CHAPTER 9

Summary and general discussion
This chapter summarises the results obtained in this thesis, and provides a general discussion in relation to research in the field of peroxisomal disorders.

A review of the biochemistry of peroxisomes, peroxisomal disorders, and the clinical and laboratory diagnosis thereof outlines the current understandings in the field of peroxisomal diseases and constitutes Chapter 1. Peroxisomes are subcellular organelles involved in various catabolic and anabolic metabolic processes, including fatty acid breakdown and synthesis of bile acids and plasmalogens. The peroxisomal disorders represent a group of genetically heterogeneous metabolic diseases caused by the dysfunction of peroxisomes. Most patients develop characteristic clinical symptoms, such as neurological features and liver disease, and laboratory diagnosis is established using several parameters that can be measured in blood samples from patients, including the accumulation of very long-chain fatty acids (VLCFAs).

Upon diagnosis of a peroxisomal disorder, clinical management of patients is limited, and until now, no curative treatment exists for most patients. Especially for patients with severe phenotypes, early disease-onset and lethality limits the scope of treatment. For patients with milder phenotypes, better survival rates allow therapeutic actions to ameliorate or at least slow down disease progression. Current treatment approaches are focused on reversing biochemical abnormalities, for instance through the restriction or replacement of specific metabolites. Follow-up of patients mainly focuses on treatable disease-related complications, such as the restoration of fat-soluble vitamin levels and general monitoring of nutritional status, anti-epileptic medication efficacy, treatment of auditory and visual problems, as well as dental abnormalities and osteoporosis. As VLCFAs can be produced endogenously in the human body, a reduction of VLCFAs in the diet does not change VLCFA levels in blood samples from patients with ALD. Although the dietary restriction of phytanic acid has not been shown to be beneficial for patients with a peroxisome biogenesis disorder, a diet low in phytol-containing products such as meat and dairy products or the reduction of phytanic acid by plasmapheresis is the current treatment option for patients with Refsum disease. For ALD, however, patients with cerebral ALD can benefit from stem-cell based gene therapy or allogeneic hematopoietic stem cell transplantation, but the treatment window is small and patients need to be monitored frequently to detect cerebral demyelination at an early time point.

In addition to the current treatment approaches, a number of compounds have been investigated in the last decade that may benefit the clinical management of patients with a peroxisomal disorder. Already in 2000, several therapeutic angles were proposed, focusing on both dietary therapies and the stimulation of peroxisome proliferation. For instance, by supplementing the long-chain polyunsaturated fatty acid docosahexaenoic acid (DHA), the often reported low levels of DHA were shown to be restored in blood. However, a double-blind placebo controlled trial did not show any effect in the two outcome measures; visual
function or growth \textsuperscript{15}. Similarly, Lorenzo’s oil, a mixture of oleic and erucic acid, was initially developed as a therapy for ALD and was shown to lower VLCFAs in plasma \textsuperscript{16,17}. However, treatment with Lorenzo’s oil did not postpone disease progression in ALD, and may at best be beneficial in asymptomatic patients \textsuperscript{18,19}. In addition, a VLCFA-restricted diet is not beneficial for patients with a Zellweger spectrum disorder (ZSD), because endogenous VLCFA production is not affected \textsuperscript{1}. One important feature of ZSD is liver dysfunction, often in combination with liver fibrosis, which is likely caused by the accumulation of the bile acid intermediates DHCA and THCA \textsuperscript{20–22}. Based on this, the bile acid cholic acid has been supplied to patients, which reduced DHCA and THCA levels in both urine and plasma, although no clinical benefits were reported \textsuperscript{23–25}. In addition, several compounds were recently reported to stimulate peroxisomal biogenesis and function \textit{in vitro}, including betaine and arginine \textsuperscript{26,27}.

Concentrations of peroxisomal markers in plasma generally do not correlate with disease severity, and can decline with age or even normalise in ZSD patients who survive into adulthood \textsuperscript{1,28}. Furthermore, a number of patients have been identified who showed no clear clinical symptoms or atypical biochemical patterns, suggesting a peroxisomal disorder \textsuperscript{29–34}. Therefore, novel or more sensitive biochemical markers would improve the diagnostics and monitoring of disease progression in peroxisomal disorders, especially in patients with a very mild phenotype. Since peroxisomes play an important role in lipid metabolism, changes in lipid profiles were investigated using a lipidomics approach in cultured skin fibroblasts from patients with a ZSD (Chapter 2) or peroxisomal single-enzyme deficiencies (PEDs) affecting β-oxidation (Chapter 3). Lipidomics is employed to study all lipids (the lipidome) in a biological system, to identify biomarkers for the diagnosis of lipid-related disorders, and to study the functions and homeostasis of lipid metabolism in the cell \textsuperscript{35,36}. As described in Chapter 2 and Chapter 3, alterations in phospholipid profiles in fibroblasts from ZSD and PED patients were found, which reflect the characteristic changes of disturbed peroxisomal function, such as the accumulation of VLCFAs and decreased plasmalogen levels. Phospholipid species containing VLCFAs accumulated, whereas species with long-chain fatty acids were decreased in cells from patients with a ZSD or PED. In addition, ether phospholipids, including plasmalogens, were significantly reduced in ZSD patient fibroblasts. Ether phospholipids were also decreased in PED patient cells with disturbed peroxisomal β-oxidation, which was a surprising finding because ether phospholipid biosynthesis was not known to require a functional β-oxidation system \textsuperscript{37}. These changes in phospholipid composition imply functional disturbances in ZSD and PED patient fibroblasts, because phospholipid homeostasis is normally tightly regulated in cells \textsuperscript{37–41}.

Based on the lipid profiles of ZSD and PED fibroblasts obtained through lipidomics, ratios of specific phospholipid species were defined, which allows for discrimination between fibroblasts from ZSD and PED patients, and healthy controls. These ratios reflect the biochemical parameters and peroxisomal functions that are affected in ZSD and PED patients, such as the accumulation of very long-chain fatty acids. The benefits of using metabolite ratios
instead of individual levels as biomarkers have been described for peroxisomal disorders and other inborn errors of metabolism \(^ {42-45}\).

Recently, C26:0-lysoPC and C26:0-carnitine have been reported as diagnostic markers for ALD, and C26:0-lysoPC analysis has been implemented in newborn screening programs for ALD \(^ {46-48}\). These markers have also been proposed for diagnosis of patients with other peroxisomal disorders, such as ZSDs and PEDs \(^ {49-51}\). The data described in this thesis, and other studies conducted in our laboratory \(^ {52}\) show that this may be legitimate for severe patients, but may not be applicable for ZSD and PED patients with a very mild phenotype. In Chapter 4, fibroblasts from patients with a mild D-bifunctional protein (DBP)-deficiency, and fibroblasts and plasma samples from ZSD patients with a mild phenotype were used to evaluate the set of phospholipid ratios that was discriminative for severe ZSD and PED fibroblasts. The proposed set of ratios for fibroblasts from patients with a severe peroxisomal disorder was not discriminative in cells with a mild phenotype, but other ratios were identified that distinguished between the mildly affected fibroblasts and control cells. Furthermore, ratios that were applicable for plasma samples from mild ZSD patients were identified. These discrepancies between the ratios discriminative for severe, but not mild patient samples, and vice versa, likely appear because of unique phospholipid species that are present in mildly affected cells, but no longer exist in cells with a severe phenotype due to the incorporation of VLCFAs. Based on these results, it is concluded that one single biomarker is likely insufficient for the diagnosis of all peroxisomal disorders. Instead, it is proposed to use a panel of ratios, which would discriminate between the different peroxisomal disorders, especially because of the heterogeneity of the different types of disorders and the spectrum of disease severity within each particular disorder.

In general, most biomarkers for peroxisomal disorders are determined in blood samples from suspected patients, including plasma, erythrocytes and dried blood spots. The advantages of using blood samples for diagnostic approaches are evident, because blood can be obtained less invasively than other biofluids, such as cerebrospinal fluid or biopsies \(^ {53}\). Furthermore, diagnostic tests based on blood samples are generally more cost-effective and applicable to high-throughput screening \(^ {54}\). Blood contains a variety of different metabolites, including nutrients, toxic intermediates, and signaling molecules such as hormones, which derive from different parts of the body. Regarding peroxisomal disorders, bile acid metabolism for example is exclusively located in the liver, and intermediate metabolites can be detected in patient plasma samples, but will not be present in fibroblasts \(^ {21}\). To explore the metabolite profiles present in plasma samples and to investigate the potential of lipidomics for the diagnosis of inborn errors of metabolism (IEM) that affect lipid metabolism, lipidomics was performed in plasma samples from patients with different peroxisomal disorders (Chapter 5). In particular, plasma samples from patients with peroxisome biogenesis disorders (ZSD, rhizomelic chondrodysplasia punctata (RCDP) type 1 and 5), and PEDs affecting peroxisomal α- and β-oxidation (Refsum disease, 2-methylacyl CoA racemase (AMACR)-,
and DBP-deficiency) were used. In general, the changes in phospholipid composition that were found in patient plasma samples when compared to healthy controls were similar to the results obtained in fibroblasts, and corresponded to the biochemical parameters that are currently used for diagnosis of these diseases, such as the accumulation of VLCFAs. Furthermore, various ether phospholipids were detected in plasma samples, including plasmalogens, and new unique lipid species for Refsum disease and AMACR deficiency were identified, which will be further investigated as potential biomarkers. Together, the results presented in Chapter 5 demonstrate that lipidomics is a powerful and sensitive tool to detect a variety of lipid species which may provide opportunities to improve current diagnostic methods. Besides, whereas the studies in the first part of this thesis focus on the (phospho) lipidome, alterations may also be present in other metabolite classes. In fact, metabolomics approaches are widely used to identify disease biomarkers and can be an effective tool to study pathogenesis and therapeutic targets for diseases. Investigating changes in the metabolome of peroxisomal disorders using untargeted metabolomics approaches will be an interesting subject for future studies.

In untargeted metabolomics approaches, the goal is to measure a wide range of metabolites to define metabolite profiles that are deviating between the different groups, such as patients and healthy controls. This approach is used to generate scientific hypotheses and to find the “needle in the metabolic haystack”. In contrast, targeted approaches focus on a specific group of metabolites which are quantified to obtain more detailed information of their biochemical behaviour. Targeted approaches can be applied for hypothesis-testing, or to generate data as input for computational modelling. Targeted metabolomics assays are also used for diagnostic approaches, in which the quantification of a small number of metabolites is often sufficient for diagnosis and disease monitoring. In Chapter 6, the identification of phytanoyl- and pristanoyl-carnitine in plasma from patients with different peroxisomal disorders is described. Phytanic and pristanic acid are known to accumulate in patients with ZSDs and RCDP type 1 and 5, whereas phytanic acid is elevated in Refsum disease. The levels of pristanic acid, on the other hand, are elevated in patients with α-methylacyl-CoA racemase deficiency. Since very long-chain fatty acylcarnitines were reported to be elevated in tissues and plasma from patients with certain peroxisomal disorders, phytanoyl- and pristanoyl-carnitine were also expected to be formed in patient cells and can be detected in plasma. As described in Chapter 6, phytanoyl- and pristanoyl-carnitine were indeed found in plasma samples from patients with different peroxisomal disorders, but only in samples from patients in whom the total plasma levels of the corresponding fatty acids, phytanic acid and pristanic acid, were clearly elevated. It is therefore concluded that phytanoyl- and pristanoyl-carnitine are not sensitive and specific enough for diagnostics of peroxisomal disorders. Upon detection of elevated levels of phytanoyl- or pristanoyl-carnitine, which may be found in untargeted metabolite profiling or in targeted acylcarnitine assays, the suspected patient requires follow-up using standard biochemical testing to diagnose a peroxisomal disorder. This is in line with a recent report that C26:0-carnitine levels are not necessarily a good biomarker for ZSD patients,
especially in patients with a milder phenotype \(^5^2\). A yet unresolved question is how phytanoyl-, pristanoyl-, and very long-chain acylcarnitines are formed in the cell. It is likely that formation is activated when CoA esters are accumulating in the cell \(^4^8\). The mitochondrial carnitine palmitoyltransferase 1 (CPT1), which primarily converts C16- and C18-CoA to carnitine esters, has been reported not to accept the branched-chain fatty acids phytanic and pristanic acid or VLCFAs as a substrate \(^5^9,^6^0\). The peroxisomal carnitine octanoyltransferase (CROT), on the other hand, has been shown to convert medium-branched-chain fatty CoA-esters to the corresponding carnitine esters \(^6^1,^6^2\). Alternatively, a yet unknown acyltransferase may perform this conversion. The exact mechanism behind the formation of phytanoyl-, pristanoyl-, and very long-chain acylcarnitines will be an interesting topic for future studies.

Whereas many targeted diagnostic methods are available for blood and urine samples, only few have been developed for research purposes and validated for in vitro studies using other material, including cultured cells. In Chapter 7, an assay to measure glutathione levels in cultured human and yeast cells is described. Glutathione is a low molecular weight molecule that is present in nearly all cell types, and which functions as a free radical scavenger \(^6^3\). Oxidative stress can trigger the oxidation of glutathione, and the ratio of reduced to oxidised glutathione (GSH/GSSG) is often used as a marker of the cellular redox state \(^6^4\). A fast assay to measure glutathione levels in fibroblasts and the yeast Saccharomyces cerevisiae was developed using tandem mass spectrometry to study the glutathione levels in cells with and without an oxidative challenge using different chemical compounds. Furthermore, fibroblasts from patients with defects in glutathione metabolism which were diagnosed with primary 5-oxoprolinuria (pyroglutamic aciduria) were studied. Elevated levels of 5-oxoproline are caused by a genetic defect in glutathione synthetase or 5-oxoprolinase, two enzymes of the \(\gamma\)-glutamyl cycle that is involved in glutathione synthesis \(^6^5\). In agreement with findings in erythrocytes and fibroblasts from patients \(^6^5,^6^6\), the GSH/GSSG ratio is normal in cells from patients with 5-oxoprolinase deficiency when compared to healthy control cells, whereas glutathione levels are depleted in fibroblasts from patients with glutathione synthetase deficiency. In addition, glutathione levels were measured in cells from healthy controls and ZSD patients incubated with VLCFAs. Whereas the GSH/ GSSG ratio was not changed in healthy control cells that were incubated with C26:0, it was significantly decreased in fibroblasts from ZSD patient when compared to untreated cells, indicating increased oxidative stress upon accumulation of VLCFAs in cells from patients with a peroxisomal disorder. In patient fibroblasts and oligodendrocytes from a mouse model with a peroxisomal disorder, increased production of ROS and oxidative stress have been reported upon treatment with VLCFAs \(^6^7,^6^8\). Furthermore, a number of PEX proteins that are involved in peroxisome biogenesis, division and protein import are susceptible to changes in the redox state, indicating that oxidative stress may affect peroxisome function \(^6^9,^7^0\). Notably, defects in peroxisome function have also been reported to affect other cell organelles, including mitochondria and the endoplasmic reticulum \(^7^1–^7^3\), suggesting functional consequences for cell metabolism in general.
CHAPTER 9  Summary and general discussion

Improved diagnosis and therapeutic approaches tailored to the individual patient are the main goals in patient management. To achieve this, a “blueprint” of human health is needed, from which deviations can be detected that point to a diseased state. To construct this “blueprint”, information from different levels of the human “composition” are needed, including genomic, proteomic, and metabolomic information. To understand pathogenesis and progression of diseases, this biological information can be investigated collectively by holistic methods. Computational modelling often makes use of genome-scale models of metabolism, which contain all currently known chemical properties and stoichiometric information of proteins, enzymes and pathways. These models can be further developed and validated by incorporation of different types of data, including gene expression and metabolite profiles, as well as biochemical and phenotypic information.

In a collaboration initiated by the Marie Curie Initial Training Network PerFuMe (Peroxisome Formation, Function, and Metabolism) network, a fibroblast-specific computational model was reconstructed for Refsum disease, a peroxisomal disorder defective in α-oxidation of the branched-chain fatty acid phytanic acid (Chapter 8). Since the pathogenesis of Refsum disease is poorly understood, a multi-OMICS study was performed, using transcriptomics, proteomics, and metabolomics experiments in fibroblasts from patients with Refsum disease incubated with phytol, a precursor of phytanic acid, to simulate the intake of phytanic acid into the human body. The constrained model was then used to study the physiological effects of phytanic acid on cellular metabolism, especially in fibroblasts with a defect in α-oxidation. This model is not only useful to study Refsum disease, but can be applied to other peroxisomal disorders and IEMs, which may help to better understand the complex metabolic networks and disease pathogenesis of these diseases.

Despite decades of research in the field of peroxisomal disorders, pathophysiology and disease progression are not well understood. The fact that the genotype does not correlate well with the phenotype, and the observation that patients with the same bi-allelic mutations may develop different phenotypes has recently raised interest into genetic and environmental factors that function as modifiers. Two decades ago, a shift in the understanding of metabolic disorders occurred, with the new insight that metabolic disorders caused by one genetic mutation are actually more complex than the “one gene, one disease” paradigm, and should be regarded as complex diseases. As such, mutations in one gene will not only disturb one metabolic pathway, but may have an impact on metabolic flux in general. In addition, both internal and external modifiers will have an influence on whole cell metabolism, and may affect other pathways. However, only a few studies focusing on modifiers in IEM have been published, mainly because studies in rare disorders are generally limited in sample size. Most studies, however, focus on either genomic or metabolic approaches to investigate modifiers. By combining different OMICS approaches, and by integrating biological information, the modifying factors as well as their impact on metabolism could be revealed. Here, systems biology approaches could provide a valuable tool for future research.
In conclusion, the work presented in this thesis demonstrates that a defect in peroxisome function results in consequences for cellular functions, which are not restricted to the peroxisome itself. Using lipidomics and metabolomics approaches, the results presented in this thesis show that the (phospho)lipid profiles determined in fibroblasts and plasma from patients with a peroxisomal disorder are profoundly altered. By developing a ratio method and targeted assays, novel biomarkers for diagnosis of patients with a peroxisomal defect were investigated. Finally, by expanding from metabolomics towards a multi-OMICS systems biology approach, new research opportunities for peroxisomal diseases were explored. In the future, studies using data sets from multi-OMICS approaches together with integrative network analysis and other systems biology approaches may provide more insight into the complex metabolic networks that are affected by diseases, identify new biomarkers, and discover modifiers that influence the phenotypic spectrum of IEMs, including peroxisomal disorders.
References


