The mouse as a model to understand peroxisomal disorders

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CHAPTER 6

ALKYL-GLYCEROL SUPPLEMENTATION RESCUES PATHOLOGY IN PERIPHERAL ORGANS OF ETHER-LIPID DEFICIENT MICE

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Alkyl-glycerol supplementation rescues pathology in peripheral organs of ether-lipid deficient mice

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ABSTRACT
Plasmalogens are ether-phospholipids which serve as endogenous antioxidants and have an important structural role in mediating membrane dynamics1, 2. Plasmalogen deficiency is the biochemical hallmark of the peroxisomal disorder Rhizomelic Chondrodysplasia Punctata (RCDP)3, but other genetic and developmental disorders, including Alzheimer’s disease and Gaucher disease have also been shown to have peripheral plasmalogen deficiencies4-6. We previously generated a Pex7 knockout (KO)7 mouse that serves as an in vivo model for RCDP type 18, 9, and shows all the biochemical and pathological characteristics of RCDP. Using this mouse model we showed that a diet enriched with 1-O-octadecyl-rac-glycerol, an alkyl-glycerol (AG), can restore plasmalogen levels and rescue the pathology caused by the plasmalogen deficiency. Plasmalogen levels were restored in most tissues examined and the pathological progress of the disorder was halted. When given prior to the occurrence of pathological changes, the AG-diet prevented or ameliorated the pathology observed in Pex7 KO pups which was related to the extent to which plasmalogen levels were restored. Our results provide in vivo evidence that AG can be used as a therapeutic agent in disorders with a deficiency in plasmalogens.

RESULTS AND DISCUSSION
The biosynthetic pathway of ether-phospholipids, including plasmalogens, involves several enzymatic steps performed in peroxisomes and the endoplasmatic reticulum8, 10. A deficiency of either of the two peroxisomal enzymes involved in plasmalogen biosynthesis, i.e., glyceronephosphate O-acyltransferase (GNPAT) and alkylglycerone phosphate synthase (AGPS) leads to a block in the biosynthesis of ether-phospholipids which results in absent or reduced levels of plasmalogens8, 11. Alkyl-glycerols (AG) can enter the plasmalogen biosynthetic pathway downstream of the peroxisomal steps and, when added to cultured cells, have been shown to restore plasmalogen levels12, 13. To determine whether AG can also restore
the plasmalogen deficiency found in Pex7 KO mice, we fed mice either a control diet or a diet containing 2% 1-O-octadecyl-rac-glycerol (AG diet) for 2 months. Measurement of plasmalogens in several tissues revealed that Pex7 KO mice fed the AG diet had normal levels of plasmalogens in erythrocytes and, in several tissues including kidney, heart and eye (Table 1). The AG diet only marginally increased plasmalogen levels in the cerebrum and cerebellum of Pex7 KO mice. This may be the reflection of an inability of AG to cross the blood-brain barrier or an increased catabolism of newly formed plasmalogens. It is also known that the metabolic heterogeneity found within different areas of the brain towards plasmalogen biosynthesis and plasmalogen incorporation into membranes results in a differential distribution between white and grey mater14-16. Moreover, detailed evaluation of the turnover of plasmalogens in brain revealed half-lives of less than 1 hour15, suggesting that in the nervous tissue longer treatments with AG may be necessary to reach a steady-state level of plasmalogens. Pex7 KO mice fed for 4 months with the AG diet showed increased plasmalogen levels in spinal cord and cerebellum (Table 1).

Next, we determined the effects of the AG diet on the pathology observed in Pex7 KO mice. In mice, testicular atrophy primarily caused by loss of cells from the spermatogonia lineage is one of the hallmarks of the pathology caused by deficiencies in the biosynthesis of plasmalogens7, 17. Histological evaluation of seminiferous tubules from Pex7 KO mice on control diet showed a Sertoli-only phenotype, with the seminiferous epithelium solely comprised of Sertoli cells and devoid of spermatogia and spermatocytes (Fig. 1a). Treatment of Pex7 KO mice with the AG diet restored plasmalogen levels in the testis and ameliorated the

<table>
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<th>Period</th>
<th>Sample</th>
<th>Control diet</th>
<th>AG diet</th>
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<td>WT KO</td>
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<td>2 months</td>
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<td>3.5 ± 0.2 N.D.</td>
<td>7.1 ± 0.5 3.9 ± 0.2</td>
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<td>Heart</td>
<td>2.3 ± 0.4 N.D.</td>
<td>9.8 ± 1.6 9.6 ± 1.1</td>
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<td>Lung</td>
<td>8.1 ± 0.9 N.D.</td>
<td>15.7 ± 2.1 17.7 ± 3.2</td>
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<td>Spinal cord</td>
<td>N.P. N.P.</td>
<td>17.2 ± 2.8 0.5 ± 0.1</td>
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Plasmalogen levels are expressed as mean ± S.D of the percentages of dimethylacetal (DMA) derivatives of C18:0 to the corresponding saturated fatty acid.

Numbers of mice tested: on control diet WT mice (n=4) and Pex7 KO mice (n=3) and on AG diet for 2 months WT mice (n=6) and Pex7 KO mice (n=6); for 4 months WT (n=3) and Pex7 KO mice (n=3). RBC- red blood cells; AG- alkyl-glycerol diet; N.D.- not detected; N.P.- not performed.
testicular pathology as evident from the presence of spermatocytes at different stages of maturation in the seminiferous epithelium (Fig. 1a), which is indicative of a restoration in spermatogenesis. Regardless of the histological improvement, however, mature spermatozoa were not detected in the seminiferous tubules or in the epididymis (data not shown). The Harderian gland synthesizes lipids, porphyrins and indoles for pheromonal and lubricatory purposes, and has been implicated in, amongst others, thermoregulatory and photoreceptor protection processes. Harderian glands contain peroxisomes and show gross abnormalities in case of peroxisomal dysfunction (Brites et al. unpublished results and Fig. 1b). Histological examination of Harderian glands from Pex7 KO mice showed atrophic secretory cells with reduced cytoplasm, lacking the characteristic lipid inclusions found in Harderian glands of WT mice (Fig. 1b). Treatment of Pex7 KO mice with the AG diet improved the histology of the Harderian gland with secretory cells showing small lipid inclusions and an increase in the cytoplasmic volume (Fig. 1b). Another hallmark of plasmalogen deficiency in mice is lipodystrophy, with extremely reduced to absent white adipose tissue. As a consequence, Pex7 KO mice showed reduced body weight when compared to WT mice (WT 33.3±1.5gr; KO 22.0±1.1gr, p=0.0004). Treatment with the AG diet led to an increase in body weight of Pex7 KO mice (WT 32.3±1.8; KO 28.7±1.7) representing a 30% gain in body weight (p=0.0039) when compared to KO mice fed the control and AG diet. The lipodystrophy of Pex7 KO mice was characterized by extremely reduced epididymal, inguinal, retroperitoneal and subscapular white adipose tissue depots whereas the size of the dorsal brown fat pads was normal. Histological analysis of epididymal white adipose tissue from Pex7 KO mice revealed abnormal adipocytes with smaller and irregular fat deposits (Fig. 1c). The AG diet in Pex7 KO mice led to an increase in body weight and the normalization of adipocyte size and fat deposition (Fig. 1c). Despite normal gross examination, histological evaluation of brown adipose tissue also showed abnormalities in Pex7 KO mice, characterized by the reduced size of the lipid inclusions (Fig. 1d). The AG diet also produced beneficial effects on brown adipocytes, characterized by the increased size of lipid inclusions (Fig. 1d). We also evaluated the effects of the AG diet on the functioning of peripheral nerves. We found that Pex7 KO mice develop a peripheral neuropathy with reduced motor nerve conductance velocity (MNCV). On the control diet, Pex7 KO mice showed increased latencies of compound muscle action potentials (CMAPs) (Fig. 1e) that resulted in a 37% reduction in MNCV when compared to WT mice on control diet (Fig. 1f). Pex7 KO mice on the AG diet showed an improvement in CMAP latencies (Fig. 1e) that resulted in an increase in MNCV. Although the AG diet did not restore the MNCV of Pex7 KO mice to normal values, it clearly improved nerve conduction, with KO mice having only a 19% reduction in MNCV when compared to WT mice (Fig. 1f).

Our combined results indicate that an AG diet has dual beneficial effects since it not only restores plasmalogen levels but it also improves the histopathological alterations in target organs of Pex7 KO mice. This led us to investigate if the AG diet could also be used to prevent the pathology caused by plasmalogen deficiency. To achieve this, we fed the AG diet to pregnant dams and determined the effects on 20-day old pups from dams fed the control or the AG diets. Regardless of the diet fed to the dams, Pex7 KO pups were born hypotonic and
measurements of body weight during the first 2 postnatal weeks showed a significant difference when compared to WT pups (20% reduction in body weight, p=0.003), suggest that AG did not cross the placental barrier or was not present in milk during lactation. Nevertheless, after the

![Figure 1](image_url)

**Figure 1** Therapeutic effects of AG diet on rescuing the pathology caused by plasmalogen deficiency. (a) Testis sections stained with hematoxylin and eosin (H&E). The hallmark of plasmalogen loss in the testis of Pex7 KO mice fed a control diet, i.e., Sertoli-only phenotype with severe loss of spermatocytes, is rescued upon feeding the AG diet. Seminiferous tubules from AG-fed Pex7 KO mice display a stratified epithelium with spermatocytes at different stages of maturation. Bars are 10μm. (b) Harderian gland sections stained with H&E. Harderian glands from Pex7 KO mice fed a control diet showed atrophy of glandular cells with reduced cytoplasmic volume and lacking lipid inclusions. After AG diet, Harderian glands from Pex7 KO mice showed a restoration in morphology that included the appearance of small lipid inclusions and increased size of glandular cells. Bars are 10μm. (c) White adipose tissue sections stained with H&E. Degenerated adipocytes with small-sized fat inclusions are characteristic features found in Pex7 KO mice. After AG diet, adipocytes from Pex7 KO mice displayed normal size and fat content. Bars are 25μm. (d) Brown adipose tissue sections stained with H&E. Adipocytes from control-fed Pex7 KO mice showed, in contrast to WT, an increased number of small fat inclusions within the cytoplasm of adipocyte cells. AG diet restored the histology of adipocytes in brown adipose tissue of Pex7 KO mice. Bars are 10μm. (e) Representative examples of compound muscle action potentials recordings after stimulation at the sciatic notch of wild type and Pex7 KO mice fed control or AG diets. Increased latencies were observed in control-fed Pex7 KO mice that were partially restored after the AG diet. (f) Calculated motor nerve conduction velocities (MNCV) of wild type and Pex7 KO mice fed control or AG diets. Bars represent the average values obtained after bilateral measurements in wild type and Pex7 KO mice. The AG diet partially restores MNCV in sciatic nerves of Pex7 KO mice.
second postnatal week the weight of Pex7 KO pups from the AG diet increased to reach that of WT pups (with KO mice having only 5 to 12.5% reduction in body weights when compared to WT mice, p=0.501). Combined with the previous results of increased body weight gain from the dietary regimen on adult mice, these results suggest that the increase in body weight of Pex7 KO pups starts upon the ingestion of the AG diet directly. Measurement of plasmalogen levels showed that the AG diet led to increased plasmalogen levels in multiple tissues of Pex7 KO pups (Fig. 2a). The restoration of plasmalogen levels in the different tissues from Pex7 KO pups varied between 45 and 65% of the WT plasmalogen levels. The AG diet during this early postnatal period also failed to increase plasmalogen levels in cerebrum and cerebellum of Pex7 KO pups (Fig. 2a). The partial restoration of plasmalogens in different tissues suggests that the steady-state levels were not reached since, the source of AG in Pex7 KO pups was primarily diet-derived and the pups ingested the AG diet for only a maximal 6-day period.

Next, we determined the effects of the AG diet on the histopathology of Pex7 KO pups. At P20, testis of Pex7 KO pups fed the control diet showed disorganization of the seminiferous epithelium with loss of spermatocytes (Fig. 2b), whereas Pex7 KO pups fed the AG diet showed normal seminiferous tubules without loss of spermatocytes (Fig. 2b) indicating that the 42% restoration in plasmalogen levels (Fig. 2a) could prevent testicular degeneration. The AG diet also prevented the development of cataracts in Pex7 KO pups. Whereas Pex7 KO pups fed a control diet showed bilateral cataracts as soon as the pups opened their eye lids (age P13), AG-fed Pex7 KO pups failed to develop cataracts or developed a small nuclear cataract (Fig. 2c). The extent of the cataract in AG-fed Pex7 KO pups was correlated with the amount of plasmalogens measured in the eye (Fig. 2d). Despite restoring plasmalogens in the eyes of adult Pex7 KO mice (Table 1), the AG diet had no effect on the cataracts of these mice because of the extensive damage that occurred prior to the treatment (data not shown).

Taken together, our data show that AG can be used in vivo to restore plasmalogen levels and to halt or prevent pathological alterations caused by plasmalogen deficiency. The therapeutic effects were found to depend on the degree to which plasmalogen levels were restored in the affected tissue and on the damage caused by the plasmalogen deficiency prior to the start of the AG supplementation. In conclusion, by restoring plasmalogens, alkyl-glycerols may be used as therapeutic agents with potential beneficial effects for peroxisomal and non-peroxisomal disorders.

METHODS
Animals. Pex7 KO mice and litter mate WT mice in a Swiss Webster background were obtained from mating Pex7 heterozygous mice and genotyped as described previously. Mice were housed under standard conditions and had free access to food and water. For tissue harvesting, mice were anesthetized with 100mg/Kg ketamine and 10mg/Kg xylazine. Blood was collected from cardiac puncture and isolated organs were snap-frozen in liquid nitrogen and stored at -80°C for further analyses. Experiments and mouse manipulations were approved by the University of Amsterdam Animals Experiments Committee.
Therapeutic approach to plasmalogen deficiency

Diet study. Alkyl-glycerol diets (AG-diet) containing 2% 1-O-octadecyl-rac-glycerol (Sigma Aldrich) dissolved in ethanol and the control diets lacking alkyl-glycerol were either manufactured by Ab Diets (Woerden, the Netherlands) or, home-made by spraying the solutions onto standard diet (Transbreed diet from Special Diets Services, UK). The ethanol was allowed to evaporate before supplying the diets to the animals.
For the treatment of adult mice, six-week-old mice were fed the control or the AG-diet for 2 and 4 months. Food intake was monitored by visual inspection of food pellet consumption and body weights were determined twice a week. For the treatment of pre-weaned pups, mating pairs were fed the control or the AG-diet from the day of the mating until the pups were 20-days old.

**Biochemical analyses.** Tissues were homogenized in PBS by sonication. The homogenates were cleared by centrifugation at 900xg for 5 minutes and protein was measured using the DC Protein Assay kit (Bio-Rad) using BSA as standard. Plasmalogens were measured as their dimethylacetal derivatives (DMA) by gas chromatography and expressed as the ratio between C18:0 DMA and methylstearate (C18:0) as previously described\(^2\).

**Histological analyses.** Pieces of harvested tissues were fixed by immersion in buffered formalin at 4°C for 48 hours, processed for paraffin embedding and sectioned on a Leica RM2255 microtome, according to routine practices. Paraffin sections, 5μm thick, were deparaffinized in HistoClear II (National Diagnostics), rehydrated using decreasing concentrations of ethanol and used for routine histological analyses including, H&E, PAS and luxol fast blue stainings. After fixation, eyes were processed for LRWhite embedding and semi-thin 1μm sections were cut with a glass knife. Eye sections were stained with Richardson’s stain, dried and mounted with DPX.

**Electrophysiology.** Mice were anesthetized (described above) and placed on a warm pad at a temperature of ~30-34°C. Recordings of compound muscle action potentials (CMAP) were obtained on a PowerLab 4/25T (AD instruments) using Chart5 software. Recording needle electrodes were placed subcutaneously in the foot pad and supramaximal stimulation of sciatic nerves was performed distally at the level of the ankle and proximally at the sciatic notch. Conduction velocities were calculated as: (proximal distance – distal distance)/(proximal latency – distal latency), with latencies corresponding to the time lapse between the stimulus and the onset of the CMAP and expressed in m/s.

**Statistical analysis.** All values are expressed as mean ± standard deviation. Statistical comparisons between two experimental groups were evaluated using the Student's paired \(t\)-test. We considered a \(p\) value <0.05 as significant.

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Therapeutic approach to plasmalogen deficiency


