. It reduces the binding ability between PEX1 and PEX6. The G843D allele frequency ranges from 25% to 37% in the different cohorts. The second mutation is the insertion 2097-2098insT, which results in a frameshift and low steady state PEX1 mRNA levels. At the protein level it leads to truncation of the PEX1 protein within the protein’s second AAA domain, abolishing its PEX1 activity. It is associated with the severe ZS phenotype. This mutation has an allele frequency of around 30%. Together, these mutations account for around 60% of all PEX1 alleles, which is about 40% of all PBD alleles. Furthermore, in the Japanese population, a common mutation in PEX10 has been found, due to a founder effect. This 2-bp deletion 814-815delCT is present in a homozygous form in all 11 Japanese PEX10 patients. Most of the other mutations found in the PEX genes are unique to one patient or family.
Chapters

**Chapter 1**

*Temperature sensitivity*

Studies in fibroblasts of patients with milder forms of PBDs (IRD and some NALD patients) have shown temperature sensitivity of biochemical parameters. In these cells, a (partial) restoration of peroxisome formation and peroxisome function was observed, when cells were cultured at 30°C instead of 37°C. This phenomenon has been reported for patients belonging to CG1 (PEX1), CG4 (PEX6), CG8 (PEX26), CG10 (PEX2), and CG13 (PEX13) and is associated with specific (point-)mutations.

**Therapy**

Because the peroxisome biogenesis disorders have a genetic origin, and multiple malformations and neocortical alterations in the brain originate prenatally, treatment of patients is limited, but there are possibilities for treatment of symptoms, especially for patients with milder phenotypes. The treatment strategies can be divided into two groups: correction of biochemical deficiencies or accumulations, and pharmacological induction of peroxisomes.

To correct plasmalogen levels, ether lipids have been administered orally, which resulted in a partial normalization of red blood cell plasmalogen levels. The high VLCFA could be (partially) corrected by a diet also used in the treatment of X-ALD, which also normalized phytanic acid levels. To correct DHA levels, cholic and chenodeoxycholic acids have been administered, which resulted in improved liver function and improvement in neurologic status. Another approach to correct DHA levels was by administering DHA ethyl ester, which resulted in a normalization of DHA levels and liver function, improvement of vision and muscle tone, as well as improvement of myelination as studied by MRI. The effect was the largest in patients who started the treatment before 6 months of age. In addition to the DHA correction, VLCFA levels decreased and plasmalogen levels increased. The decreased levels of α-tocopherol that are found in many patients can be corrected by oral vitamin E suppletion.

Induction of peroxisomes has been tried by the administration of the peroxisomal proliferator clofibrate. Although this did have effects in rodents, it did not have any result in humans. The peroxisome proliferator sodium 4-phenylbutyrate has only been tested in human fibroblasts yet, but did show promising results. After treatment of PBD fibroblasts, an approximate two-fold increase in peroxisome number was observed. In NALD and IRD fibroblasts there was an increase in very-long-chain fatty acid β-oxidation and plasmalogen concentrations, and a decrease in very-long-chain fatty acid concentrations. No clinical studies with this compound have been performed yet.

In conclusion, although many defects in PBD patients originate prenatally, symptomatic treatment was found to be beneficial in patients, and new strategies like the administration of 4-phenylbutyrate, are being investigated that might result in the upregulation of peroxisomes in patients.

**Outline of this thesis**

It is evident that our knowledge of peroxisome biogenesis, peroxisomal functions and the peroxisome biogenesis disorders has expanded enormously since the discovery of peroxisomes in the 1950s. Many peroxins, indispensable for peroxisome formation, have
been identified and a ‘corner of the veil has been lifted’ about how these peroxins function. Much has been learned about the metabolic pathways that function inside the peroxisomes. Furthermore, many PBD patients have been described, and the initial idea of three or more separate peroxisomal disorders had been abandoned, and it is now clear that the disorders form a disease spectrum and originate from defects in the same genes, which is why they are now collectively called the peroxisome biogenesis disorders.

This marked genetic heterogeneity and the presence of many unique mutations among patients with a PBD are causing complications in genotype-phenotype studies. Therefore, in chapter two propose to use a different approach to predict the life expectancy of the patients: by examining the effects of the defective genes on peroxisome function, rather that the mutations themselves. Therefore we conducted a search for suitable biochemical parameters, which would be best in predicting the severity of patients. In chapter three and four, we report novel mutations in the PEX2 and PEX12 genes of PBD patients, shedding new light on the differences in importance of the zinc-binding domains in the proteins encoded by these genes. Chapter five describes eight PBD patients with a very unusual biochemical phenotype, characterized by abnormal peroxisomal plasma metabolites, but normal to very mildly abnormal parameters in cultured skin fibroblasts, including a mosaic catalase immunofluorescence pattern, which so far made complementation analysis impossible. We developed a novel complementation technique and characterized the patients. In chapter six the reinvestigation of a unique patient is described, who was reported with a presumed deficiency in THCA-CoA oxidase, but instead was found to be suffering from a mild PBD, although peroxisomes in fibroblasts appeared normal. Chapter seven describes a rapid, non-invasive alternative technique to determine the presence of peroxisomes in patient cells: immunofluorescence microscopy analysis in lymphocytes, which can be isolated from the same blood samples as used for metabolite analyses. In chapter eight, we report all mutations in the different PEX genes that have been determined in our laboratory so far, combined with those reported in literature.

References

Chapter 1


General introduction


