Metabolomics to study functional consequences in peroxisomal disorders
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CHAPTER 1

General Introduction
Discovery and Biochemistry of Peroxisomes

Peroxisomes were first described in 1954 and obtained their name based on the discovery that they contain enzymes to generate and degrade hydrogen peroxide from organic substrates \(^1\,^2\). After that, these organelles have long been underrated and were considered to have no major functional role in human physiology. The discovery in 1973 that peroxisomes were absent in specific tissues of patients with Zellweger Syndrome (ZS) raised scientific interest in these organelles \(^3\). About ten years later, the first biomarkers for diagnostic screening of patients were reported \(^4\,-\,^6\), and in 1992, the first genetic defect causing ZS was identified \(^7\). Today, we know that peroxisomes are crucial organelles in eukaryotic cells, and involved in a variety of catabolic and anabolic metabolic processes \(^8\), including the α- and β-oxidation of various fatty acids, the biosynthesis of ether phospholipids, bile acids and docosahexaenoic acid (DHA; C22:6-ω3), and the detoxification of glyoxylate and reactive oxygen species such as hydrogen peroxide \(^9\).

In humans, peroxisomes harbour a variety of enzymatic reactions, many of which are unique for peroxisomes, while several are also present in other organelles \(^8\,^10\). Fatty acid degradation, for example, occurs both in peroxisomes and mitochondria, but the substrates for mitochondrial β-oxidation are fatty acids of short, medium and long chain length (≤C20), whereas the breakdown of very long-chain fatty acids (VLCFAs, ≥C22), branched-chain fatty acids such as phytanic and pristanic acid, long-chain dicarboxylic acids, and di- and trihydroxycholestanoic acid only occurs in peroxisomes \(^11\). VLCFAs enter the peroxisome mainly via the transmembrane proteins ATP-binding cassette transporter D1 (ABCD1), and alternatively via ABCD2 or ABCD3 \(^12\,-\,^14\). Before entering the β-oxidation pathway, VLCFAs first need to be activated to their coenzyme A (CoA) esters, followed by degradation in a cycle containing four basic steps, including dehydrogenation, hydration, dehydrogenation and thiolytic cleavage \(^15\) (Figure 1). This sequence of reactions is catalysed by a number of peroxisomal enzymes, acyl-CoA oxidase 1 (ACOX1), D-bifunctional protein (DBP), and the peroxisomal thiolases sterol carrier protein X (SCPx) and 3-ketoacyl-CoA thiolase (ACAA1) \(^10\,^16\,-\,^18\). Each cycle of β-oxidation produces one acetyl-CoA and one acyl-CoA shortened by two carbon atoms, which is either again substrate for another round of β-oxidation, or can be transported out of the peroxisome for complete breakdown in the mitochondria \(^8\,^19\).

Different types of fatty acids can serve as a substrate for β-oxidation in peroxisomes, including straight- and branched-chain fatty acids, mono- and polyunsaturated fatty acids. Fatty acids containing a methyl or other functional group at the 3-carbon instead of the 2-carbon atom, however, are first catabolised by peroxisomal α- or ω-oxidation \(^20\,^21\). Phytanic acid is a branched-chain fatty acid with the methyl group at the 3-carbon, and is the classical substrate for α-oxidation, during which one carbon atom is removed \(^10\,^20\). After phytanic acid is activated to phytanoyl-CoA and imported into the peroxisome via ABCD3, the peroxisomal enzymes phytanoyl-CoA hydroxylase (PHYH) and 2-hydroxyacyl-
CoA lyase remove the carboxyl carbon and produce the branched-chain aldehyde pristanal, which is subsequently oxidised to pristanic acid by pristanal dehydrogenase (Figure 1). Pristanic acid is a branched-chain carboxylic acid with the methyl group at the 2-carbon, and is a substrate for β-oxidation. Before pristanic acid can be β-oxidised, its CoA-ester is first converted from the (R)-configuration into its (S)-configuration isomer by the enzyme 2-methylacyl CoA racemase (AMACR).

An alternative route to α-oxidation is ω-oxidation. For degradation of phytanic acid (and VLCFAs) via this pathway, the fatty acids are converted to an ω-hydroxy fatty acid, followed by oxidation to a dicarboxylic fatty acid, which in turn is a substrate for β-oxidation. Of note, ω-oxidation has been described as a rescue pathway for FA disorders affecting peroxisomal α- or β-oxidation, which can be upregulated to support breakdown of FAs such as phytanic acid and VLCFAs.

Peroxisomal β-oxidation is also involved in anabolic processes, such as the production of bile acids in hepatocytes. The bile acid intermediates di- and trihydroxycholestanolic acid (DHCA and THCA) are synthesised from cholesterol, and enter the peroxisome as CoA-esters via ABCD3. Next, AMACR converts the bile acid intermediates DHC-CoA and THC-CoA from their (25R)-configuration to their (25S)-isomers, which can then undergo peroxisomal β-oxidation. After one round of β-oxidation, the CoA-esters of chenodeoxycholic acid and cholic acid are produced, which are enzymatically converted to taurine and glycine conjugates by the enzyme bile acid-CoA:amino acid N-acyltransferase (BAAT) (Figure 1). The bile acids are then exported from the peroxisome and excreted into the bile. Another product of β-oxidation is DHA, which is synthesised by one round of β-oxidation from its precursor tetracosahexaenoic acid (C24:6-ω3). DHA is the most abundant ω-3-PUFA in brain and retina tissues, and DHA is deficient in peroxisomal disorders affecting β-oxidation.

Other important lipids that are generated in the peroxisome are ether phospholipids. Whereas phospholipids contain an ester bond at the sn-1 position, ether phospholipids, including plasmalogens, contain a fatty alcohol at position sn-1 that is linked to an (vinyl) ether bond. Ether phospholipid biosynthesis is initiated in the peroxisome by the enzymes glycerone phosphate O-acyltransferase (GNPAT) and alkyl glycerone phosphate synthase (AGPS) (Figure 1). The fatty alcohol is provided by one of the two fatty acyl-CoA reductases (FAR1 and FAR2) localised at the outer membrane of the peroxisome. Deficiencies in one of the proteins involved in plasmalogen synthesis cause different types of the peroxisomal disorder rhizomelic chondrodysplasia punctata (RCDP).
Figure 1. Biochemical pathways in peroxisomes. The main metabolic pathways located in the peroxisome are shown. Peroxisomal enzymes and transporter proteins are indicated in grey boxes. Substrate and product metabolites are indicated without boxes. Enzymes belonging to the three main metabolic pathways, in particular fatty acid α- and β-oxidation, and ether phospholipid synthesis, are encircled by dashed lined boxes. A variety of fatty acids can be degraded by peroxisomal β-oxidation, including very long-chain fatty acids (VLCFAs), dicarboxylic acids (DCAs), the bile intermediates di- and trihydroxycholestanolic (DHCA and THCA), and pristanic acid. These substrates are first converted to the corresponding coenzyme A (CoA) esters before entering the peroxisome via the indicated peroxisomal transporter proteins. Since peroxisomal β-oxidation only accepts substrates present in the (2S)-configuration, (2R)-isomers of DHCA, THCA and pristanic acid first need to be converted to their corresponding (2S)-isomers. Phytanic acid is first degraded to pristanic acid by α-oxidation, which in turn is a substrate for peroxisomal β-oxidation. After each round of β-oxidation, a chain-shortened fatty acyl-CoA is produced which may be a substrate for another round of β-oxidation, or can be transported into the mitochondria for complete degradation by mitochondrial β-oxidation. The first steps of ether phospholipid synthesis are located in the peroxisome, which introduces the characteristic ether bond of these species. The following steps of ether phospholipid synthesis occur in the endoplasmic reticulum. Abbreviations: ACAA1, 3-ketoacyl-CoA thiolase; ACOX1, Acyl-CoA oxidase 1; ACOX2, acyl-CoA oxidase 2; ABCD1, ABC transporter D1; ABCD2, ABC transporter D2; ABCD3, ABC transporter D3; ADHAPR, acyl/alkyldihydroxyacetonephosphate reductase; AGPS, alkyldihydroxyacetonephosphate synthase; AGT, alanine-glyoxylate aminotransferase; AMACR, 2-methylacyl-CoA racemase; BAAT, bile acid-CoA:amino acid N-acyltransferase; brAcyl, branched-acyl; CA, cholic acid; CDCA, chenodeoxycholic acid; DBP, D-bifunctional protein; DCA, dicarboxylic acids; DHAP, dihydroxyacetone phosphate; DHCA, dihydroxycholestanolic acid; FAR1, fatty acyl reductase 1; GNPAT, dihydroxyacetonephosphate acyltransferase; GP, glycerophosphate; HACL1, 2-hydroxyphytanoyl-CoA lyase; LBP, L-bifunctional protein; PHYH, phytanoyl-CoA 2-hydroxylase; PrDH, pristanal dehydrogenase; SCPx, sterol carrier protein X; THCA, trihydroxycholestanolic acid; VLCFA, very long chain fatty acids.
Peroxisomal Disorders

Peroxisomal disorders are multi-systemic, and often display neurological features. The prototype is the cerebo-hepato-renal syndrome or ZS, which is characterised by the absence of peroxisomes and a severe clinical presentation. By studying ZS patient material, a variety of biomarkers have been identified in plasma and erythrocytes, which led to the discovery of several additional peroxisomal disorders. In general, peroxisomal disorders can be subdivided into two groups, including (1) the peroxisome biogenesis disorders (PBDs) and (2) the single peroxisome enzyme deficiencies (PEDs) (Figure 2). Combined, all peroxisomal disorders together have an estimated incidence of 1:5000 newborns, of which X-linked adrenoleukodystrophy (ALD) is the most frequently occurring disorder.

Since often only one of the metabolic pathways of peroxisomes is impaired in the PEDs, this group can be sub-classified based on the affected pathway. PEDs that are associated with β-oxidation of VLCFAs are ALD, ACOX1-, ACOX2-, and DBP deficiency, as well as AMACR- and peroxisomal sterol Carrier X protein (SCPx) deficiency. Recently, the deficiency of Acyl-CoA binding domain containing protein 5 (ACBD5) has been described as a peroxisomal disorder with a defect in VLCFA breakdown. Refsum disease, on the other hand, is caused by a mutation in the gene encoding phytanoyl-CoA hydroxylase (PHYH) and results in impaired α-oxidation. PEDs leading to a defect in ether phospholipid synthesis are described as RCDP Type 2 – 4, including GNPAT deficiency (RCDP Type 2), AGPS deficiency (RCDP Type 3) and FAR1 deficiency (RCDP type 4). Furthermore, primary hyperoxaluria Type 1 is a peroxisomal disorder of glyoxylate metabolism, and acatalasemia is a disorder of peroxisomal hydrogen peroxide metabolism presented with a mild clinical phenotype. Finally, ABCD3 (PMP70) deficiency and BAAT deficiency are peroxisomal bile acid synthesis disorders that also belong to the group of PEDs.

PBDs are caused by bi-allelic mutations in one of the 14 PEX genes, resulting in a defect in peroxisome formation, leading to multiple peroxisomal enzyme defects. Based on the clinical features and biochemical parameters, PBDs can be subdivided in the Zellweger spectrum disorders (ZSD) and RCDP Type 1 and 5. Prior to the finding that these disorders have shared biochemical features, the ZSDs were traditionally described as three distinct clinical phenotypes, including ZS, neonatal adrenoleukodystrophy (NALD), and infantile Refsum disease (IRD). Currently, these disorders are part of a wide spectrum of different phenotypes- the ZSDs- with ZS being at the most severe, and IRD at the milder end of the spectrum. In addition, very mild phenotypes, such as Usher Syndrome and isolated cerebellar ataxia and neuropathy have been included into the spectrum. Very recently, Heimler Syndrome was found also to be a PBD with a very mild phenotype.
Besides PBDs and PEDs, a number of peroxisomal defects have recently been identified, in which the maintenance of peroxisomes is impaired, including PEX11β-deficiency, and deficiencies in the proteins dynamin-like protein 1 (DLP1), and mitochondrial fission factor (Mff) \(^{57,60-62}\) (Figure 2). The Pex11 protein family is involved in peroxisome proliferation, and Pex11β-deficiency caused rather mild clinical phenotypes along with almost no biochemical abnormalities \(^{38,57}\). DLP1, Mff and Fission 1 (FIS1) are involved in fission of peroxisomes and mitochondria, and case reports of patients with DLP1- and Mff-deficiency mostly describe a severe clinical phenotype with both peroxisomal and mitochondrial biochemical features \(^{60,61,63,64}\).

Although patients with bi-allelic mutations in either of the 13 PEX genes have been identified, the majority (approximately 60%) of ZSD patients have mutations in the PEX1 gene \(^{65}\). In general, severe genetic defects in one of the PEX genes required for peroxisome membrane assembly – PEX3, PEX16 and PEX19 – result in the complete absence of peroxisomal structures in the cell and cause a severe phenotype \(^{66-68}\). In cells from ZSD patients with a severe defect in any of the other known PEX genes, however, peroxisomal membrane remnants (so-called “peroxisomal ghosts”) are still present, while cells from milder affected ZSD patients often have a number of cells that contain intact functional peroxisomes among other cells that do not (so-called “peroxisomal mosaicism”) \(^{9,69}\). Although some genotype-phenotype correlation exists \(^{70,71}\), the clinical severity and progression of the disease is difficult to predict for individual patients, and biochemical abnormalities are not clearly correlated with clinical features \(^{9,66}\). Furthermore, similar clinical presentations as described for a PBD may also be caused by single-enzyme deficiencies, especially those which are involved in peroxisomal fatty acid β-oxidation \(^{72}\). About 15% of all patients with a clinical phenotype compatible with a ZSD are actually affected with a peroxisomal single-enzyme deficiency, and biochemical and genetic tests are required for the correct diagnosis \(^{56,73}\). Notably, this includes DBP- and ACOX1 deficiency, of which the first patients were described as pseudo-ZS and pseudo-NALD, respectively \(^{16,31,74}\).

While patients with the classical ZS have clear clinical and biochemical abnormalities, and their diagnosis is not considered difficult, identifying ZSD patients with much milder phenotypes is more challenging \(^{39}\). Despite the variety of disease-specific metabolites that principally can be measured in blood samples, only minimal abnormalities have been found in a number of patients with a very mild ZSD phenotype, many of whom were only diagnosed at a later age \(^{9,58,75-77}\).
Figure 2. Schematic overview of peroxisomal disorders. Peroxisomal disorders can be sub-divided in peroxisome biogenesis disorders (PBDs) and peroxisome single-enzyme deficiencies (PEDs). In the PBDs, peroxisome formation is disturbed, whereas for PEDs, only one particular peroxisomal pathway is affected.

Clinical Symptoms and Biochemical Features

In general, most PD patients share a pattern of clinical symptoms, including poor vision (retinopathy) and hearing deficits (sensory neural deafness), liver diseases, adrenal dysfunction and problems affecting the central and peripheral nervous system, resulting in symptoms such as developmental delay, myelopathy, and peripheral neuropathy. Based on the age of presentation, ZSD patients are divided into three different groups. While the neonatal-infant presentation resembles the classical ZS with poor prognosis and survival usually not beyond the first year of life, the childhood presentation is more heterogeneous and prognosis depends on the affected organ system and the occurrence of progressive cerebral demyelination. However, most patients die before adolescence. In the adolescent-adult group, the symptoms are less severe, but abnormalities in vision and sensorineural hearing deficits are common features in this group, whereas the degree of developmental delay is highly variable. Patients with presentation in (childhood and) adolescence usually have a better prognosis, with long periods of stabilisation or no disease progression. Prognosis decreases if they develop progressive liver disease or leukodystrophy. The described
common features for the recognition of ZSD are, however, insufficient for the clinical diagnosis of patients with a very mild phenotype, although these also often show vision or sensorineural hearing deficits. 58,73.

After clinical suspicion of a PD, biochemical tests covering the full spectrum of peroxisomal metabolites should be performed in blood and urine samples from the patient (Figure 3). Peroxisomes are involved in both catabolic and anabolic pathways, and the absence of intact functional peroxisomes causes major consequences for metabolism 9. A variety of metabolic aberrations can be detected in blood and urine samples from PD patients, including the levels of very long-chain fatty acids (VLCFAs), phytanic and pristanic acid, pipecolic acid, THCA and DHCA in plasma, and plasmalogen levels in erythrocytes 8,50,69. Recently, the determination of Lyso-PC(26:0) and C26:0-acylcarnitine in dried blood spots has been added to the range of biochemical tests available for the diagnosis of peroxisomal disorders 69,79. These metabolites were originally found to be a good marker for patients with ALD, and are used for the diagnosis of ALD in newborn screening programs 80,81.

Subsequently, peroxisomal biomarkers should ideally be examined in fibroblasts derived from a skin biopsy of the patient 39,69 (Figure 3). The studies in fibroblasts are especially important if atypical, or only minimal abnormalities are found in the patient’s blood. A growing number of patients exists who have normal peroxisomal metabolite levels in plasma, including a normal VLCFA profile 69,72,77,82. In addition, the levels of VLCFAs have been reported to normalise with age in mild ZSD patients 78. In some cases, such as for patients with Heimler Syndrome, a peroxisomal disorder was initially excluded based on the biochemical findings in plasma, and those patients were later identified through whole exome sequencing 58,59. In those cases, the study of patient fibroblasts is essential for the proper diagnosis, and usually shows mild, but clear abnormalities. In addition, diagnosis of the specific disorder using blood samples only may be difficult, especially since the levels of for example VLCFAs may be artificially elevated as a result of a ketogenic diet or the consumption of peanuts 83,84, whereas other metabolites such as pristanic and phytanic acid are diet-dependent, but not yet elevated in neonatal and infant patients 20,69,85. In addition, the determination of some characteristic metabolites, including bile acids and plasmalogens, is not performed in many laboratories 69.

Since most functions of peroxisomes are expressed in fibroblasts, studying cultured cells derived from patient skin biopsies is ideal for the diagnosis of peroxisomal disorders. For instance, peroxisomal functions such as α- and β-oxidation, and ether phospholipid synthesis can be analysed by studying the oxidation of individual fatty acids in fibroblasts, including C26:0, pristanic acid and phytanic acid using radio-labelled substrates 8,41,86,87. In addition, β-oxidation defects can be detected using deuterated substrates, such as d3-C22:0 57,88. Furthermore, individual enzyme assays and immunoblot analysis can be performed for a number of proteins, including DHAPAT, ACOX1, PHYH and DBP 9,16,17,89. Using immunofluorescence microscopy, the morphology of peroxisomes can
be analysed using specific antibodies against catalase (peroxisomal matrix enzyme) and ABCD3/PMP70 (peroxisomal membrane protein) \(^{90,91}\). This tool allows the identification of subtle abnormalities under the microscope, which is especially helpful for the diagnosis of patients with a very mild phenotype and only minor alterations in peroxisome function and morphology \(^{69,92}\). Together, the study in patient-derived fibroblasts allows the confirmation of the results from the biochemical tests obtained in blood samples. Furthermore, genetic analysis can be performed in fibroblasts as well. To rapidly identify the affected gene, several approaches have been developed for genetic diagnostics, including transfection complementation assays followed by sequencing of the implicated PEX gene \(^{65,93}\). Alternatively, a screening algorithm was developed in which the most frequently mutated exons of the different PEX genes are genetically analysed \(^{9,94}\). In addition, new techniques, such as whole exome/genome sequencing, may be helpful to identify peroxisomal patients \(^{58,82,95,96}\). However, the results from next-generation or whole exome sequencing may not always be clear \(^{97}\), and could require additional biochemical testing in blood or fibroblasts to confirm the diagnosis, and to establish severity of the disease-causing mutations for clinical management \(^{69}\).

**Figure 3.** Schematic overview of laboratory analysis. In the traditional approach, biochemical parameters are determined in blood and/ or urine samples from patients with clinical symptoms suggesting a peroxisomal disorder. When abnormal parameters are found, biochemical analysis and additional tests should ideally be
performed in cultured skin fibroblasts from the patient. This is especially important if none, or only minor abnormalities are found in blood or urine samples. Finally, the suggestion of a peroxisomal disorder is confirmed by molecular analysis of targeted genes or a panel of relevant genes, leading to a genetic diagnosis. In recent years, a growing number of patients has been identified via Next Generation Sequencing (NGS), such as Whole Exome Sequencing. This approach is especially advantageous for patients with non-suggestive clinical symptoms. Nevertheless, performing biochemical analyses is still necessary to confirm a peroxisomal disorder following NGS.

**Abbreviations:** very long-chain fatty acids (VLCFAs), bile intermediates di- and trihydroxycholestanolic (DHCA and THCA).

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**Metabolomics for inborn errors of metabolism**

Metabolomics, the study of metabolites and metabolism, is an emerging tool in the clinical field\(^98,99\). The idea to study human bio-fluids for medical purposes is not novel, but goes back to the ancient Greeks, who used the sensory characteristics of urine to diagnose medical conditions, for example that sweet urine points towards diabetes\(^100\). In principle, metabolite profiling allows the discovery and monitoring of hundreds of disease biomarkers from a single blood sample\(^101,102\). In addition, metabolomics provides a platform to study the emergence and progression of disease by studying changes in metabolism\(^103\). Although the term metabolomics had been introduced in the last century\(^98,104\), advances in instrumentation in the last decade allowed for more sensitive measurements and the detection of hundreds to thousands of metabolites in one screen\(^105\). The advantage of metabolomics is that, in contrast to genomics approaches, metabolic profiling gives insight into the current status of metabolism, instead of possible outcomes and risk factors that are predicted by the genome\(^99\).

Accordingly, metabolomics offers a close view on the phenotype, because metabolites are the direct read-out of gene expression and protein variation\(^106\). In addition, whereas the genetic cause of disease is often known in the patient, metabolomics is a powerful tool to support the understanding of biochemical processes affected by the genetic mutation\(^100,107\). Thus, where classical laboratory diagnosis involves several biochemical assays using different platforms, metabolic profiling may offer a fast screening method for metabolites that are affected by the genetic mutation in the cell\(^108\). This may improve patient management, by providing faster results, earlier diagnosis and better patient monitoring\(^100\).

The most commonly used bio-fluids for patient diagnostics are urine, serum, and plasma\(^109–111\). Other liquid samples are saliva and breath, which can be obtained non-invasively\(^112,113\). In addition, the use of dried blood spots (DBS) has increased in the last years\(^114–117\), likely because of its advantages when compared to liquid samples, such as efficient storage and transportation, low costs, and in particular because only small amounts of blood are needed\(^100\). The latter is especially crucial for newborns, and newborn screening programs almost exclusively use DBS for the diagnosis\(^102,115,118,119\).
Metabolomics relies on the separation of metabolites of interest using chemical and physical techniques. The challenge of metabolomics is therefore to combine a suitable physical technique with an appropriate chemical method to yield individual metabolites that can be separated, identified, and quantified. The most commonly used techniques are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). NMR spectroscopy offers fast and reproducible results while not destroying the sample, whereas MS is more cost-effective and has been shown to be more sensitive than NMR, thereby covering a larger proportion of the metabolome. Furthermore, separation techniques such as gas (GC) or liquid chromatography (LC) can be coupled to MS, which enhances the sensitivity and providing complementary molecular information. In principle, two metabolomics workflows exist, untargeted and targeted approaches. In the untargeted approach, as many metabolites as possible are measured, with the aim to identify metabolites or metabolite profiles that discriminate between the groups, such as potential biomarkers. In the targeted approach, on the other hand, a specific group of metabolites, for example amino acids or acylcarnitines, are identified and quantified to obtain more detailed information about their biochemical behaviour. Especially for diagnostic approaches, quantifying a few relevant metabolites could be sufficient to diagnose and monitor the disease.

In the last century, scientists such as Sir Hans Krebs and Otto Warburg started to discover and map essential metabolic pathways. In that time, the term ‘inborn errors of metabolism’ (IEM) was established, which dates back to a publication by Sir Archibald Garrod in 1902. His hypothesis was that every human is chemically individual, and the biochemical fingerprint that can be found in physiological fluids, such as in urine, reflects human variation. Although he was not able to confirm his theory due to a lack of techniques at that time, Sir Garrod argued that specific phenotypes are associated with specific biochemical metabolites, and that IEM are “extreme examples of variations of chemical behaviour.” Actually, recent advances in MS techniques and the implementation of metabolomics with other large-scale omics platforms have discovered genetically influenced metabotypes, and are monitoring pathophysiological changes in the metabolism of IEM patients. For instance, MS is widely used for newborn screening programs in the world, and the number of publications is increasing in which metabolomics has been used to discover biomarkers for different IEM. However, recent understandings consider IEM as more complex diseases than the original paradigm of “one gene, one enzyme, one disease.” Indeed, any protein affected by a genetic mutation may alter the metabolic flux completely, and metabolic pathways are intertwined in complex networks rather than being linear routes. Since metabolomics combines advanced biochemical and analytical approaches with cutting-edge bioinformatics and computational methods, it has potential to improve the diagnosis and characterisation of diseases, and to unravel the pathophysiology of IEM. By studying diseases in a multidisciplinary fashion, and by linking clinical data to different OMICS-techniques, including metabolomics and genomics, this may initiate a process from hypothesis-based screening and diagnosis to data-driven personalised medicine.
Outline of this thesis

This thesis focusses on metabolomics approaches performed in cultured cells and blood samples from patients with peroxisomal disorders. By applying both targeted and untargeted metabolomics, the aim of these approaches was to study the functional consequences of the primary genetic defects causing peroxisomal disorders, and to investigate potential biomarkers. The first part of this thesis provides a general introduction to peroxisomes and diseases associated with peroxisome dysfunction, and reviews the current diagnostic workflow to identify patients (Chapter 1). In addition, this first chapter briefly introduces the topic of metabolomics.

The second part of this thesis outlines lipidomics studies in fibroblasts from patients with ZSD (Chapter 2), which belong to the peroxisome biogenesis disorders, and from patients with single-enzyme deficiencies (Chapter 3). We performed lipidomics using high-resolution mass spectrometry (HRMS), and present a data processing pipeline that annotates approximately 1000 phospholipid species. By studying lipid metabolism in an untargeted, global approach, we investigated changes in phospholipid composition and lipid profiles. These changes not only comprised known biomarkers for peroxisomal disorders, such as the accumulation of VLCFAs or reduced levels of plasmalogens, but also indicated functional disturbances in those patient cells. In addition to lipidomics profiling, we developed a method to create ratios of different phospholipid species that discriminated between the patient and the control cells. In Chapter 4, we compared the lipid profiles and evaluated the set of ratios in mild DBP-deficient and mild ZSD fibroblasts, and plasma samples from relatively mild ZSD patients.

Compared to fibroblasts, plasma samples have considerable advantages for diagnostic approaches, as the drawing of blood from a patient is less invasive than a skin biopsy. Furthermore, plasma samples contain metabolites derived from origins throughout the whole body. To investigate the potential of lipidomics for diagnostic approaches, we studied the phospholipid composition in plasma samples from patients with different peroxisomal disorders (Chapter 5). The lipid profiles obtained in these plasma samples were generally comparable to those obtained in patient fibroblasts, and were similar to the biochemical parameters used for the diagnosis of peroxisomal disorders.

In contrast to untargeted metabolomics approaches, targeted assays focus on a small number of metabolites, which can be studies in more detail. In Chapter 6, we describe the identification of phytanoyl- and pristanoyl-carnitine in plasma from patients with different peroxisomal disorders. Elevated levels of phytanic acid and pristanic acid in patients lead to the formation of phytanoyl- and pristanoyl-carnitine, and we assessed the value of these metabolites for patient diagnostics. In addition, we developed an assay to study oxidative stress in cultured cells by measuring glutathione in patient fibroblasts and the yeast
Saccharomyces cerevisiae (Chapter 7). We used different compounds to induce oxidative stress or to block glutathione synthesis, and used fibroblasts from patients with a ZSD and glutathione synthetase deficiency to validate the assay.

In a collaboration initiated by the Marie Curie Initial Training Network PerFuMe (Peroxisome Formation, Function, and Metabolism) network, we reconstructed a fibroblast-specific genome-scale model for Refsum disease, with the aim to study the physiological effects of phytanic acid on cellular metabolism (Chapter 8). In a multi-OMICS approach, we performed transcriptomics, proteomics, and metabolite profiling in fibroblasts from controls and Refsum disease that were incubated with a precursor of phytanic acid. These data were then used to constrain or validate the fibroblast-specific model.

The final part of this thesis summarises all chapters, and provides concluding remarks and future perspectives (Chapter 9).
References


