Identification and characterization of novel peroxisomal disorders

Falkenberg, K.D.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 1

Introduction
Peroxisomes

Peroxisomes are vital organelles found in virtually every human cell\(^1\). Measuring approximately 1 µm in diameter, these tiny organelles consist of a single bilayer membrane enclosing a matrix with a large variety of metabolic enzymes\(^2\). Peroxisomes constitute a safe harbor for metabolic processes by providing a suitable environment and bringing substrates and enzymes in close vicinity. Despite recent advances in our understanding of peroxisomes, many aspects of peroxisomal function, metabolism and proteins remain unresolved to date.

Peroxisomal metabolic functions

A wide variety of metabolic processes takes place in peroxisomes. Peroxisomal enzymes are involved in catabolic processes, like the degradation of fatty acids, purines, D-amino acids and hydrogen peroxide, as well as in anabolic processes, like the biosynthesis of ether-phospholipids and bile acids\(^3\). The following paragraphs outline several peroxisomal pathways, with special emphasis on pathways involved in peroxisomal diseases.

\(a)\) VLCFA \(\beta\)-oxidation and transport

Very long-chain fatty acids (VLCFAs) are fatty acids with carbon chains longer than 20 carbons. They can be derived from the diet (for instance due to consumption of peanut butter)\(^4\), but are usually produced by elongation of long-chain fatty acids in the endoplasmic reticulum (ER)\(^5,6\). VLCFAs and their derivatives are required for specific cellular functions, for some of which they cannot be substituted for by shorter chain fatty acids\(^7\). Very long-chain sphingolipids, for example, determine the properties of membranes in which they are embedded and are involved in myelin stability and liver functions\(^8-10\).
**Figure 1 - Peroxisomal metabolic pathways**

This scheme, modified from 11, depicts the main metabolic pathways occurring in the peroxisome, including relevant participating peroxisomal proteins (boxed), as well as the interplay with other organelles. Enzymes involved in peroxisomal beta oxidation (yellow boxes) process various substrates, which are indicated by different colors, for instance dicarboxylic acids (DCA, bordeaux), VLCFAs (sVLCFA and unVLCFA, green), bile acid intermediates (DHCA and THCA, orange) and pristanic acid (blue). Defects in underscored proteins have been reported as single enzyme deficiencies.

ABCD1, ATP-binding cassette sub-family D 1; ABCD2, ATP-binding cassette sub-family D 2; ABCD3, ATP-binding cassette sub-family D 3 (alternatively known as 70 kDa peroxisomal membrane protein or PMP70); ACAA1, Peroxisomal 3-ketoacyl-CoA thiolase; ACOX1, Acyl-CoA oxidase 1; ACOX2, Branched acyl-CoA oxidase; AGPS, Alkyl-glycerone-phosphate synthase (formerly known as alkyl-dihydroxyacetone phosphate synthase or ADHAPS); AMACR, α-methylacyl-CoA racemase; BAAT, Bile acid-CoA:amino acid N-acyltransferase; br, branched chain; CA, Cholic acid; CAT, catalase; CDCA, Chenodeoxycholic acid; CoA, Coenzyme A; DBP, D-bifunctional protein (alternatively known as multifunctional protein 2 or MFP2); DCA, dicarboxylic acids; DHAP, Dihydroxyacetone phosphate; DHCA, Dihydroxycholestanolic acid (= bile acid intermediate); ER, Endoplasmic reticulum; FAR1, Fatty acyl-CoA reductase 1; GNPAT, Glycerone-phosphate O-acyltransferase (formerly known as Dihydroxyacetone phosphate acyltransferase or DHAPAT); HACL1, 2-hydroxyacyl-CoA lyase; LBP, L-bifunctional protein (alternatively known as multifunctional protein 1 or MFP1); PHYH, Phytanoyl-CoA hydrolase; PrDH, Pristanal dehydrogenase; SCPx, Sterol carrier protein-X thiolase; sVLCFAs, saturated VLCFAs; THCA, Trihydroxycholestanolic acid (= bile acid intermediate); unVLCFA, unsaturated VLCFAs.
Chapter 1

Fatty acid homeostasis is a highly dynamic process and depends on the balance of fatty acid synthesis and degradation. Whereas most fatty acids are directly degraded in mitochondria, VLCFAs first have to be “shortened” to long-chain fatty acids in peroxisomes in order to become substrates for mitochondrial β-oxidation\(^{12}\). Current evidence holds that the synthesis of VLCFAs takes place at the ER membrane by chain-elongation of long-chain fatty acids, after which they are transported as VLCFA-CoAs to peroxisomes. Since only small solute molecules can enter the peroxisomes via peroxisomal pores\(^ {13}\), the import of VLCFAs is dependent on ABC transporter proteins located in the peroxisomal membrane (Figure 1). The main transporter protein involved in the peroxisomal VLCFA import is ABCD1\(^ {14,15}\), but also alternative entry routes via other peroxisomal ABC transporters, like ABCD2\(^ {16}\) and ABCD3\(^ {17,18}\), can be employed by VLCFAs. The current import model proposes, that VLCFAs are bound by the ABC transporter in the form of acyl-CoA esters and imported into the matrix following ATP binding and hydrolysis\(^ {19–21}\).

After the import of VLCFAs into the peroxisomal matrix, they become substrates for peroxisomal β-oxidation, by which two carbons from their carboxyl end are removed per cycle (Figure 1, green pathway). β-oxidation of VLCFAs involves several consecutive steps which are catalyzed by the enzymes acyl-CoA oxidase (ACOX1, first step), D-bifunctional protein (DBP, second and third step) and both sterol carrier protein X (SCPx) and 3-Ketoacyl-CoA thiolase, for short “thiolase” (ACAA1), for the last step\(^ 3\).

b) Other functions of peroxisomes

Also other substrates are processed via peroxisomal β-oxidation, whereby this process can be catalyzed by the same enzymes as for VLCFAs, or by alternative enzymes, like for instance branched acyl-CoA oxidase (ACOX2)\(^ {22}\). Substrates include branched chain fatty acids, like pristanic and the bile acid intermediates DHCA and THCA (Figure 1, orange pathway), which partly undergo additional processing steps.

......
before entering the β-oxidation pathway. Phytanic acid, for instance, can only be
degraded in the β-oxidation pathway after it has been α-oxidized to pristanic acid
(Figure 1, light blue pathway)\textsuperscript{23,24}. DHCA, THCA and pristanic acid - when in the
2\((R)\)-orientation - first undergo racemization by the 2-methyl-acyl-CoA racemase
(AMACR) prior to β-oxidation\textsuperscript{25}. Moreover, the first steps in ether-phospholipid
biosynthesis – catalyzed by the enzymes GNPAT and AGPS (glycerone-phosphate
O-acyltransferase and alkyl-glycerone-phosphate synthase, respectively) – take place
in peroxisomes, before the intermediates are further processed in the ER (Figure
1, purple pathway)\textsuperscript{26}. Essential ether-phospholipids are, for example, plasmalogens
which play vital roles in plasma membranes and cell signaling and are especially
found in myelin, heart muscle and blood cells\textsuperscript{27–29}.

Peroxisomal metabolic processes also play a major role in balancing the redox
state of the cell: The formation of reactive oxygen species, like hydrogen peroxide,
as byproducts of the peroxisomal degradation of purines and fatty acids increases
oxidative stress in cells, whereas the peroxisomal enzyme catalase degrades hydrogen
peroxide, thereby reducing oxidative stress (Figure 1, light green)\textsuperscript{30}.

c) The interaction of peroxisomes with other organelles

Peroxisomes do not operate in isolation, but in close interaction with other
cellular organelles, like mitochondria, ER, lysosomes or lipid droplets\textsuperscript{31}. Peroxisomal
metabolism is directly connected to mitochondrial metabolism, and various products
are exchanged between these organelles\textsuperscript{32}. As mentioned before, long-chain fatty
acids produced in peroxisomal VLCFA oxidation are transported to mitochondria
to be fully oxidized (see Figure 1). Furthermore, peroxisomes are exchanging
metabolites with the ER: VLCFAs, which are elongated at the ER membrane, are
transported into peroxisomes for oxidation\textsuperscript{32}, whereas the intermediates of ether
phospholipid synthesis, that are generated in the peroxisome, are transferred to the
ER for formation of the final products\textsuperscript{27}.
In recent years, evidence of physical interactions between various organelles via protein tethers at membrane contact sites has emerged\textsuperscript{31}. Whereas already multiple participating proteins have been discovered in yeast model systems, in mammalian cells only one peroxisomal tether has been identified to date\textsuperscript{33,34}. The peroxisomal membrane protein ACBD5 is interacting with ER-bound proteins VAPA and VAPB, forming a physical tether between the organelles, which supports peroxisomal \(\beta\)-oxidation by improving VCLFA transport and limits peroxisomal motility within the cell. Chapter 2 describes the identification of ACBD5 deficiency as a novel peroxisomal disorder, a study conducted prior to these reports\textsuperscript{35}.

**Life cycle of peroxisomes**

Peroxisomes have a limited life span\textsuperscript{36,37}. Cells tightly control their biosynthesis and degradation in order to adapt the peroxisomal number to the current energy state and metabolic requirements\textsuperscript{38}. The next paragraphs describe the life cycle of a peroxisome, from its biogenesis to its degradation.

**a) The de novo biogenesis of peroxisomes**

A mature peroxisome basically consists of a lipid membrane vesicle, with peroxisomal membrane proteins integrated in its membrane and its matrix filled with peroxisomal proteins, including for instance the metabolic enzymes involved in peroxisomal \(\beta\)-oxidation. New peroxisomes are generated either by *de novo* biogenesis or by the proliferation of existing peroxisomes, whereby only the former process is of relevance for this thesis and thus discussed in more detail (see \textsuperscript{39} for a recent review on peroxisome proliferation).

The current model of peroxisome biogenesis proposes that a phospholipid bilayer vesicle originates from the ER and is subsequently equipped with the protein import machinery for peroxisomal membrane and matrix proteins. In humans, this machinery
Introduction

consists of 14 proteins, so called peroxins or PEX proteins, 13 of which are crucial for peroxisome biogenesis, while PEX11β is involved in peroxisomal division40. The PEX proteins PEX3 and PEX16, which are essential for the import of peroxisomal membrane proteins (PMPs), are already present in the original ER membrane41–43. Other PMPs are integrated into the peroxisomal membrane either directly from the cytosol where they are bound by the PMP transporter PEX19, transported to the peroxisomal membrane and inserted into the membrane via PEX3 or PEX1644,45 or via ER-derived vesicles46. PMPs inserted in the peroxisomal membrane can be transporter proteins, like ABCD1, but can also fulfill other functions, like the additional members of the PEX protein machinery which are required for the import of peroxisomal matrix proteins.

Peroxisomal matrix proteins mostly present with the peroxisomal targeting signal PTS1, consisting of the consensus sequence serine-lysine-leucine (SKL), which is recognized by the receptor protein PEX5 (Figure 2)47,48. PEX5 binds the PTS1 protein and transports it to the peroxisomal membrane, where it docks at the PEX13/PEX14 complex and releases its protein cargo into the matrix49,50. Afterwards, PEX5 is ubiquitinated by the ubiquitin-protein E3 ligase RING complex consisting of PEX2, PEX10 and PEX12. The nature of the PEX5 ubiquitination determines its further fate51,52: When poly-ubiquitinated, PEX5 becomes a target for the proteasome and is degraded53,54. This poly-ubiquitination might not only target PEX5 but the whole peroxisome for degradation55,56 (or ‘pexophagy’, see also chapter 5). In contrast, when PEX5 is mono-ubiquitinated, it can cycle back into the cytosol to bind and transport another PTS1 protein57,58. To date it is unknown, which factors or cell stimuli decide on the ubiquitination state and thus the ultimate fate of PEX559.

Recycling PEX5 back into the cytosol is an ATP-dependent process involving the AAA+ ATPases PEX1 and PEX6 60. The cytosol-facing PEX1/PEX6 complexes are anchored to the peroxisomes by the PMP PEX26, which interacts with PEX661. Although AAA+ ATPases typically present with two ATPase domains (D1 and D2),
each containing a Walker A and B motif with a second region of homology (SRH), PEX6 only contains the conserved D2 domain\textsuperscript{62}. Interestingly, the yeast Pex6p-D2 domain was found to be responsible for the majority of ATPase activity in the Pex1p/Pex6p complex\textsuperscript{63}. PEX1 and PEX6 form hetero-hexameric complexes, and structural analyses of yeast Pex1p/Pex6p complexes have shown that the complex consists of alternating PEX1 and PEX6 units forming a central channel and containing ATP binding pockets at their interface\textsuperscript{63–66}. Binding of ATP is necessary for the interaction of PEX1 and PEX6, whereas the cycling of PEX5 back into the cytosol is dependent on the hydrolysis of the bound ATP\textsuperscript{61,67,68}.

**Figure 2 – Peroxisome biogenesis**

This scheme presents the import machinery of peroxisomal membrane (left panel) and matrix proteins (right panel), encoded by 13 different *PEX* genes. See main text for more details.

5L, Long isoform of PEX5; PMP, Peroxisomal membrane protein; PTS1, Matrix protein presenting peroxisomal targeting signal 1 (or SKL); PTS2, Matrix protein presenting peroxisomal targeting signal 2.
Introduction

While PTS1-dependent transport represents the majority of peroxisomal matrix protein import, three human peroxisomal matrix proteins have been identified with a PTS2 domain (Figure 1): thiolase (ACAAT1, involved in β-oxidation), phytanoyl-CoA 2-hydroxylase (PHYH, involved in α-oxidation) and AGPS (involved in ether-phospholipid synthesis). PTS2 proteins are recognized by the transporters PEX7 and a longer splice variant of PEX5, PEX5L, and transported into the peroxisomal matrix in a similar fashion as PTS1 proteins (see Figure 2).

b) The degradation of peroxisomes (pexophagy)

The macro-autophagic degradation of peroxisomes is generally referred to as ‘pexophagy’. In this process, the peroxisome is targeted for degradation by ubiquitination of its membrane proteins, like PEX5 or PEX3. The adaptor proteins NBR1 and p62 bind to the ubiquitinated proteins and subsequently recruit a phagophore to the peroxisomes. Phagophores are membrane structures containing autophagy factors, like for example LC3-II, which can interact with the adaptor proteins. The phagophore engulfs the peroxisome and the thus formed autophagosome is transported to a lysosome. The lysosome then fuses with the autophagosome and the peroxisome is degraded (reviewed in ). Interestingly, all adaptor proteins identified to date are not exclusively involved in pexophagy, but also in the degradation of other cellular compounds, like for instance protein aggregates. The only protein, which had – based on its similarity to the yeast pexophagy receptor Atg37 – been proposed to be strictly specific and essential for pexophagy, is ACBD5 (see also chapter 2).

Besides the normal turnover of peroxisomes, the cell might be faced with different metabolic challenges requiring the targeted degradation of peroxisomes, for example oxidative stress or ATP limitation. ATP limitation might potentially induce pexophagy by causing the dissociation of PEX1/PEX6 from peroxisomes, leading to PEX5 poly-ubiquitination, or a decreased PEX5/PEX14 interaction promoting PEX14 interaction with the autophagy factor LC3-II.
Chapter 1

Peroxisomal Disorders

The crucial importance of peroxisomes becomes especially evident when faced with the consequences of their defects in patients suffering from peroxisomal disorders\textsuperscript{5}. To date, over 15 different peroxisomal disorders have been described, all of them the consequence of a defective peroxisomal protein\textsuperscript{22,79}. These inherited diseases are very rare and can be broadly divided into two groups: single enzyme deficiencies (SEDs), that are caused by the defect in a peroxisomal protein which affects exclusively the pathway the protein functions in, and peroxisome biogenesis disorders (PBDs), which are caused by the impairment in the biosynthesis of peroxisomes.

The classical diagnostic procedure of patients, whose clinical symptoms indicate a peroxisomal disease, includes the measurement of peroxisomal parameters in blood and fibroblasts of the patient, as well as of peroxisomal enzyme activities (see Table 1)\textsuperscript{79–81}. In this way, the cause of the peroxisomal defect can be narrowed down and can eventually be confirmed by identifying pathogenic mutations in respective candidate genes by DNA sequencing. More recently, the use of Next Generation Sequencing (NGS) in combination with subsequent functional studies has become a valuable asset to identify disease-causing mutations in patients, for whom either the classical procedure was unsuccessful, who suffer from a to date uncharacterized peroxisomal disease, or who were initially – based on their clinical phenotype – not considered as a peroxisomal disease case (see for example chapter 4).
Peroxisomal single enzyme deficiencies

Peroxisomal Single Enzyme Deficiencies (SEDs) are caused by a defect of a single peroxisomal protein, which impairs exclusively the metabolic pathway it is involved in. This results in a characteristic clinical phenotype and is reflected in biochemical abnormalities in patients’ blood and fibroblasts.

Defects in proteins involved in the same pathway may result in an overlapping biochemical and clinical phenotype. For instance, both ABCD1 and ACOX1 are specifically involved in the oxidation of VLCFAs (Figure 1, yellow pathway). Thus, defects in either of the two proteins result in the sole accumulation of C26:0 and other VLCFAs in patients suffering from ACOX1 deficiency or from an ABCD1 defect (also known as X-linked adrenoleukodystrophy or X-ALD). Although the biochemical profiles of ACOX1 and X-ALD patients can therefore be nearly indistinguishable from each other (Table 1), the clinical phenotype is clearly different, with X-ALD manifesting in adrenal insufficiency, fatal cerebral demyelination or progressive spinal cord disease and ACOX1 deficiency patients displaying a clinical phenotype comparable to PBD patients\textsuperscript{79,82}. The clinical phenotype of ACOX1 deficiency patients is also very similar to DBP deficiency patients, but the involvement of DBP protein in the β-oxidation of multiple substrates (e.g. bile acid intermediates) causes more biochemical abnormalities in DBP deficiency patients than only an increased VLCFA level\textsuperscript{83,84}.

In chapter 2, the identification of another defect in peroxisomal VLCFA oxidation is reported, ACBD5 deficiency. Like X-ALD and ACOX1 deficiency patients, the ACBD5 deficiency patient exclusively displayed increased VLCFA concentrations, whereas her clinical phenotype was more similar to ACOX1 deficiency patients\textsuperscript{35}.
Peroxisome biogenesis disorders

Peroxisome biogenesis disorders (PBDs) are caused by biallelic pathogenic mutations in one of the PEX genes, which impair peroxisome biogenesis and thus affect multiple peroxisomal pathways. Defects in PEX7 or the long isoform of PEX5 impair the import of the three PTS2 proteins, resulting in Rhizomelic Chondrodysplasia Punctata (RCDP) type 1 or 5, respectively. Defects in one of the other 13 PEX genes impair the formation (or division) of peroxisomes including all peroxisomal metabolic pathways, and are the cause of so-called Zellweger Spectrum Disorders, or ZSDs. Whereas severe defects in the PEX proteins involved in PMP import (PEX3, PEX16 or PEX19) lead to the complete absence of peroxisomal structures, an impaired matrix protein import due to defects in the other PEX proteins gives rise to the appearance peroxisomal membranes with and empty matrix, so called ghosts.

ZSDs are rare diseases inherited in an autosomal recessive manner. Consequently, both copies of a PEX gene need to be affected in order to be disease-causing, and one functional PEX allele is usually sufficient to prevent a disease phenotype. Recently, however, we encountered seven ZSD cases caused by a single heterozygous mutation, which was situated on an allele over-represented due to allelic expression imbalance (or AEI, see chapter 3).

a) Clinical spectrum

ZSD patients suffer from heterogeneous clinical symptoms, ranging from neurosensory deficits and developmental delay to muscle hypotonia and liver abnormalities, whereby the severity of symptoms can vary immensely. Patients at the most severe end of the spectrum already present in the neonatal period or as an infant with facial dysmorphism, liver dysfunction, adrenal insufficiency, neuronal defects, seizures and severe muscle hypotonia. Death occurs usually...
within the first year of life. At the other end of the spectrum, patients affected
with a mild form of ZSD are often diagnosed later in life\textsuperscript{91}, and suffer mainly from
sensory defects, like deafness or visual impairment, and neurological deficiencies.
Their disease progression can vary substantially and can in some cases be quite
stable, so that patients may reach adulthood and even achieve university degrees.

Most recently, Heimler Syndrome was identified as the mildest ZSD form to date,
manifesting in nail and teeth abnormalities and sensorineural hearing loss (see
chapter 4)\textsuperscript{92,93}.

b) Diagnosis

Since ZSD symptoms are so heterogeneous and show overlaps with other metabolic
disorders (compare e.g. \textsuperscript{94}), the differential diagnosis of milder ZSD forms can be
difficult and patients have been missed or misdiagnosed in the past\textsuperscript{5,92,95}. As soon as
a patient is suspected to be affected with a ZSD, however, a multitude of laboratory
analyses is available for diagnostic testing (Table 1).

Blood and fibroblasts can be analyzed for accumulations of several peroxisomal
substrates, like VLCFAs, phytanic and pristanic acid and bile acid intermediates\textsuperscript{96,97},
with an increased level of lyso-PC C26:0 being the most sensitive marker known
to date\textsuperscript{98}. Further studies of the enzyme activities of GNPAT (formerly known as
DHAPAT), thiolase and ACOX1, as well as measurements of peroxisomal oxidation
activities by loading the cells with labelled fatty acids, can confirm a peroxisomal
defect\textsuperscript{99,100}. In the mildest ZSD cases, however, almost all these tests might show
no or only slight abnormalities\textsuperscript{92,101}. Hence, the most conclusive assay indicating a
peroxisome biogenesis defect is the immunofluorescence microscopy analysis of
peroxisomal matrix protein import in patients’ fibroblasts, which is impaired in even
the mildest cases (with catalase being the most sensitive marker)\textsuperscript{102}.
Table 1 - Clinical phenotype of selected peroxisomal disorders including diagnostic

<table>
<thead>
<tr>
<th>disease</th>
<th>gene</th>
<th>neurological (CNS)</th>
<th>sensory</th>
<th>musculoskeletal</th>
<th>others</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZSDs</td>
<td>PEX genes</td>
<td>hypotonia</td>
<td>seizures</td>
<td>developmental delay</td>
<td>developmental regression</td>
</tr>
<tr>
<td>RCDP1</td>
<td>PEX7</td>
<td>+</td>
<td>L</td>
<td>L/A</td>
<td>+</td>
</tr>
<tr>
<td>RCDP5</td>
<td>PEX5_L</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>X-ALD</td>
<td>ABCD1</td>
<td>-</td>
<td>L</td>
<td>-</td>
<td>L</td>
</tr>
<tr>
<td>ACOX1 def.</td>
<td>ACOX1</td>
<td>+</td>
<td>L</td>
<td>L/A</td>
<td>+</td>
</tr>
<tr>
<td>DBP def.</td>
<td>HSD17B4</td>
<td>+</td>
<td>L</td>
<td>L/A</td>
<td>+</td>
</tr>
</tbody>
</table>

PBDs, Peroxisome Biogenesis Disorders; SEDs, Single Enzyme Deficiencies; ZSDs, Zellweger spectrum disorders; XALD, X-linked adrenoleukodystrophy; ACOX1 def., Acyl-CoA oxidase 1 deficiency; DBP def., D-bifunctional protein deficiency; RCDP, Rhizomelic Chondrodysplasia Punctata; PEX5_L, long isoform of PEX5; CNS, Central Nerve System;

+, Symptom present; -, Symptom not present; L, Late childhood onset; A, adolescent or adult onset.

Tables adapted from 79.
analyses used for differential diagnosis

<table>
<thead>
<tr>
<th>Peroxisomal metabolites</th>
<th>Enzyme activity</th>
<th>Matrix protein import</th>
<th>Peroxisomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLCFAs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lyso PC C24:0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>phytanic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pristanic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pimelic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bile acid intermediates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>THCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plasmalogens C16:0, C18:0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Glycerone-phosphate Oacyltransferase (formerly known as DHAPAT); PMP, Peroxisomal membrane protein; P, In plasma; F, In fibroblasts; E, In erythrocytes; U, In urine; DBS, In dried blood spot; c, Concentration measurement; ox, Oxidation assay; a, Activity assay; im, Immunofluorescence microscopy assay; ib, Immunoblot analysis; ↑, Increased level (≠ reduced oxidation); ↓, Decreased level/activity; -, normal.

<table>
<thead>
<tr>
<th>disease</th>
<th>method (material)</th>
<th>disease</th>
<th>method (material)</th>
<th>disease</th>
<th>method (material)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZSDs</td>
<td>c(P,F)3,4</td>
<td>RCDP1</td>
<td>c(DBS)5</td>
<td>RCDP5</td>
<td></td>
</tr>
<tr>
<td>-/-↑</td>
<td>-/-↑</td>
<td>-/-↑</td>
<td>-/-↑</td>
<td>-/-↑</td>
<td>-/-↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X-ALD</td>
<td></td>
<td>ACOX1</td>
<td></td>
<td></td>
<td>DBP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>def.</td>
<td></td>
<td></td>
<td>def.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* abnormal peroxisomal morphology / reduced number / no membranes (in PEX3, 16 or 19 defect).
Chapter 1

When these diagnostic assays indicate a ZSD in a patient, the defective PEX protein causing the disease can be identified using two approaches. Complementation assays can be performed by expressing functional copies of each PEX protein one by one in patient’s cells and analyzing the re-appearance of peroxisomes\textsuperscript{103}. Candidate PEX genes identified this way are subsequently sequenced to detect compound heterozygous or homozygous pathogenic mutations. Alternatively, all PEX genes can be sequenced directly to identify the potential disease-causing mutations by NGS (e.g. Whole Exome Sequencing or peroxisomal gene panel sequencing)\textsuperscript{89,104}. While defects in all 13 PEX genes have been described to result in ZSDs\textsuperscript{103,105}, most ZSD cases are caused by mutations in PEX1 (60\%) or PEX6 (15\%) in European populations\textsuperscript{103}.

c) Treatment options

To date, there is no curative treatment for peroxisomal disorders. Interventions are merely supportive, often focusing on replenishing deficient compounds or preventing the accumulation of potentially toxic peroxisomal substrates. All previous approaches, however, showed only limited positive impact on the disease course\textsuperscript{5}. One famous example is the use of Lorenzo’s oil for the treatment for X-ALD, which decreases VLCFA levels in plasma, without improving the disease progression, however\textsuperscript{106,107}.

Several in vitro studies have used cultured primary fibroblasts derived from ZSD patients to test the effect of chemical compounds and culturing conditions on peroxisomal functions. An improvement of peroxisome biogenesis was for instance observed when treating the cells with betaine, L-arginine or glycerol, or when incubating them at lower temperatures (30°C)\textsuperscript{108–110}. The underlying pathways causing this positive effect are difficult to resolve, considering the wide-range effect of all those treatments on cellular metabolism\textsuperscript{111–114}. Potential modes of action might be via an effect on cellular signaling pathways regulating peroxisomal proteins\textsuperscript{115,116} or on the oxidative state of the cell\textsuperscript{110,117}. Also a direct impact of these treatments on folding, abundance or activity of the defective PEX protein is possible (see also next
Introduction

1. Most recently, the inhibition of autophagy in (immortalized) cells of ZSD patients was reported to have a positive effect on peroxisome biogenesis\textsuperscript{56}. Chapter 5 presents subsequent studies investigating the effect of pexophagy inhibitors and L-arginine on peroxisomal performance in ZSD patient cells in more detail.
mRNA abundance as a factor in disease severity

The severity of a ZSD can be influenced by the residual functionality but also the abundance of mutated PEX proteins in the patient, with higher protein levels usually associated with a milder phenotype\textsuperscript{109,118}. The level of a protein depends on the rate of its synthesis and degradation, the former being crucially influenced by the level of its encoding mRNA\textsuperscript{119}. mRNA abundance is, in turn, dependent on the rate of gene transcription and mRNA processing, as well as its stability and degradation rate\textsuperscript{120}. The following chapter gives a brief overview on mechanisms influencing mRNA abundance, with special emphasis on the role of polyadenylation signals, since they are of special interest for chapter 3.

\textbf{a) Regulation of mRNA abundance}

A crucial prerequisite for the transcription of genes is their accessibility to the RNA Polymerase II-transcription machinery (POLII), which depends on the chromatin and methylation state of the DNA and is tightly regulated\textsuperscript{121–123}. The rate of the subsequent transcriptional initiation and elongation is then determined by the interplay of DNA binding \textit{trans} elements, like transcription factors, with \textit{cis} acting elements on the DNA, i.e. the promotor region of the gene, and downstream and upstream situated silencer and enhancer sequences (Figure 3)\textsuperscript{120}. For example, a gene with a strong promoter and multiple binding sites for activating transcription factors can be highly efficient in recruiting POLII and promoting its progress, which results in a high transcription rate.

Before being transported out of the nucleus and translated to proteins in the cytosol, the generated pre-mRNA needs to undergo processing steps, whose rates also influence the final abundance of mature mRNA\textsuperscript{124}. Processing steps, occurring both co- or post-transcriptionally, include mRNA splicing, the addition of the 7-methylguanosine cap to the 5’ mRNA end and the polyadenylation of its 3’ end (PolyA tail)\textsuperscript{123,125}. 

.......
Figure 3 - Regulation of cellular mRNA level
This scheme represents the different processes involved in regulating the rate of mRNA synthesis, processing and degradation. See main text for more details.
UTR, untranslated region; miRNA; microRNA, lncRNA long non-coding RNA; POLII, RNA-polymerase II-transcriptional machinery; TF, transcription factor; PAS, polyadenylation signal.

The regulation of the DNA chromatin state, the transcription and the processing of mRNA are complexly coupled, interdependent processes. Promotor elements and elongation speed, for example, affect (alternative) splicing of mRNA\textsuperscript{126,127}, whereas
mRNA processing steps can, in turn, influence chromosome integrity\textsuperscript{128} and transcriptional initiation and elongation\textsuperscript{129,130}. Alterations in a single process might thus also affect interdependent processes and eventually have broad consequences on the final mRNA synthesis rate.

After maturation, the mRNA is exported into the cytosol for translation, where it becomes also eventually subject to degradation\textsuperscript{131}. Quality control systems, essential for degrading defective mRNAs and thereby inhibiting the synthesis of dysfunctional proteins, check the mRNA for indications of impaired integrity, for example improper processing or incorrect sequences like premature stop codons\textsuperscript{132}. But also the half-life of an entirely integer mRNA is only limited\textsuperscript{131}. Its physical proneness to degradation can, among others, be influenced by its secondary structure\textsuperscript{133,134} or the length of its PolyA tail\textsuperscript{135}. Furthermore, mRNAs may present sequences for trans factors, like stabilising or destabilising RNA binding proteins and non-coding RNAs, as for instance microRNAs\textsuperscript{131,136}. These small single stranded RNAs can bind to sequences in the 3’ untranslated region (UTR) of the mRNA and target the mRNA this way for degradation\textsuperscript{137}.

In conclusion, the final mRNA level in a cell is determined by the balance of mRNA synthesis and degradation.

\textbf{b) Influence of alternative polyadenylation on mRNA abundance}

The polyadenylation and cleavage of mRNA, two essential mRNA processing steps, are dependent on polyadenylation signals (PAS) located downstream of the stop codon. PAS consist of the hexamer AAUAAA or variations thereof, which reside 10-30 bp upstream of the cleavage site and recruit (in interaction with auxiliary sequences) the multimeric cleavage and polyadenylation specificity factor to the cleavage site, hence determining the length of the 3’-UTR\textsuperscript{124}.

Over 50% of mRNAs have multiple polyadenylation sites and are thus subject to
alternative polyadenylation, which can result in mRNA isoforms with different 3’-UTR lengths\textsuperscript{138,139}. Which of the PAS is used for mRNA cleavage and polyadenylation, depends among others on the strength of the PAS and auxiliary sequences (e.g. the PAS similarity to the consensus AAUAAA hexamer\textsuperscript{140}) and the speed of transcriptional elongation\textsuperscript{141}. A preference of the more distal or proximal PASs can also be controlled by global stimuli regulating DNA interacting \textit{trans} elements, for instance during cell differentiation\textsuperscript{142}. Moreover, a universal shortening of mRNA 3’-UTRs due to alternative polyadenylation has been reported in some cancer cells, resulting in increased mRNA levels\textsuperscript{143–145}.

The use of different polyadenylation signals can influence the total cellular mRNA level in multiple ways. On the one hand, the choice of the polyadenylation signal might have a direct impact on the speed and efficiency of transcription and processing by affecting the transcriptional termination\textsuperscript{124,146} and coupled processes like transcriptional initiation and elongation\textsuperscript{147}, pre-mRNA splicing\textsuperscript{148} and the mRNA export into the cytosol\textsuperscript{149}. On the other hand, alternative polyadenylation might also affect mRNA stability, if the usage of different PAS results in mRNA isoforms with varying 3’-UTR lengths\textsuperscript{145,150}. Longer 3’-UTR ends may present more binding sites for factors regulating mRNA stability, like RNA binding proteins\textsuperscript{151}, long non-coding RNA\textsuperscript{152}, or – the best studied example – microRNAs\textsuperscript{137}. An increased level of mRNAs with a shortened 3’-UTR length was often supposed to be the result of their increased stability\textsuperscript{143–145}, but recent studies suggest that mRNA stability might only play a minor role\textsuperscript{153}.

Taken together, the final mRNA level in a cell is determined by a sheer myriad of factors, of which many may be influenced by alternative polyadenylation\textsuperscript{123}. Thus, when faced with differential levels of mRNAs that use different PASs, the search for the specific cause is challenging, because it is complicated by the interdependency and complexity of mRNA synthesis and degradation processes (see also chapter 3).
Chapter 1

This Thesis in a Nutshell – Outline

The major aim of this thesis was to identify the cause of disease of patients suffering from unidentified peroxisomal defects and thus to improve the laboratory diagnosis of future patients. Understanding the underlying disease mechanisms also provides valuable insights into the functioning of peroxisomes and the phenotypical consequences of their defects, which can eventually help to identify potential treatment options.

The introductory chapter 1 presents essential background information about peroxisomes, their biogenesis and functions, as well as the phenotypes and mechanisms of known peroxisomal diseases.

Chapter 2 reports the discovery of ACBD5 deficiency, a novel peroxisomal disorder, which was found to affect a young girl from the United Arab Emirates. She suffered from neurological symptoms and vision impairment, but the cause of her afflictions was unknown. In her cells, we detected an accumulation of VLCFAs, which pointed to a peroxisomal defect, since VLCFAs are normally degraded in peroxisomes. By genetic analyses, we discovered that the girl had a homozygous ACBD5 gene mutation, resulting in the complete absence of this peroxisomal protein. We confirmed that the loss of ACBD5 is the underlying cause of the patient’s disease by using two functional assays: We introduced functional ACBD5 into cells of the patient, which rescued the peroxisomal defect, and we eliminated ACBD5 in a model cell line using CRISPR-Cas9 genome editing, which lead to similar VLCFA accumulations as observed in the patient. In conclusion, we identified ACBD5 deficiency as a novel peroxisomal single enzyme deficiency, leading to VLCFA accumulations.

Chapter 3 describes a novel genetic disease mechanism for the autosomal recessive Zellweger Spectrum Disorders (ZSDs). These diseases are caused by defects in peroxisomal biogenesis, which are usually the result of biallelic mutations in one
of 13 different PEX genes\textsuperscript{80}. We were thus surprised to identify seven unrelated ZSD patients from all over the world, who only displayed a single heterozygous PEX6 mutation (p.Arg860Trp). This pathogenic mutation not only abolished the function of the encoded PEX6 proteins, but also had a dominant-negative effect on functional wild-type PEX6 proteins. However, since several of the asymptomatic parents presented with the identical PEX6 mutation, a dominant disease mechanism (with this mutation as the sole cause of the ZSD) had to be excluded. Instead, we noticed that the mRNA of the mutant PEX6 allele was in all patients more abundant than the wild-type PEX6 mRNA, whereas the mRNA expression of PEX6 alleles in the asymptomatic parents was equal. We discovered that this imbalance in the allelic expression (AEI) of PEX6 was a common phenomenon, also frequently occurring in control individuals. The AEI correlated with a common heterozygous PEX6 deletion eliminating a polyadenylation signal, which hence potentially represents the underlying cause of the differential PEX6 expression. Taken together, the ZSD of the seven patients is caused by the over-representation of a dominant-negative PEX6 allele, which is the result of the common PEX6 AEI.

ZSDs represent a spectrum of diseases, ranging from rather mild to very severe phenotypes. The disease severity partly correlates with the genotype, i.e. the severity of the PEX mutations, with for example non-sense mutations which result in the entire loss of the protein leading to the most severe cases\textsuperscript{154}. Recently, we discovered that Heimler syndrome, a rare syndrome of previously unknown disease cause, is actually a very mild ZSD, and caused by mutations in peroxisome biogenesis genes PEX1 and PEX6 (\textbf{chapter 4}). Using an unbiased sequencing approach (NGS) we identified PEX1 or PEX6 mutations in Heimler Syndrome patients from six different families, and subsequently confirmed a peroxisomal biogenesis defect in material of the patients. We noted that several of the PEX1 and PEX6 mutations only impaired the protein function to a certain degree, leaving each of the patients still with substantial residual peroxisome biogenesis activity, which explains the mildness of their phenotype. This

......
way, we could not only solve the unknown cause of Heimler syndrome, but also could expand the phenotypical spectrum of ZSDs at its mildest end.

To date, there is no curative treatment for ZSDs. Several chemical compounds, for instance L-arginine and glycerol, have been described to improve peroxisomal functions when applied in vitro to cells with a PEX defect, but none of them has proven to be effective in vivo\textsuperscript{108,110}. Most recently, autophagy inhibiting compounds, including hydroxychloroquine (HCQ), were reported to have a beneficial effect on cells with the mild PEX1-Gly843Asp mutation. This was based on the theory that these compounds reduce the rapid autophagic degradation of peroxisomal membranes and thus increase the opportunity for the residually active peroxisome machinery to correctly import peroxisomal proteins\textsuperscript{56}. In chapter 5 we have studied the effect of autophagy inhibition and analyzed if autophagy inhibition might also be the underlying mechanism of the beneficial effect of L-arginine. To this end, we used diverse assays, that analyze peroxisomal functions in different PEX1-Gly843Asp cell lines. Unexpectedly, we noted in all cells that autophagy inhibition did not improve peroxisomal functions and biogenesis, but actually seemed to slightly worsen them. Our results thus strongly advise against treating ZSD patients with autophagy inhibitors, and in particular with HCQ.

Finally, all major results of this thesis are summarized and discussed in chapter 6, in which also conclusions for future perspectives are drawn. This chapter additionally contains Dutch and German summaries of this thesis.
References


Introduction


