The mouse as a model to understand peroxisomal disorders
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CHAPTER 1

GENERAL INTRODUCTION
INTRODUCTION

After the first description of peroxisomes by Rhodin [106], the importance of these organelles was first highlighted by Goldfisher’s discovery that patients suffering from Zellweger syndrome lack discernible peroxisomes [36]. At that time, the first link between peroxisomes and metabolic diseases was also made [35]. These discoveries initiated detailed studies on peroxisome biogenesis, peroxisomal metabolism and the role of peroxisomes in human health. Since then, impaired peroxisomal function has been identified in more than twelve human disorders [121,136] and we have identified more than 95% of the peroxisomal proteome [45,68,143]. We also have a better understanding on how peroxisomes are formed and how proteins are imported into peroxisomes. For a detailed reading on these topics, I recommend several outstanding reviews that present and discuss the latest advances in the field of peroxisome biogenesis [29,85,86,99,122,123] and biochemistry [108,126,135]. In this introductory chapter I will review three peroxisomal disorders that were within the focus of my research.

RHIZOMELIC CHONDRODYSPLASIA PUNCTATA
The name Rhizomelic Chondrodysplasia Punctata (or RCDP), denotes a syndrome characterized by the presentation of bone abnormalities (Chondrodysplasia). On X-ray imaging ectopic stippled calcifications (Punctata) can be found in the vertebral bodies and epiphyseal cartilages of developing bones, as well as coronal clefts in vertebral bodies [34]. Shorter limbs are the consequence of a shortening of proximal long bones (Rhizomelic) that affects both arms and legs [137]. RCDP became a peroxisomal disorder with the discovery of a severe deficiency of plasmalogens in RCDP patients [40].

RCDP is a complex disorder in terms of clinical presentation, pathology and genetics. Although most RCDP patients die within the first years of life, some RCDP patients survive well into their teenage years [7,137]. The most common cause of death during childhood relates to respiratory problems (e.g. aspiration, infection). The clinical presentation of RCDP includes: (i) short stature, primarily caused by symmetric shortening of proximal limb bones; (ii) contractures; (iii) atypical facial appearance (with prominent forehead, anteverted nares, long philtrum); (iv) bilateral cataracts; (v) growth retardation; and (vi) neurologic impairments (spastic tetraplegia, epilepsy, mental retardation). Examination of RCDP patients using magnetic resonance imaging (MRI) may also reveal delayed myelination, ventricular enlargement, cerebellar atrophy and neuronal migration defects. Severe and mild presentations have been characterized within the RCDP spectrum and may have an outcome in the survival rate [139], and may be related to the degree of the biochemical defect [7,124].

The mechanisms behind the pathology in RCDP are not well understood. It is puzzling to realize that the bone defects are prominent in proximal bones (i.e. femur and humerus) but largely absent from distal bones (ulna, radius, tibia and fibula). The puzzling nature of this defect is that all these bones are formed through the process of endochondral ossification [59,82,95] and mechanistically it is difficult to explain why only a small subset of bones is
affected. The shorter length of both femur and humerus suggests that the ossification process is delayed and autopsy studies showed abnormal chondrocytes and abnormal turnover of cartilage [34,88,138]. Regarding the mechanism of cataract development even less is known, but histological analysis revealed swollen and degenerating fiber cells with cellular proliferation in the anterior lens epithelium [34]. In a few cases of RCDP where the central nervous system has been analyzed at autopsy, key findings include neuronal degeneration, impaired neuronal migration and reduced myelination [1,34,92,93].

Genetically, RCDP is divided in three types: (i) RCDP type-1, caused by mutations in the PEX7 gene; (ii) RCDP type-2, caused by mutations in the GNPAT gene; and (iii) RCDP type-3, caused by mutations in the AGPS gene. Clinically, all forms of RCDP are indistinguishable requiring metabolic and genetic analysis to correctly determine which form a given patient belongs [134].

RCDP TYPE-1, the most frequent type, is caused by mutations in the PEX7 gene [12-14,78,79,97]. The PEX7 gene encodes Peroxin 7, a cytosolic protein responsible for the import into peroxisomes of proteins carrying a peroxisomal targeting signal type-2 (PTS2) [31,58,80,96]. So far, only three proteins have been identified carrying a bonafide PTS2: (1) acetyl-Coenzyme A acyltransferase 1 (ACAA1; also called peroxisomal 3-oxoacyl-Coenzyme A thiolase or thiolase), (2) alkylglycerone phosphate synthase (AGPS; also called alkyl-dihydroxyacetonephosphate synthase, ADHAPS or alkyl-DHAP synthase) and (3) Phytanoyl-CoA 2-hydroxylase (PHYH). In RCDP type-1, as a consequence of the mutations in Peroxin 7, all PTS2 proteins are not imported into peroxisomes and remain in the cytosol [10,20,39,83].

The effect of this mislocalization in the cytosol is, to some extent, readily seen at the biochemical level [41] because in the cytosol, these PTS2 enzymes are easily degraded or unable to perform their function. Biochemically, RCDP type-1 patients are characterized by: (1) impaired biosynthesis of plasmalogens that results in extremely reduced levels of plasmalogens in cells and tissues, (2) impaired α-oxidation of phytanic acid that results in accumulation of this fatty acid in plasma and tissues, and (3) accumulation of very-long-chain fatty acids (VLCFA) in erythrocytes, lymphoblasts and platelets.

In RCDP type-1 the plasmalogen deficiency is thought to be the underlying cause of the pathology because (i) the severity of the disease presentation correlates with the residual levels of plasmalogens [7] and, (ii) the clinical presentation of RCDP type-1 patients is similar to that of RCDP type-2 and -3 (see below). Nevertheless, the impaired α-oxidation of phytanic acid may also modulate the severity of the pathology since the accumulation of phytanic acid may cause additional abnormalities [7,92].

Although thiolase is not properly imported into peroxisomes, RCDP type-1 patients do not have a generalized accumulation of VLCFA [39]. The mechanism behind this observation is thought to involve sterol carrier protein X (SCPx), since this peroxisomal enzyme can accommodate both straight and branched-chain fatty acids [115,127]. The observation that some blood cells of RCDP type-1 patients accumulate VLCFA [114] suggests that the compensatory role of SCPx may be cell and tissue dependent.
RCDP TYPE-2 is caused by mutations in the GNPAT gene [81], that encodes for glyceronephosphate O-acyltransferase (GNPAT, also known as acyl-CoA:diacylglycerol O-acyltransferase (DGAT)), the first enzyme of the plasmalogen biosynthesis pathway (see chapter 2). RCDP type-2 patients are characterized by impaired biosynthesis of plasmalogens that results in extremely reduced levels of plasmalogens in cells and tissues. No other biochemical abnormality has been detected in RCDP type-2 patients. The clinical presentation of RCDP type-2 patients does not differ from that of RCDP type-1 patients.

RCDP TYPE-3 is caused by mutations in the AGPS gene [9,21-23] that encodes AGPS, the second enzyme in the plasmalogen biosynthetic pathway (see chapter 2). RCDP type-3 patients are characterized by impaired biosynthesis of plasmalogens that results in extremely reduced levels of plasmalogens in cells and tissues. No other biochemical abnormality has been detected in RCDP type-3 patients. The clinical presentation does not differ from that of RCDP type-1 and type-2 patients.

Currently there is no therapeutic approach to treat RCDP patients. For RCDP type-1, some care is taken to follow-up the plasma levels of phytanic acid. Usually RCDP type-1 patients receive a diet free of phytanic acid or its precursor, phytol [125]. This approach is similar to that used as a therapeutic approach in Refsum’s patients [32,66,67], to lower the levels of phytanic acid. The defect in plasmalogens found in patients suffering from all types of RCDP, may be rescued using alkyl-glycerols. It has been known for a long time, that alkyl-glycerols can circumvent the peroxisomal steps involved in the plasmalogen biosynthesis pathway and normalize the levels of plasmalogens in fibroblasts of RCDP patients [17,113]. Alkyl-glycerols have been used in some patients with Zellweger syndrome, but the outcome varied and did not show major improvements [17,18,113,144]. These limited results may have hindered the use of alkyl-glycerols in RCDP patients, but one must be aware that Zellweger patients have a multitude of biochemical defects [37,134] that may have obscured any beneficial effects caused by the restoration of plasmalogens.

**REFSUM’S DISEASE**

Refsum’s disease is the disorder first delineated by Dr. Sigvald Refsum in 1945 as “heredopathia atactica polyneuritiformis” [100]. This syndrome was characterized by: (i) polyneuritis, ataxia, muscular atrophy and increased amounts of albumin and globulin in the cerebrospinal fluid; (ii) retinitis pigmentosa and night blindness; (iii) hearing abnormalities; (iv) electrocardiographic disturbances, bone abnormalities and ichthyosis [101,102]. More than 60 years have passed and the original description remains the most comprehensive and only recently was anosmia described as a common presentation in Refsum’s patients [33,141]. Refsum’s disease is a progressive disorder thus adding important challenges for correct diagnosis. Usually the tetrad of symptoms that may help in the initial diagnostic triage for Refsum’s disease is: (1) retinitis pigmentosa, (2) peripheral neuropathy, (3) cerebellar atrophy and (4) high protein content in cerebrospinal fluid. The symptomatology in Refsum patients has
been carefully evaluated and studied during the progression of the disease [140,141]. From these studies we can infer that the most common and frequent presentation is retinitis pigmentosa, followed by anosmia and neuropathy. Refsum’s disease is a progressive disorder that despite having its first symptoms during late childhood [104] develops into a more severe presentation during adulthood [16]. This progressive nature of the disorder was explained after the discovery that Refsum’s patients were characterized by the accumulation of a branched-chain fatty acid, i.e., phytanic acid (3,7,11,15-tetramethyl-hexadecanoic acid) [50].

Refsum’s disease was classified as a lipid storage disorder in which the accumulation of phytanic acid would have detrimental effects in the target tissues. The tissue distribution of phytanic acid in Refsum’s patients was first evaluated in nervous tissues [61] where it was found that phytanic acid accumulated preferentially in peripheral nerves when compared to central white and gray matter. At this time it was again proposed that the degree of phytanic acid accumulation would be roughly proportional to the extent of pathology. To explain the characteristic neuropathy with a demyelination component, the authors proposed that in Schwann cells (the cells responsible for myelination of the peripheral nervous system) phytanic acid would not only disrupt the packing of myelin sheaths but also influence the ability of Schwann cells to correctly re-myelinate the damaged nerves. Follow-up studies [145] did not support these predictions since: (i) biopsies of nerves from Refsum patients showed minor accumulations of phytanic acid which were greater in the epineurium and perineurium when compared to the levels found in the endoneurium and, (ii) tissue distribution of phytanic acid in a variety of tissues from Refsum patients showed that nervous tissues had the lowest degree of phytanic acid accumulation when compared to liver, heart and kidney [30].

Although it is still unsolved by which mechanism phytanic acid leads to the observed pathology, the accumulation of phytanic acid found in many of the target tissues in addition to the certain degree of correlation with the pathology, favors the “molecular distortion” hypothesis [61,146]. This hypothesis suggests that the incorporation and accumulation of phytanic acid in biological membranes would affect the ordered structure of the membranes due to the branched structure of phytanic acid. In myelin this altered membrane structure could affect membrane stability and/or function of membrane proteins. Recent studies have shown that phytanic acid impairs mitochondrial respiration [51,105,111,112] and this observation suggests that phytanic acid may modulate the pathology in Refsum’s disease through a mechanism that involves toxicity. Because these recent findings have not been validated in samples from Refsum patients, I will not discuss them further. However I would like to point out that there may not be a uniform/generalized mechanism through which phytanic acid accumulation leads to the observed pathology in Refsum patients.

The accumulation of phytanic acid in Refsum patients suggested that Refsum’s disease is a disorder of fatty acid α-oxidation [27], since the branched-chain structure of phytanic acid, in particular the methyl group at the third position, blocks the degradation of this fatty acid through the more common β-oxidation process. Through α-oxidation, one carbon atom is removed from phytanic acid resulting in the production of pristanic acid (2,6,10,14-tetramethylpentadecanoic acid). Although pristanic acid is also a branched-chain fatty acid, the methyl group at the second position does not hinder degradation through β-oxidation. The search for
the enzymes responsible for the \(\alpha\)-oxidation of phytanic acid underlined the discovery of the genetic basis of Refsum’s disease. Refsum’s disease is caused by mutations in the \(\text{PHYH}\) gene [46,69]. The \(\text{PHYH}\) gene encodes the protein phytanoyl-CoA hydroxylase (\(\text{PHYH}\)), the enzyme responsible for hydroxylation of phytanoyl-CoA into 2-hydroxyphytanoyl-CoA. This first step in the \(\alpha\)-oxidation of phytanic acid is followed by the decarboxylation of 2-hydroxyphytanoyl-CoA to form pristanal that is then converted to pristanic acid by the action of an aldehyde dehydrogenase [47,48,125,128]. So far, phytanic acid accumulation has not been identified in other human disorders. Moreover, although the process of phytanic acid \(\alpha\)-oxidation involves 3 distinct steps, Refsum patients are characterized by mutations in the \(\text{PHYH}\) gene. An apparent exception to this “rule” is the observation that a few patients with the clinical presentation of Refsum’s disease were found to have mutations in the \(\text{PEX7}\) gene [43,124]. From the genetic point of view, this small subgroup of Refsum patients would be considered RCDP type-1 patients but due to the biochemical abnormalities these patients have an atypical presentation that resembles more Refsum’s disease than RCDP [124]. From the medical point of view this small group of patients is very interesting because, as discussed above, if we would be able to rescue or improve the plasmalogen levels in RCDP patients, we would be able to treat the major defects presented by RCDP patients.

Therapeutic approaches in Refsum patients have so far focused on preventing the accumulation of phytanic acid through a diet free or with low amounts of phytanic acid and its precursor, phytol [28,32,38,66,67,103,120,142]. In Refsum patients with high plasma levels of phytanic acid [15] plasma-exchange is also an option to rapidly clear phytanic acid from blood [32,38]. The dietary approach aims at halting the progression of the disease, and although having beneficial effects it requires a committed compliance by the patients and but it does not restore the deficient \(\alpha\)-oxidation of phytanic acid. An alternative method to degrade phytanic acid has been identified in Refsum patients, by the discovery of urine-secreted 3-methyl-adipic acid [142]. 3-methyl-adipic acid is a dicarboxylic acid that can be formed through \(\omega\)-oxidation of phytanic acid [52]. The identification of the enzymes involved in the \(\omega\)-oxidation of phytanic acid [53,54] may now provide a new therapeutic approach in Refsum’s disease. If \(\omega\)-oxidation of phytanic acid can be induced (e.g. through drug administration) then the combined approach of limiting phytanic acid intake with increased \(\omega\)-oxidation may provide an improved therapy that can maintain very low levels of phytanic and improve clearance [56,129].

**X-LINKED ADRENOLEUKODYSTROPHY**

X-linked adrenoleukodystrophy (X-ALD) is a complex disorder first identified by Siemerling and Creutzfeldt [117] with a adreno-testiculo-leukomyelo-neuropathic presentation. X-ALD is primary characterized by adrenal insufficiency, testicular atrophy and the destruction of myelin, particularly in the central nervous system, although it can also affect the peripheral nervous system [90]. The disorder has been found to have different clinical presentations that are easily divided in three groups: (1) patients suffering only from adrenal insufficiency, called Addison-only [5,57]; (2) patients suffering from a cerebral form of X-ALD [90,110] and (3) patients suffering from a spinal cord form of X-ALD that is denominated adrenomyeloneuropathy (AMN) [3,65,90,91,94,110]. The cerebral form of X-ALD is the most severe form of the
disease and is characterized by a rapidly progressive cerebral inflammatory demyelination. It primarily affects children between the ages of 3 to 10 years old although it can also develop in adolescents (onset between 10 and 21 years of age). Based on severity AMN may be considered a milder variant because it develops slower than the cerebral form. Nevertheless, AMN patients gradually develop spastic paraparesis and may even develop a mild to moderate cerebral involvement similar to that of the cerebral X-ALD variant. The most intriguing aspect of X-ALD relates to these different clinical presentations of the disorder. What dictates the occurrence of the cerebral X-ALD versus the slow progression into AMN? How is the disease modulated? Answers to these questions have long been awaited but they should provide us with better insights in the neurobiology of the disease.

At the biochemical level X-ALD patients were found to have a drastic accumulation of VLCFA in all target tissues [44,49,109]. This accumulation of VLCFA was linked to a defect in the β-oxidation of these fatty acids [89,118,130] and at that time the defect was thought to be caused by an impairment in the synthetase that would activate VLCFA to be β-oxidized in peroxisomes [131,132]. It was puzzling to discover that the molecular basis of X-ALD involves mutations in the *ABCD1* gene [6,76,77]. The nature of this discovery was puzzling since ALDP, the protein encoded by the *ABCD1* gene, is a transmembrane protein belonging to the ATP-binding-cassette (ABC) transporter family [11,24,133], but lacks homology to any known synthetase and is predicted to act as a transporter. Today more than 400 different mutations have been described in X-ALD patients (www.x-ald.nl) ranging from missense mutation to large deletions. The identification of these mutations in X-ALD families also showed that there isn’t a well defined genotype-phenotype correlation [26]. This is especially striking when we consider that multiple presentations can be found within the same kindred [19,64] and even between twins [25,55,119]. These studies combined with other observations have led to the proposal that the disease may be influenced by modifier genes [2,8,42,63], that could modulate the disease progression or could alter the propensity to acquire a given form of X-ALD. Although some candidate genes have been identified [2] it still remains largely unresolved how these genes/proteins modulate the pathology and the progression of X-ALD.

Therapeutic approaches for X-ALD are hindered by the different variants and by the severe nature of the pathology [71]. In cases of Addison-only, appropriate hormone replacement therapy prevents morbidity and mortality associated with adrenal insufficiency. But treatment options become scarcer with the progression from an Addison-only presentation to one of the other variants of X-ALD.

The best known therapeutic approach to X-ALD has been the "Lorenzo's oil", a mixture of glyceryl trioleate and glyceryl trierucate [70,107]. This dietary supplement was found to normalize the level of VLCFA in plasma and peripheral tissues of X-ALD patients [62,72-74,87]. "Lorenzo's oil" was primarily given to pre-symptomatic X-ALD patients or patients showing initial signs of cerebral involvement. Many trials have been performed using "Lorenzo’s oil" but recent reviews of long term treatment indicate that it may have reduced beneficial effects towards halting or preventing the severe cerebral involvement [75]. These results although disappointing possibly reflect the observation that in brain tissue of X-ALD patients undergoing treatment with "Lorenzo's oil", levels of VLCFA are largely unchanged.
Nevertheless, “Lorenzo’s oil” is able to decrease or normalize VLCFA levels in peripheral tissues of X-ALD patients. The mechanism behind the normalization of VLCFA levels through the "Lorenzo's oil" diet relies on the inhibition of medium and long-chain fatty acid elongation to produce VLCFA. This mechanism and "Lorenzo's oil" may still be crucial when developing other therapeutic approaches.

Although "Lorenzo's oil" is the best known treatment, the most effective therapeutic approach in X-ALD is bone marrow transplantation (BMT) [4,60,84,116]. BMT performed in X-ALD patients having mild initial signs of cerebral involvement as been shown to stabilize and even alleviate the neurological abnormalities. The mechanisms through which the hematopoietic stem cells are able to halt or alleviate the pathology are largely unknown [56]. Nevertheless, since the demyelination observed in the cerebral form of X-ALD has a strong immunologic and inflammatory component it is hypothesized that with BMT, the donor cells (e.g. macrophages/microglia, and T-cells) could enter the central nervous system and provide metabolic and immunologic improvement. Although BMT may be the most efficient treatment for X-ALD it possesses limitations that hinder the wider application (e.g., the finding of appropriate donors, survival rates post-BMT, graft rejection, etc).

**OUTLINE AND SCOPE OF THE THESIS**

The present work relates and revolves around the human disorder RCDP type-1 and three peroxisomal functions: plasmalogen biosynthesis, phytanic acid α-oxidation and very-long-chain fatty acid β-oxidation. The work was aimed to study and understand other peroxisomal disorders, namely X-linked adrenoleukodystrophy and Refsum’s disease through the use and characterization of mouse models (reviewed in the addendum to Chapter 1).

In this body of work we have studied the molecular basis of RCDP type-1 patients (Chapter 3). In order to study and understand the disorder we generated and characterized a mouse model for RCDP type-1, i.e., the Pex7 knockout mouse (Chapter 4). Based on the work obtained and our general interest in plasmalogens (Chapter 2) we have also investigated the consequences of multiple peroxisomal deficiencies by generating and characterizing a Pex7:Abcd1 double-knockout mouse (Chapter 5). Furthermore, we have studied the beneficial effects of alkylglycerol as a therapeutic agent for plasmalogen deficiency (Chapter 6). Finally, we have also generated and characterized a mouse model for Refsum’s disease (Chapter 7).

**REFERENCES**


General introduction


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