Molecular biology and pharmacogenetics of x-linked adrenoleukodystrophy
Kemp, S.

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Chapter 1

General introduction
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History
In 1923, Siemerling and Creutzfeldt reported a boy who had developed a bronzed skin at the age of four years, became deeply pigmented and subsequently developed severe neurological dysfunction and died at the age of seven years.¹ Postmortem examination revealed atrophy of the adrenal cortex and extensive cerebral demyelination with a perivascular inflammatory response. By 1963, nine cases had been described in the literature. Since all patients had been male, Fanconi and coworkers proposed X-linked recessive inheritance.² The name adrenoleukodystrophy was introduced in 1970, based on the striking association of a leukodystrophy with adrenocortical insufficiency.³ In 1976, a more slowly progressive adult form of the disease was described.⁴ A year later, five more cases were described by Griffin et al.⁵ who proposed that this form be named adrenomyeloneuropathy (AMN) because mainly adrenal cortex, spinal cord and peripheral nerves were involved. X-linked adrenoleukodystrophy (X-ALD) is the most common peroxisomal disorder with an incidence of approximately 1 in 50,000.

Clinical manifestations
X-ALD includes at least six different clinical phenotypes, based on the age of onset and the organs principally affected.⁶ Different phenotypes frequently co-occur within the same kindred or even within the same nuclear family. The most devastating phenotypes are characterized by a sudden onset of cerebral demyelination with a relentless and rapid progression (Fig 1 and Fig 2). The onset of cerebral demyelination most frequently is in childhood (CCALD: mean age 7 ± 2 years). Less frequently, the age of onset is in adolescence or adulthood. The first neurological symptoms are behavioral changes, poor school performance, impaired auditory discrimination and impaired visual acuity. Most patients with cerebral demyelination are in a vegetative state or have died within three years of the onset of symptoms. In CCALD patients, cerebral magnetic resonance imaging (MRI) typically reveals extensive demyelination in the occipital periventricular white matter usually starting in the splenium of the corpus callosum (Fig 1).⁷

Adrenomyeloneuropathy (AMN) is a milder phenotype. The onset most frequently is in the third to fifth decade (mean age 28 ± 9 years). AMN is slowly progressive with initial symptoms limited to the spinal cord and peripheral nerves (Fig 3). Patients gradually develop a spastic paraparesis, often combined with impaired vibration sense, sphincter dysfunction and impotence.⁶,⁸ Approximately 70% of AMN patients have adrenocortical insufficiency (Addison’s disease).⁹ An equal percentage of affected males have signs of testicular insufficiency,
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occasionally even in the absence of other symptoms.\textsuperscript{10} It has been reported that about 55% of AMN patients have no subsequent cerebral involvement (pure AMN) and that approximately 45% will eventually develop cerebral involvement (AMN-cerebral phenotype).\textsuperscript{6}

Primary adrenocortical insufficiency without evidence for nervous system involvement (‘Addison-only’ phenotype) occurs in approximately 10% of X-ALD patients.\textsuperscript{6}

Some patients have the genetic and biochemical defect (see below), mostly identified by family screening, but are free of adrenocortical insufficiency and have no neurological abnormalities. These patients are still at high risk for developing neurologic symptoms. However, some patients remain asymptomatic into their sixties.\textsuperscript{6}

Figure 1. (left) A 7 year old boy with CCALD. First neurological symptoms, impairment of auditory discrimination, were noted at the age of 6 years. Shortly thereafter his visual acuity deteriorated and he developed seizures and a spastic tetraparesis. He died two years after the onset of neurological symptoms. (middle) A cerebral T2-weighted MR image of an 8 year old boy with impaired visual acuity and seizures due to CCALD. The destruction of the occipital periventricular white matter is evident. (right) For comparison, a cerebral T2-weighted MR image of an age related healthy control (photos were adapted, with permission, from reference 8).

CCALD and AMN are the two most common X-ALD phenotypes. Together they account for more than 75% of all X-ALD cases. Initially, CCALD was reported to be the most frequent phenotype of X-ALD. However, systematic analyses in the Netherlands and France have shown that AMN is the more common form (ref. 11; P. Aubourg, personal communication). In the Netherlands, AMN accounts for 46% of all cases and CCALD for 31%.\textsuperscript{11}
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Figure 2. The relentless and rapid metamorphosis of a healthy 6 year old boy into a demented and spastic 17 year old adolescent. Retrospectively, adolescent cerebral was diagnosed after X-ALD was diagnosed in a cousin (photos were adapted, with permission, from reference 8).

Figure 3. A 44 year old man with AMN, showing a posture resulting from a slowly progressive spastic paraparesis with an onset 15 years ago (photo adapted, with permission, from reference 8).

Approximately 50% of women heterozygous for X-ALD have neurological abnormalities demonstrable by neurological examination but are without clinical symptoms. About 20% of carriers may develop symptoms that are similar to AMN. The age of onset is usually in the fourth decade (mean age $37.8 \pm 14.6$ years), the symptoms are milder and the progression is slower compared to affected males.\textsuperscript{8,12} Unlike male patients, heterozygotes only rarely develop adrenocortical insufficiency. Frequently, X-ALD carriers are misdiagnosed as having multiple sclerosis (MS). Exceptionally, heterozygotes may develop rapidly progressive cerebral demyelination.\textsuperscript{8} At least three cases are known in which extreme non-random X-inactivation, with the result that all the heterozygous cells express the mutant ALD allele, is the suspected cause for cerebral involvement (S. Kemp & K. D. Smith, unpublished observations).
Biochemistry

The first insight in the biochemistry of X-ALD came when it was found that the adrenal glands and cerebral white matter contained characteristic lipid inclusions. These inclusion bodies contained cholesterol esterified with saturated very long-chain fatty acids (VLCFA, ≥ 22 carbon atoms). Cholesterol esters in normal brain contain mostly C16 to C20 fatty acids (long-chain fatty acids); those in X-ALD brain contain large amounts of VLCFAs. Accumulation of VLCFAs increases membrane microviscosity, resulting in disruption of membrane structure and stability, and impaired capacity of cultured adrenal cells to respond to ACTH stimulation.

Biochemically, X-ALD can be diagnosed by elevated levels of saturated VLCFA in all tissues and plasma. Long-chain fatty acid levels (12-20 Cs) are normal. Virtually all male patients have elevated tetracosanoic acid (C24:0) and hexacosanoic acid (C26:0) levels in plasma and increased C24:0/C22:0 and C26:0/C22:0 ratios. The greatest excess occurs in the cholesterol ester fraction of brain white matter and adrenal cortex. Some male patients have C26:0 levels that are borderline normal or even in the normal range. However, using a conversion based on three plasma VLCFA measures all male patients can be identified. In contrast to hemizygotes, 15% of obligate X-ALD carriers have VLCFA levels that are in the normal range.

Fatty acids are metabolized through β-oxidation (chain shortening by two carbon atoms per cycle), but prior to degradation they must be activated to their coenzyme-A (CoA) thioesters. The activation of fatty acids is catalyzed by acyl-CoA synthetases. Acyl-CoA synthetases have peak-specific activities for either short-chain (2 - 4 C atoms), medium-chain (6 - 10 Cs), long-chain (12 - 20 Cs) or very long-chain (≥ 22 Cs) fatty acids. However, most acyl-CoA synthetases have overlapping chain length specificity. In mammals, the level of expression of the acyl-CoA synthetases appears to be tissue specific. Long-chain acyl-CoA synthetase (LACS) activity is present in all tissues, but LACS expression is highest in liver, kidney, adipose tissue and heart. In rat, at least two organ-specific isozymes have been identified, of which one enzyme is brain specific. Very long-chain acyl-CoA synthetase (VLCS) activity has been detected in: liver, brain and skin fibroblasts. Recently, a VLCS from rat liver was identified. Subsequently, the homologous VLCSs from both mouse and human were identified. Mouse VLCS shares 93% amino acid identity with rat VLCS, and human VLCS shares 82% identity with both mouse and rat VLCS. According to mRNA analysis, the mouse, rat and human VLCS are liver and kidney specific. Based on sequence homology, at least three additional VLCS homologous proteins
are present in both human and mouse (P. A. Watkins and S. Kemp, *unpublished observations*).

On the subcellular level, LACS activity is associated with microsomal membranes, peroxisomes and mitochondria. Long-chain fatty acids are metabolized in both mitochondria, peroxisomes and endoplasmic reticulum (microsomes); there does not appear to be an organelle specific LACS. VLCFAs are oxidized exclusively in peroxisomes. However, VLCS activity is present in both peroxisomes and microsomes. Incorporation of fatty acids into complex lipids is restricted to the microsomes. Using immunocytochemical analysis it was demonstrated that the VLCS is located exclusively in peroxisomes and microsomes. VLCFA levels in X-ALD patients are the result of impaired VLCFA metabolism. It was demonstrated that peroxisomal VLCFA activation is strongly reduced, whereas microsomal VLCFA activity is normal. Hashmi et al. showed that cultured X-ALD fibroblasts were impaired in the β-oxidation of C24:0 but oxidized C24:0-CoA at normal rate. These latter observations made the peroxisomal VLCS the most likely candidate for X-ALD.

**Genetics of X-ALD**

Genetic linkage with glucose-6-phosphate dehydrogenase (G6PD) pointed the X-ALD locus to the extremity of the long arm of the X-chromosome, Xq28.In 1993, the ALD gene was identified using positional cloning strategies. The ALD gene is 21 kb long and contains ten exons. It came as a surprise that its product, ALDP, does not share homology with any known acyl-CoA synthetase. Based on sequence homology ALDP belongs to a different family of proteins, the ATP-binding cassette (ABC) superfamily of transmembrane transporter proteins. ALDP consists of 745 amino acids and contains a membrane domain with six transmembrane segments in the amino-half and an ATP-binding domain in the carboxy-half of the protein. Immunocytochemical studies demonstrated that ALDP is a peroxisomal membrane protein, which is in agreement with the observed biochemical abnormality in X-ALD patients.

Evidence that the gene identified by positional cloning is indeed the ALD gene came initially from identification of mutations in the ALD gene. To date, over 250 mutations have been identified in the ALD gene (Reviewed in Chapter 5). Mutations have been found in all X-ALD patients thoroughly examined. Complementation studies in fibroblasts derived from X-ALD patients demonstrated that expression of wild type ALD cDNA restores VLCFA β-oxidation confirming that ALD and not VLCS is the gene responsible for X-ALD. Furthermore, stable expression of ALD cDNA in X-ALD fibroblasts corrects VLCFA levels to
normal levels. However, the function of ALDP and its role in relation to either VLCFA metabolism and/or VLCS activity remains to be unveiled. Immunocytochemical studies have shown that in 70% of all X-ALD patients ALDP cannot be detected by immunoassay. All mutations other than missense mutations disrupt the stability of ALDP.

There is no correlation between the phenotypic variability observed in X-ALD patients and either VLCFA levels in plasma or fibroblasts, or the residual VLCFA β-oxidation activity present in X-ALD skin fibroblasts. The clinical variation in X-ALD can not be explained by the degree of biochemical abnormality, at least in plasma and fibroblasts. However, the relative levels of VLCFAs at the time of disease onset in the affected tissues, such as brain and adrenal gland, are unknown. Interestingly, there is also no correlation between the different phenotypes in X-ALD and the mutations identified in the ALD gene. The most severe phenotypes can be found in patients with a missense mutation in which ALDP was demonstrable by immunological assays, and mild phenotypes in patients with large deletions and no demonstrable protein product (Reviewed in Chapter 5). The most common ALD mutation, a two base pair deletion in exon 5 found in approximately 12% of X-ALD families, has been associated with all X-ALD phenotypes. Segregation analysis suggests that the phenotypic variability is due to an autosomal modifier gene. However, unidentified environmental factors may also be involved, as indicated by phenotypic variability in a set of monozygotic twins.

Function of ALDP

ALDP appears not to be required for either anchoring of human VLCS to the peroxisomal membrane or for translocation of VLCS into the peroxisome, since peroxisomes from X-ALD fibroblasts contain normal levels of VLCS in the absence of ALDP. In humans, over-expression of VLCS cDNA alone in X-ALD fibroblasts does not improve VLCFA β-oxidation, while over-expression of both VLCS and ALD cDNA in X-ALD fibroblasts increased VLCFA β-oxidation synergistically. This indicates that both proteins interact functionally to regulate peroxisomal VLCFA β-oxidation; however, the nature of this functional interaction remains to be solved.

Functional ABC transporters contain two membrane spanning regions and two ATP-binding domains. In some cases, the entire transporter is transcribed by a single gene, like the multidrug resistance P-glycoprotein (MDR), the cystic fibrosis transmembrane conductance regulator (CFTR) or the yeast pheromone transporter (STE6). In other cases, a functional ABC transporter is formed by
heterodimerization of two ABC half-transporters, like TAP1 and TAP2, which are
associated with antigen processing,\textsuperscript{71} or Pxa1p (Pat2p)\textsuperscript{72} and Pxa2p (Pat1p)\textsuperscript{73} two
yeast proteins which form the yeast peroxisomal long-chain fatty acid transporter.\textsuperscript{74,75} ALDP with one membrane spanning region and one ATP-binding
domain has the structure of a half-transporter. Three additional mammalian
peroxisomal membrane ABC half-transporters which are closely related by nucleic
acid and protein sequence have been identified: ALDRP, an ALDP-related
protein;\textsuperscript{76,77} PMP70, a 70-kDa peroxisome membrane protein;\textsuperscript{78,79} and PMP69 (or
P70R), a 69-kDa peroxisome membrane protein.\textsuperscript{80,81} The function(s) of the
peroxisomal ABC half-transporters and their interaction with VLCS is unknown,
but their marked sequence similarity suggests that they might have related and/or
overlapping function(s) in peroxisomal fatty acid metabolism. This notion is
supported by two observations: 1) X-ALD cells lacking ALDP have approximately
20% residual activity for VLCFA $\beta$-oxidation,\textsuperscript{60} which could result from one or
more of the other peroxisomal ABC half-transporters. 2) Over-expression of
PMP70 cDNA has been shown to partially restore VLCFA $\beta$-oxidation in X-ALD
fibroblasts,\textsuperscript{82} and over-expression of ALDR cDNA completely restored VLCFA $\beta$-
oxidation,\textsuperscript{83} indicating that the other peroxisomal ABC half-transporters can
substitute for the absence of ALDP. In vitro ALDP homodimerization and
heterodimerization of ALDP with either ALDRP or PMP70 has been demonstrated
in co-immunoprecipitation assays.\textsuperscript{84} Thus, it is likely that ALDP is a peroxisomal
transmembrane transporter, that functions either as a homodimer or as a
heterodimer with any of the three additional peroxisomal ABC half-transporters.

In the yeast \textit{Saccharomyces cerevisiae} the two orthologs of ALDP, Pxa1p and Pxa2p
have been demonstrated to heterodimerize to form a functional ABC
transporter.\textsuperscript{75} This transporter is involved in the transport of activated long-chain
fatty acids from the cytoplasm, across the peroxisomal membrane, into the interior
of the peroxisome.\textsuperscript{74} Recently, it was demonstrated that Pxa2p is responsible for the
transport of long-chain fatty acyl-CoA esters across the peroxisomal membrane.\textsuperscript{85} In the fruit fly \textit{Drosophila melanogaster}, the uptake of metabolic precursors that
are necessary for the synthesis of the brown and red eye pigments in pigment cells
is controlled by the presence of different dimer combinations of ABC half-
transporters in the cell membrane of these cells. Red color pigment precursors are
imported by the guanine transporter that contains heterodimers of subunits encoded
by the \textit{white} and \textit{brown} genes. Whereas, proteins from the \textit{white} and \textit{scarlet} genes
heterodimerize to form the tryptophan transporter, involved in the import of
metabolites for the synthesis of brown color pigments.\textsuperscript{86-90} Thus, in analogy to the
processes in yeast and fruit fly it is possible that ALDP, either as a homo-
heterodimer, is involved in the transport of activated VLCFAs from the cytoplasm into the peroxisome and that different peroxisomal ABC half-transporter dimer combinations may be involved in the import of specific fatty acids or other substrates, or may result in tissue specificity.

The X-ALD mouse
In 1997, three laboratories reported the construction of a knock-out mouse model for X-ALD. The X-ALD mouse exhibits reduced β-oxidation of VLCFAs in cultured fibroblasts and significant increased levels of VLCFAs in all tissues measured and in cholesterol esters from adrenal glands. The highest increase in VLCFA levels is observed in brain and adrenal glands; however, the increase is not as marked as in X-ALD patients. Lipid cleft inclusions were observed in adrenocortical cells, similar to those observed in X-ALD patients. In contrast to X-ALD patients, X-ALD mice do not have increased VLCFA levels in plasma. Despite the biochemical similarities to human X-ALD patients, X-ALD mice do not show any sign of neurological involvement and/or adrenocortical insufficiency. Even though the X-ALD mouse does not resemble cerebral ALD, it provides a good animal model for the future testing of potential therapeutic strategies for X-ALD. For example, the in vivo efficacy of treatment can be monitored by determination of its effect on VLCFA levels in the brain and adrenal gland, tissues that are primarily affected in X-ALD. Crossing of X-ALD mice with mice strains of different genetic background may result in a more severe X-ALD phenotype, and may facilitate the discovery of the modifier gene that is assumed to cause the wide phenotypic variation.

Therapy
Steroid replacement therapy for adrenocortical insufficiency, found in most X-ALD patients, is effective and of great importance. Although it does not alter the course of the neurologic disease, if left untreated adrenocortical insufficiency may be lethal.

Dietary therapy
The discovery that VLCFAs in the brain are the result of both dietary origin and endogenous synthesis, i.e. through elongation of medium and long-chain fatty acids, provided the rationale for the development of dietary therapies. Mono-unsaturated fatty acids compete with saturated fatty acids for the microsomal elongation system, hence interfering with the biosynthesis of VLCFAs. Addition of mono-unsaturated fatty acids, especially oleic acid (C18:1), to cultured fibroblasts
obtained from X-ALD patients resulted in a decrease of VLCFAs. The most widely used dietary therapeutic approach utilizes a 4:1 mixture of two unsaturated fatty acids, glyceryl trioleate (oleic acid in triglyceride form, or GTO) and glyceryl trierucate (erucic acid (C22:1) in triglyceride form, or GTE). This mixture of GTO and GTE is better known as “Lorenzo’s oil”, named after Lorenzo Odone, the first CCALD patient treated with the oil. Treatment of X-ALD patients with “Lorenzo’s oil” resulted in normalization of VLCFA levels in plasma within four to six weeks. The dramatic effect on VLCFA levels resulted in a number of therapeutic trials carried out in several different countries around the world, with over 300 X-ALD patients involved. Because of the rapid progression of the cerebral form of X-ALD, the impressive biochemical effect in plasma, the rareness of the disorder, the considerable phenotypic variation and the lack of a predictable marker for the different phenotypes, open studies were preferred to randomized, placebo controlled, and double blinded studies. This has complicated the evaluation of the efficacy of “Lorenzo’s oil” tremendously. However, despite the dramatic effect on plasma and tissue VLCFA levels, it has no apparent effect on the clinical course of the disease. “Lorenzo’s oil” could not stop or reverse neurological progression, nor did it improve endocrine function in five patients with AMN. Postmortem analysis of tissues of four X-ALD patients who had used “Lorenzo’s oil” for various lengths of time showed that, although substantial amounts of erucic were present in liver and adipose tissue, no increase in erucic acid levels in the brain could be observed. Furthermore, although VLCFA levels were reduced in liver and adipose tissue, the VLCFA levels in various brain lipids were indistinguishable between three of the treated patients and patients who had never used “Lorenzo’s oil”, raising the question whether the oils reach the brain at all.

An international collaborative trial of dietary therapy with “Lorenzo’s oil” in 250 neurologically asymptomatic boys with X-ALD is ongoing. The aim of this study is to determine whether initiation of the diet in boys without neurological involvement can reduce the frequency and/or severity of subsequent neurologic disabilities.

Immunosuppression
Cerebral demyelination is associated with a severe inflammatory reaction mediated by as yet unknown cytokines or immune mechanisms. Attempts to suppress or modify this inflammatory reaction with β-interferon, cyclophosphamide, cyclosporin, immunoglobulins, pentoxifylline, or β-thalidomide have failed.
Bone-marrow transplantation

Postmortem examination of the brain of cerebral ALD patients demonstrated perivascular mononuclear infiltrates compatible with an immune reaction.\textsuperscript{111,112} This inflammatory reaction has been linked to excess of VLCFAs in myelin lipids in the region of the brain where demyelination takes place.\textsuperscript{112-114} Macrophages of patients with X-ALD are unable to degrade VLCFAs. In 1984, it was demonstrated that normal bone-marrow derived cells can degrade VLCFAs.\textsuperscript{23} This provided the rationale for bone-marrow transplantation (BMT). In the same year, the first X-ALD patient with rapidly progressive CCALD received bone-marrow transplantation.\textsuperscript{115} The transplantation resulted in correction of plasma VLCFA levels within two months. The patient died 141 days after the transplantation due to an adenovirus infection without showing signs of neurological improvement. No additional BMTs were performed during the next four years. In 1990, the first reversal of both neurological and neuroradiological (MRI) manifestations were reported.\textsuperscript{116} An eight year old boy with mild neurologic involvement received bone-marrow from his nonidentical twin. Within two years after the BMT the neurological deficits and the white matter lesion had disappeared. At present, the boy is alive and well: his cerebral MRI, cognitive function are normal. However, he has developed mild peripheral nerve involvement, suggesting the onset of AMN (P. Aubourg, personal communication).

To date, over 100 BMTs have been performed worldwide in X-ALD. More cases have been reported in which patients who were transplanted at the first sign of MRI abnormalities showed stabilization or even improvement in neurological functions.\textsuperscript{117,118} All patients that responded well to BMT showed mild neurologic involvement at the time of transplantation. No benefit from transplantation could be achieved if the patient was already in the rapidly progressive stage of neurological deterioration.

It is not known by which mechanism BMT benefits patients; the most likely explanation is scavenging of VLCFAs by microglia cells derived from the new, normal bone-marrow.\textsuperscript{119} Although the origin of microglia is still debated, monocytes derived from peripheral hematopoietic cells probably cross the blood-brain barrier and transform into microglia.\textsuperscript{120} Within the central nervous system, microglia and astrocytes are the predominant cells expressing the X-ALD protein.\textsuperscript{121} Microglia cells resemble macrophages,\textsuperscript{122} and abnormal function of microglia cells may affect oligodendrocytes, the cells that produce myelin in the central nervous system.

BMT appears to be the most effective therapy for the variants of X-ALD with cerebral involvement. However, BMT cannot be performed in every patient
with cerebral demyelination, as a HLA-matched donor may be unavailable. The mortality and morbidity of the BMT procedure greatly confines the use of BMT as a therapy for X-ALD. Therefore, additional therapeutic approaches for X-ALD are warranted.

**Gene therapy**

*In vivo* gene therapy with retroviral-mediated gene transfer is not likely to be successful for cerebral disorders. Retroviral integration depends on active proliferating cells and the turnover of oligodendrocytes and microglia cells in the mature brain is very slow.

The parvovirus adeno-associated virus (AAV) does not require cell division for successful integration, which makes this virus a potential candidate for *in vivo* gene therapy for cerebral disorders. It is a relative safe virus, AAV infection has never been associated with disease.\(^{123}\) Wild-type AAV integrates at a site-specific locus, located on chromosome 19q13.3-qter.\(^{124}\) Its limitations however, are loss of site-specific integration on chromosome 19 of recombinant AAV and the size restriction (4.5 kb) for the insertion of target DNA.\(^{125,126}\) If these problems can be solved, AAV may be a very powerful tool for *in vivo* gene transfer into tissues with a low cell division rate, like the mature brain.

A more promising approach may be *ex vivo* gene therapy. Bone-marrow cells are isolated from the patient and transduced *in vitro* with a retroviral vector containing a copy of the normal *ALD* cDNA and returned to the patient by autologous transplantation with the genetically corrected hematopoietic cells. *In vitro* retroviral-mediated transfer of *ALD* cDNA has been demonstrated to result in correct targeting of viral-vector encoded ALDP to the peroxisomal membrane.\(^57\) Transduced fibroblasts showed normal VLCFA β-oxidation and normal ALDP expression two months after transduction. Successful retroviral transfer of normal *ALD* cDNA to peripheral blood and bone-marrow derived stem cells (CD34+ cells) from X-ALD patients was demonstrated recently.\(^127\) However, the transduction efficiency in these experiments was only 20%. *Ex vivo* retroviral gene therapy with hematopoietic stem cells is not yet possible for X-ALD, because of the limited efficiency of transduction of the hematopoietic stem cells. ALDP is a peroxisomal integral membrane protein, and can therefore not be transferred from transduced cells to non-transduced cells. For a favorable effect *in vivo* a much higher transduction efficiency of the hematopoietic stem cells with *ALD* cDNA is needed. In brain disorders where the disease gene produces a protein that is not membrane bound, i.e. the glucocerebrosidase protein, the transduction efficiency required to obtain a favorable *in vivo* effect can be lower. Studies in normal mice have shown
that up to 20% of microglia cells in the brain can be replaced within four months after transplantation with autologous bone-marrow cells expressing the human glucocerebrosidase gene from a retroviral vector. Studies to determine the in vivo efficacy of ex vivo gene therapy with transduced CD34+ cells in X-ALD mice are underway (P. Aubourg, personal communications).

Pharmacological approaches

Alternative pharmacological therapeutic approaches for X-ALD have been initiated as well. In 1998, Singh and coworkers demonstrated that treatment of fibroblasts derived from X-ALD patients with lovastatin or sodium phenylacetate (NaPA) resulted in reduction of VLCFAs and increased VLCFA β-oxidation. An additive effect was obtained when a mixture, at lower concentrations, of both drugs was administered to the cells. It was also demonstrated that lovastatin and NaPA inhibit the induction of nitric oxide synthase and proinflammatory cytokines such as tumor necrosis factor-α and interleukin-1β in astrocytes, microglia and macrophages from rat. These proinflammatory cytokines may be involved in the induction of demyelination as observed in CCALD. Recently, the in vivo efficacy of lovastatin was demonstrated, treatment of five X-ALD patients with lovastatin resulted in normalization of plasma total VLCFAs. At present the mechanism of lovastatin action and its effect on VLCFA levels in tissues, the brain in particular, is unknown.

Recently the potential of pharmacological gene therapy for X-ALD with another drug, 4-phenylbutyrate (4PBA) was demonstrated (Ref 83 and Chapter 6). 4PBA and NaPA are related compounds, NaPA is the first breakdown product of 4PBA. Treatment of cell lines from X-ALD patients and X-ALD knockout mice with 4PBA resulted in correction of both C24:0 and C26:0 levels as a result of increased VLCFA β-oxidation. The mechanism of 4PBA action involves increased gene expression of ALDR and the induction of peroxisome proliferation. ALDRP and ALDP are 66% identical and are functionally redundant; over-expression of ALDR cDNA in X-ALD fibroblasts restores VLCFA metabolism. The in vivo potential of dietary 4PBA treatment was demonstrated in X-ALD mice. Treatment resulted in reduction of VLCFA levels in the two principally affected organs in X-ALD, brain and adrenal gland (see Chapter 6 for details).

Both pharmacogenetic approaches are currently under intense investigation, small-scale pilot studies in X-ALD patients are ongoing. It is unlikely that lovastatin and NaPA/4PBA have similar mechanisms of action, since the combination of lovastatin and NaPA resulted in an additive effect. NaPA is very
likely to have a similar mechanism of action as 4PBA, while lovastatin might work through increase of the intracellular second messenger cAMP.

Scope of this thesis
The initial aim of this thesis was the genetic and molecular analysis of the Dutch X-ALD patient population. In Chapters 2 and 3 the identification and systematic analyses of mutations in 29 Dutch X-ALD kindreds is described. Most of the X-ALD kindreds have a unique mutation, the only frequent mutation found in the ALD gene is described in Chapter 2. In Chapter 4 the effect of X-ALD mutations on the stability of the ALDP was investigated and the importance of these findings for carrier detection and prenatal diagnosis is discussed. Chapter 5 reviews the X-ALD mutations identified thus far, the evolutionary relationship among the peroxisomal ABC half-transporters, protein-protein interactions of ALDP with these proteins, and discusses the evidence for the existence of a second, ALDP-dependent VLCS. And in Chapter 6 the effect of 4-phenylbutyrate (4PBA) treatment on the VLCFA levels in X-ALD cell lines (in vitro) and X-ALD knockout mice (in vivo) and the potential mechanisms of 4PBA action are described and discussed.

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