X-linked adrenoleukodystrophy in Norway

Clinical and epidemiological aspects

Thesis for the degree of PhD

by

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Til Ida og Maja

Nå er boka til Pappa ferdig…

"Lorenzo's Oil" by Source. Licensed under Fair use via Wikipedia -
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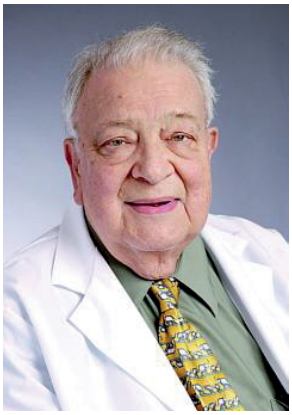
Preface

When at parties, being asked what I do for a living, my answer has for some years now been: ”X-linked adrenoleukodystrophy”. Blank faces is the usual response. However, I’ve had some success mentioning the 1992 film “Lorenzo’s Oil”, which heartbreakingly pictured the most severe form of this disease, and the parents’ struggle to find a cure for their beloved son. I have sometimes wondered whether the name of the disease ought to be changed to “Lorenzo’s disease”, to capitalize on the fame of that movie. But it’s rapidly becoming old – what, the kids nowadays haven’t even heard about yesterday’s great actors like Susan Sarandon, Nick Nolte and Peter Ustinov.

Ustinov played the role of Hugo W. Moser, and one of the most problematic aspects of the film is the unflattering way he is portrayed. Actually, Dr. Moser soars as the undisputed giant of X-ALD research. His interest in the disease, his care for the patients, and the breadth of his engagement, is unrivalled. His footprints are all over what we know today about X-ALD. If a less “medical” term was needed, we might as well name it “Moser’s disease”.



Hugo W. Moser (1924-2007)

There are two other problems with this important Hollywood movie: Firstly, in order to provide the expected happy ending, the film ends by hinting that Lorenzo’s parents, Augusto and Michaela Odone, were actually able to save their son. Almost single-handedly, they researched the 4:1 combination of glyceryl trioleate and glyceryl trierucate later known as Lorenzo’s Oil. Unfortunately, the truth of the story is that even though the oil was able to normalize Lorenzo’s levels of very long chain fatty acids, he remained permanently brain damaged, and lived in a vegetative state until his death at age 30 in 2008. Even today, the scientific basis for offering Lorenzo’s Oil to X-ALD sufferers remains disputed.

The other problem with using Lorenzo Odone as the image of X-ALD is that his variant of the disease, the childhood cerebral ALD, while being the classical form, is actually not the most common presentation. This is not a disease constrained to youngsters, or even to males. On the contrary, a modern view of X-ALD would rather describe it as a slowly progressive myelopathy due to axonal degeneration of the long tracts in the spinal cord, usually accompanied by a peripheral neuropathy, and in the majority of males by endocrine symptoms of primary adrenal and testicular failure.
Moreover, even female "carriers" are affected, although with milder symptoms, at a later age, and without the endocrine symptoms seen in males. Superimposed on this slowly progressive course, then, male patients may develop a rapidly progressive cerebral demyelination, most often in childhood – like in Lorenzo's case. However, recent research (supported by my own findings in this dissertation) shows that cerebral disease may develop in a disturbingly high fraction of adult males as well.

Therefore, the image of X-ALD as primarily a dramatic disorder of young boys is somewhat misleading. The adrenomyeloneuropathy may actually be seen as the true "basic" phenotype of X-ALD, with the cerebral disease as a sort of epiphenomenon, possibly triggered by external factors.

Despite the dubious gains and sad setbacks illustrated by Lorenzo Odone's case, one important lesson must be remembered: Although the cause of X-ALD is written in the genes of the patients, it's also a metabolic disorder, where much is known about the biochemistry and the pathogenesis, and where actual treatment has been found and is effective. Furthermore, the era of gene therapy is on its way. X-ALD is indeed a treatable peroxisomal disorder, and the future holds promises for both patients and caregivers.

Acknowledgements

First I want to thank the Norwegian X-ALD patients, for whom this work has been done. I hope they and future patients may benefit from the insights we have gained.

I did my research as part researcher/part clinical neurologist at Oslo University Hospital Ullevål, from 2011 as a clinical teacher in neurology at the University of Oslo. I thank my superiors at the hospital, Elisabeth G Celius, Espen Dietrichs and Sigrun K Brækken, for giving me the opportunity to carry out this lengthy project. I am also grateful to the Medical Faculty, University of Oslo, for their patience with a clinical researcher who took the long way round.

In this thesis the term "alternative paternity" is used. However, there can be no doubt that my first supervisor, Ola H Skjeldal, is the father of this project. I first met Ola in 2003, when he took me in on a European research program on Adult Refsum Disease. Although that project atrophied due to lack of patients, it introduced me to peroxisomal disorders and to a network of European researchers that paved the way for my subsequent research.

Ola showed his wisdom when, seeing me stuck, he changed the focus of our research to X-linked adrenoleukodystrophy, and brought in Chantal ME Tallaksen as my co-, later main, supervisor. Chantal, then, became the true mother of my project. There must be few PhD students who have had such a kind and caring supervisor as I have had. I know I may have abused your open door, Chantal, but without it, I'm not sure this project would have been fulfilled. I am deeply grateful.

As all PhD students, I am indebted to my co-authors. In particular Lars Retterstøl, who helped out a neurologist trying to understand some genetics. Also, I thank the milieu at the Registry for organ-specific autoimmune disorders (ROAS) at Haukeland University Hospital, represented by Martina M Erichsen, Anette SB Wolff and Eystein S Husebye, and the Oslo MS Registry at Ullevål, represented by Elisabeth G Celius, for giving me access to these important patient registries that form the basis of Papers 3 and 4. Michael Abdelnoor helped me with the

epidemiology. In Paper 2, I was fortunate to be able to cooperate with some of Norway's leading experts on small fiber neuropathy, Ellen Jørum and Svein I Mellgren. Besides contributing to that article, Bernhard Nilsen helped me give my teaching of medical students that special buzz.

The survey of Norwegian X-ALD subjects has truly been a "dugnad", and I am indebted to the colleagues all over the country who contributed. Karin BM Mikaelsen came late, but strong, with the patient in Paper 5. A special thanks to Magnhild Rasmussen and Anne Grethe Myhre. Similarly, I'm grateful towards the laboratories performing VLCFA analyses; in Norway (Berit Woldseth at OUS) and abroad (Marinus Duran at AMC, Richard Jones at KKI, and Jan-Eric Månsson at Sahlgrenska), who helped me with the critical task of identifying X-ALD probands. I thank Ronald JA Wanders and Sacha Ferdinandusse at the AMC for their kindness helping out a stray colleague from a small research group far to the north.

I would like to thank Ottar Kruse for his invaluable support at a time when everything looked hopeless. With your help, I found strength within.

My parents, Anne and Per Richard, have probably shaped me as a person – and now as a researcher – more than I realize. I'm really grateful for the way you and your spouses have helped us out during these years; being there as caring grandparents for our children, helping us shape them as well.

If I have achieved nothing else, at least I have fathered and got the chance to know Ida and Maja, my beautiful daughters. That alone makes it all worth it. You are the meaning of my life, you're my inspiration…

Last in this list, yet most important to me – my beloved wife Katrine. You are my foundation, and with your calm and quiet belief in me, I've been able to keep on going despite doubt and frustration. For me, home&family is what I treasure most, and that is all about you, Katrine. I thank you deeply for your love and patience.

Morten A Horn, Oslo, June 2015

Abbreviations

Abcd1	The gene corresponding to the human gene *ABCD1* in mice
ABCD1	ATP-binding cassette, superfamily D, member 1
ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit hyperactivity disorder
ALD	Adrenoleukodystrophy
ALDP	ALD protein; encoded by *ABCD1*, defective in X-ALD
Adol-CER	Cerebral ALD with onset in adolescence
Adult-CER	Cerebral ALD with onset in adulthood
AMN	Adrenomyeloneuropathy
AO	The Addison Only phenotype
ASYMP	The asymptomatic phenotype
CASS	Composite autonomic scoring scale
CCER	Childhood cerebral ALD
CER	Cerebral ALD; demyelinating leukodystrophy
CoA	Coenzyme A
GTE	Glyceryl trioleate; part of LO
GTE	Glyceryl trierucate; part of LO
HCST	Hematopoietic stem cell therapy; bone marrow transplantation
HSP	Hereditary spastic paraparesis
IENFD	Intraepidermal nerve fiber density

Lyso-PC	Lysophosphatidyl choline
MLPA	Multiplex ligation-dependent probe amplification
LO	Lorenzo's oil (4:1 mixture of GTO/GTE)
MRI	Magnetic resonance imaging
MS	Multiple sclerosis or Mass spectrometry
NADR	National Addison's disease registry (abbreviation used here only)
NFS	Neurologic function score
NIPT	Non-invasive prenatal testing
NSAIDs	Non-steroidal anti-inflammatory drugs
OMSR	Oslo Multiple sclerosis registry (abbreviation used here only)
OUS	Oslo University Hospital
PAI	Primary adrenal failure; Addison's disease
PGD	Preimplantation genetic diagnosis
PPMS	Primary progressive multiple sclerosis
QSART	Quantitative sudomotor axon reflex test
QST	Quantitative sensory testing
SIGNS	Phenotype category: neurological signs of myeloneuropathy
SIRT1	NAD-dependent deacetylase surtuin-1
VLCFA	Very long chain fatty acids (> 22 carbon atoms)
X-ALD	X-linked adrenoleukodystrophy
21OHAb	Anti-21-hydroxylase autoantibodies

List of tables and figures

Publications included

Paper 1

Horn MA, Retterstøl L, Abdelnoor M, Skjeldal OH, Tallaksen CME. Adrenoleukodystrophy in Norway: High rate of *de novo* mutations and age-dependent penetrance. Pediatr Neurol 2013;48:212-219.

Paper 2

Horn MA, Nilsen KB, Jørum E, Mellgren SI, Tallaksen CME. Small nerve fiber involvement is frequent in X-linked adrenoleukodystrophy. Neurology 2014;82:1678-1683.

Paper 3

Horn MA, Erichsen MM, Wolff ASB, Månsson JE, Husebye ES, Tallaksen CME, Skjeldal OH. Screening for X-linked adrenoleukodystrophy among adult men with Addison's disease. Clin Endocrinol (Oxf.) 2013;79:316-320.

Paper 4

Horn MA, Woldseth B, Skjeldal OH, Celius EG, Tallaksen CME. X-linked adrenoleukodystrophy as differential diagnosis of primary progressive multiple sclerosis. (manuscript submitted to Multiple Sclerosis Journal)

Paper 5

Horn MA, Mikaelsen KBM, Ferdinandusse S, Jørum E, Mellgren SI, Retterstøl L, Wanders RJA, Tallaksen CME. Mild phenotype in an adult male with X-linked adrenoleukodystrophy – case report. (manuscript submitted to the Clinical Case Reports journal).

1 Introduction

X-linked adrenoleukodystrophy (X-ALD) [1, 2] is a genetic metabolic disorder characterized by the deficient metabolism of very long chain fatty acids (VLCFAs) [3]; that is saturated unbranched fatty acids with more than 22 carbon atoms. All subjects carry a mutation in the *ABCD1* gene [4]. The disease affects mainly males (hemizygotes), but similar to other X-linked disorders, it is increasingly recognized [5] that females (heterozygotes) may be clinically affected as well. The main neurological manifestations [4] include a progressive, fatal, inflammatory cerebral leukodystrophy (cerebral adrenoleukodystrophy, CER) most frequently appearing in young boys, and a slowly progressive, non-inflammatory axonal myelopathy and peripheral neuropathy (adrenomyeloneuropathy, AMN) [6] that occurs in most adult males. Many females develop myeloneuropathy as well, but usually at a later stage and with milder symptoms than seen in males [5, 7-9]. Furthermore, the disorder manifests with primary adrenal failure (PAI) in about 70 % of males [10], usually not seen in females [11]. Males may also show signs of testicular failure [12], and will typically have scanty scalp hair and early balding [13].

1.1 Historical perspectives

The historical background of X-ALD is complex, in particular because it was initially thought to be solely a childhood disorder of cerebral leukodystrophy – the manifestations of adulthood were recognized as late as in the 1970s [6]. The first definite cases of X-ALD were probably described in 1910 [14], with a pair of male siblings who at the age of six and eight years developed the typical fatal picture of rapidly progressive intellectual deterioration, spastic gait disturbances, loss of speech and autonomic control, and dark skin. At autopsy, the brain showed what Schilder [15] described as “encephalitis periaxialis diffusa”, with severe, diffuse myelin loss. In 1923, Siemerling and Creutzfeldt described a similar case [16], in which they also found evidence of adrenal involvement.

During the first and middle part of the 20th century, there was much uncertainty as to the cause and classification of childhood disorders of rapid neurological

deterioration with diffuse demyelination demonstrated on autopsy. The term "Schilder's disease" or "Schilder's diffuse sclerosis" was applied to several disorders now seen as separate disease entities, among them cases now believed to be X-ALD [17]. The term "adrenoleukodystrophy" (ALD) was introduced by Blaw in 1970 [18].

An X-linked mode of inheritance was suggested in the 1960s by Fanconi [19] and Blaw [20]. In the 1970s, pathological evidence of fatty deposits in adrenal glands and cerebral tissue was demonstrated by Schaumburg [21], and in 1976 Igarashi [3] demonstrated that these lipids consisted of cholesterol esters with saturated fatty acids with very long carbon chains; the VLCFAs. The ability to measure VLCFA levels in plasma, introduced in 1981 [22], revolutionized classification and diagnosis of X-ALD.

Singh [23, 24] showed that the accumulation of VLCFAs in blood and tissues was related to impaired β-oxidation of these acids in the peroxisome. This made it clear that X-ALD belonged to the peroxisomal disorders, where it now constitutes the third subgroup (single peroxisome substrate transport deficiencies) [25]. Notably, VLCFA accumulation was also discovered in other peroxisomal disorders, particularly the severe childhood disorders now known as the Zellweger Spectrum Disorders (ZSD); Zellweger Syndrome (ZS), Neonatal Adrenoleukodystrophy (NALD) and Infantile Refsum Disease (IRD). Importantly, NALD [26] is *not* the neonatal form of X-ALD; as the other ZSDs it is a clinically distinct disease entity genetically separate from X-ALD.

In 1910, von Neusser [27] first described the association between adrenal failure and spastic paraplegia. In 1976, Budka [6] suggested that this phenotype was an adult variant of the adrenoleukodystrophy of childhood. Griffin and Schaumburg coined the term adrenomyeloneuropathy (AMN) [28, 29]. By 1994, AMN was shown to be the most common form of X-ALD in the Netherlands [30].

The gene for X-ALD was mapped to chromosome Xq28 in 1981 [31], and in 1993 the *ABCD1* was identified as the gene involved in X-ALD [32]. Rather than

encoding an enzyme responsible for VLCFA breakdown, the ALD protein (ALDP) was shown to be involved in transport of VLCFA-CoA esters across the peroxisomal membrane. Subsequently, a vast number of unique mutations (currently about 700) have been identified in the *ABCD1* gene, the only gene found to be involved in X-ALD. An archive of *ABCD1* mutations is maintained at the www.x-ald.nl website (managed by Kemp [33]). The latest offshoot of the discovery of the genetic cause of X-ALD is the recent attempts at treating CER with gene therapy [34].

1.2 Clinical features of X-ALD

The "classical" form of X-ALD is the devastating cerebral form typically seen in young boys, vividly presented in the film "Lorenzo's Oil". However, increased experience with and systematic studies of X-ALD populations have, during the last decades, changed our view of X-ALD on three important areas: Firstly, it is now increasingly recognized that AMN may be the basic and most frequent phenotype of X-ALD [4]. Secondly, recent research [8, 9, 35] has established that most or all females with X-ALD will develop some degree of neurological involvement, with an age-dependent penetrance. Thirdly, evidence is accumulating that CER may develop beyond childhood, even in full grown males with AMN [35-37], possibly triggered by external factors such as head trauma [38].

The traditional classification of X-ALD phenotypes has been based on division into several phenotype categories (see Table 1), set up by Hugo Moser [1]. A critique of this classification system is provided in section 3.2 (Phenotype assignment). The main problem with this old way of subdividing X-ALD subjects is that we now see the disease as being dynamic: affected subjects will typically move from one phenotype category to another as the disease progresses. Which category a given subject is allocated to depends not only on disease severity, on the pathogenicity of the mutation: to a large extent it is a function of age.

Table 1 X-linked adrenoleukodystrophy phenotypes in males.

Phenotype	Description	Estimated relative frequency
CCER	Onset at 3–10 years of age. Progressive behavioral, cognitive and neurologic deficit, often leading to total disability within 3 years. Inflammatory brain demyelination	31–35%
Adolescent	Similar to CCER, but somewhat slower progression. Onset age 11–21 years	4–7%
AMN	Onset 28 ± 9 years, progressive over decades. Involves spinal cord mainly, distal axonopathy inflammatory response mild or absent. Approximately 40% of patients have or develop cerebral involvement, with varying degrees of inflammatory response and more-rapid progression	40–46%
Adult cerebral	Dementia, behavioral disturbances. Sometimes focal deficits, without preceding AMN. White matter inflammatory response present. Progression parallels that of CCER	2–5%
Olivo-ponto-cerebellar	Mainly cerebellar and brainstem involvement in adolescence or adulthood	1–2%
Addison-only	Primary adrenal insufficiency without apparent neurologic involvement. Onset common before 7.5 years. Most patients eventually develop AMN	Varies with age. Up to 50% in childhood
Asymptomatic	Biochemical and gene abnormality without demonstrable adrenal or neurologic deficit. Detailed studies often show adrenal hypofunction or subtle signs of AMN	Diminishes with age. Common <4 years. Very rare >40 years

Abbreviations: AMN, adrenomyeloneuropathy; CCER, childhood cerebral adrenoleukodystrophy.

Table 1. Phenotype categories of X-ALD, as set up by Hugo Moser in 1991. From Moser H et al, Nat Clin Pract Neurol 2007;3:140-151 (reproduced with permission from the publisher).

This modern view on X-ALD is illustrated by Figure 1 from the recent review article by Engelen [4]. Our own research on X-ALD has led us to the same way of looking at X-ALD: A life-long disease in which the genetic and biochemical disturbances (possibly a toxic effect of chronically high levels of VLCFAs, leading to membrane instability, oxidative stress and other deleterious effects [39-41]) cause progressive damage to affected tissues (the central and peripheral nervous system, endocrine tissues like the adrenal cortex and testicles, hair follicles). The acute cerebral demyelination, once seen as the classical form of X-ALD, may actually be a secondary phenomenon, superimposed on the slowly progressive axonopathy in a proportion of male subjects.

Interestingly, the *Abcd1* knockout mouse model [42-44] is deemed suboptimal [4], because it does not develop CER; hence it cannot be used to study the pathological process of or therapeutic interventions for this most severe manifestation of X-ALD. However, the mouse does develop a gait disorder (at 18 months) indicative of myelopathy, in the same slow fashion as AMN develops in humans [45]. This may be seen as supportive of the view that AMN is the basic phenotype in X-ALD.

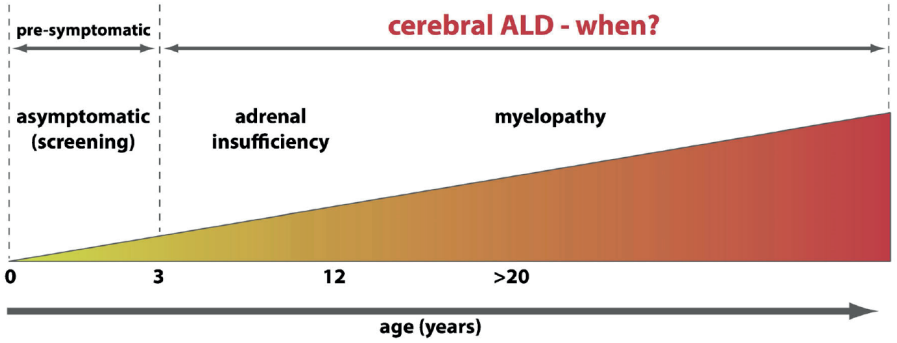


Figure 1. Modern view on the evolution of phenotypes in X-ALD. From Engelen M et al, Curr Neurol Neurosci Rep 2014;14:486 (reproduced with permission from the publisher).

Addisonism. PAI due to X-ALD may develop insidiously, or present with an acute, sometimes life-threatening, Addison crisis [46, 47]. Typical symptoms are fatigue, weakness, weight loss, gastrointestinal complaints, salt craving and hyperpigmentation of the skin [48]. Frequently, latent PAI is disclosed by laboratory testing in boys or adult males diagnosed with X-ALD due to neurological symptoms or pedigree screening [10]. A typical mode of onset of X-ALD is the Addison only (AO) phenotype, with slow or acute onset of PAI in a previously healthy young boy. X-ALD is a major cause of PAI of childhood onset in boys [49-53], and is often diagnosed after an Addison crisis – frequently leading to identification of several other affected members in the extended family. Onset of PAI can be at any age, before or after onset of neurological symptoms.

In the literature, it is commonly stated that PAI is present in about 70 % of males, however, the exact basis for that assertion is hard to find. One study of asymptomatic boys identified through pedigree screening found that 80 % had some laboratory evidence of latent PAI [10]. Engelen [4] states that almost all males will develop PAI during life, but the source is unclear. In females, symptoms of PAI are very uncommon (1 %), although subtle laboratory abnormalities may be found in the majority of heterozygotes [11].

Hypogonadism. Powers [54] described lamellar inclusions in the Leydig cells, indicating that the testicles are affected in X-ALD. This was supported by several studies [12, 55, 56] showing laboratory abnormalities and clinical features suggesting some degree of testicular failure in the majority of adult males.

Clinically, some X-ALD males show striking symptoms of hypogonadism, such as small testicles, impotence, and infertility, whereas others appear to have normal testicular function. Among adult Norwegian males with X-ALD, we encountered four who had produced offspring, seven who had not (including three seeking medical aid for infertility), and five who were too young to classify (data not published). Stradomska [57] found no evidence of reduced fertility among adult Polish males with AMN. The clinical significance of the testicular involvement in X-ALD needs to be further elucidated.

1.2.2 Neurological features

Cerebral demyelination. The most striking manifestation of X-ALD is the devastating cerebral demyelination, the leukodystrophy. It most typically occurs in boys between four and eight years of age, comprised in the childhood cerebral ALD (CCER, onset of CER < 10 years of age) phenotype, affecting about one third of males [2]. With diminishing frequency, CER may present in adolescent boys (Adol-CER, onset 10-21 years), and even in young adults with no apparent signs of preexisting myelopathy (Adult-CER, onset >21 years in males without AMN).

However, Kumar [58] found MRI evidence of cerebral involvement in 46 % of adult males with AMN. Research from the American and Dutch groups [36, 37] has shown that during an observation period of about 10 years (range 1 to 30), between 19 and 63 % of AMN males developed cerebral demyelination, with a poor prognosis similar to that seen in boys with CER. Thus, even though early childhood obviously is a high-risk age for CER, long-term follow-up of X-ALD males indicates that CER may occur at any age, and may eventually affect the majority of males. This new realization has implications for preventive measures,

follow-up and possibly therapy directed at CER (like hematopoietic stem cell transplantation (HCST), see section 1.7).

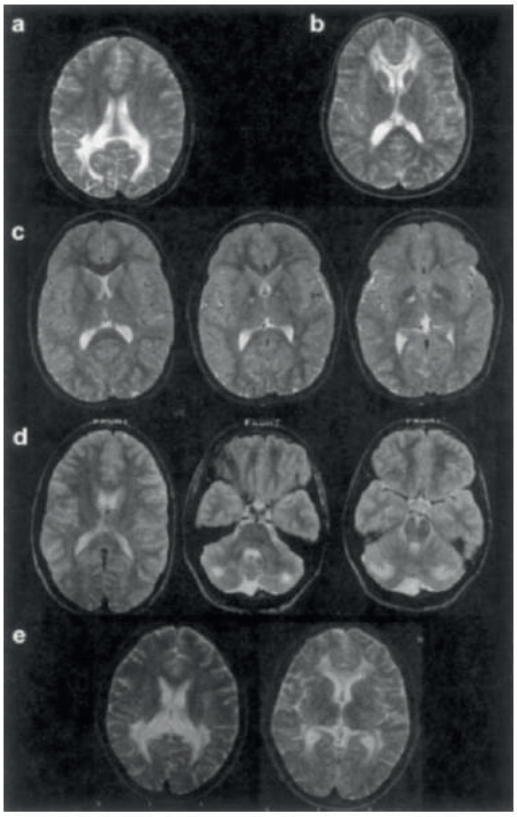


Figure 1. Different patterns recognized in male patients with cerebral X-linked adrenoleukodystrophy. (a) Pattern 1: white matter in the parieto-occipital lobe or splenium of corpus callosum; (b) pattern 2: white matter in the frontal lobe or genu of corpus callosum; (c) pattern 3: primary involvement of frontopontine or corticospinal projection fibers without affection of periventricular white matter; (d) pattern 4: primary involvement of cerebellar white matter; (e) pattern 5: combined but separate initial involvement of frontal and parieto-occipital white matter.

Figure 2. MRI patterns of cerebral demyelination in X-ALD. From Loes et al, Neurology 2003;61:369-374 (reproduced with permission from the publisher).

Clinically, CER presents insidiously at first, with subtle cognitive decline and impairment of visuomotor skills [2]. Boys typically show decline in school performances and behavior, often misinterpreted as attention deficit hyperactivity disorder (ADHD). As the disease progresses, typically within months, overt neurological deficiencies become apparent. At this stage, MRI is usually performed, disclosing the diagnosis of X-ALD. During the subsequent months, neurological deterioration is rapid and dramatic; most boys end up tetraspastic, demented, blind, deaf, unable to speak or swallow. Death ensues after two to four years, depending on

level of care, usage of life-prolonging measures, and presence of complications to the bedridden state.

On MRI (see Figure 2), CER starts most commonly in the splenium of the corpus callosum (occipital pattern, 80 % of cases), while in a minority, the first lesions appear in the genu of the corpus callosum (frontal pattern, 20 % of cases) [59]. With disease progression, the lesions spread in a symmetric, confluent pattern, eventually involving most of the white matter of the brain. Severity of the MRI lesions are graded using the Loes score (0-39 points) [60], with 10 being an important threshold beyond which HCST is not beneficial [61]. The frontal pattern of demyelination may give rise to a picture dominated by neuropsychiatric symptoms; we have seen this in some of our own patients.

In some cases "arrested cerebral ALD" [62] occurs, where the progression of the demyelinating lesions halts inexplicably, and the patient may remain stable for years, even decades. This is estimated to occur in about 10 % of childhood and adult cerebral cases [2, 63], although the frequency of this phenomenon is dependent on whether all subjects with *ABCD1* mutations are actually identified. In theory, there might exist cases of arrested CER, possibly due to *de novo* mutations and therefore not captured through pedigree screening, who could remain undiagnosed until they develop AMN in adulthood. The observation that CER may halt spontaneously complicates decision-making when asymptomatic boys are shown to have emerging MRI lesions indicating CER, and HCST must be considered. Gadolinium enhancement of the MRI lesions appears to indicate a higher risk of progression [64].

Myeloneuropathy. As noted, one of the major changes in the concept of X-ALD has been the realization that AMN [6] is not simply the "adult form of X-ALD". Rather, this seems to be the basic phenotype, affecting most or all subjects with a pathogenic *ABCD1* mutation, regardless of gender, but heavily dependent on age [4]. Although the pathological process of long tract degeneration may take place in most subjects, the clinical problem of myeloneuropathy, in terms of symptoms and

disability, may not necessarily affect all (note, however, the strikingly high proportion of symptomatic females in the Dutch study: 88 % at age > 60 years [8]).

Clinically, the myeloneuropathy of AMN presents as a slowly progressive spastic paraplegia, with or without signs or symptoms of peripheral neuropathy (like drop foot, prominent in one of our own patients). In patients, the effects of the myelopathy frequently overshadow symptoms of peripheral neuropathy. However, neurophysiologic studies show pathology in a majority of AMN subjects, usually an axonal sensorimotor neuropathy [65], although some subjects present with a primary demyelinating neuropathy [66].

Onset of neurological symptoms of AMN is usually in the 3rd or 4th decade in males. Moser [7] described mean age at onset as 28 $\pm$ 9 years. However, this is based on data from before 1991, when AMN was only recently identified as a variant of X-ALD, and not yet recognized as the most common form [30]. Possibly, a renewed population-based survey of X-ALD males would have yielded different results. As mentioned in several review articles on X-ALD [7, 30, 67, 68] there exist male cases of mild X-ALD (the subject of our Paper 5). The variability of severity, age of onset and rate of progression may be larger than recognized initially, when the most striking cases may have been preferentially included.

For females, Moser [69] described mean age at onset of symptoms at 37 $\pm$ 14.6 years. Similarly, the cohort of South Brazilian heterozygotes described mean age of onset of symptoms at 39.4 $\pm$ 10 years (range 21-59) [9]. However, as discussed in section 5.2.2, that cohort might be skewed towards inclusion of more severely affected females. In the Dutch study [8], the proportion of symptomatic females was radically higher in the age group 40-59 than in those < 40 years of age (82 vs. 18 %).

Progression of the myeloneuropathy of AMN is usually described as slow, although in some subjects (observed in our cohort) there may be a rather abrupt mode of onset, with significant impairment of gait and ambulation in the span of some few years. In the Norwegian cohort of AMN males, the median age of

wheelchair dependency was 50 years (range 34-70, unpublished data). Long-term prognosis of AMN is a murky area, not least because of the impact of CER that may be superimposed on AMN [37], thereby interrupting the natural course of the myeloneuropathy. Furthermore, there is a lack of detailed descriptions of elderly AMN patients.

1.2.3 Other features

Hair loss. A longstanding clinical observation is that X-ALD males, and to some extent females, have scanty scalp hair and early balding. König [13] described hair status in 16 consecutive adult AMN males with mean age 34 years (range 27-62). 12/16 had male-pattern hair loss, whereas the remainder had hair loss with other patterns. In 11/16 the remaining scalp hair was scarce and thin. 11/16 had loss of eyebrows and/or eyelashes. In 10/15 subjects with androgenetic alopecia, the hair loss had started during adolescence or young adulthood (15-22 years), and hair loss usually preceded other manifestations of X-ALD. Alopecia has also been observed in X-ALD heterozygotes [13, 70]. The mechanism of hair loss in X-ALD remains to be elucidated; however, it is of interest that hair follicles are among the tissues where ALDP is expressed [71].

1.3 Epidemiology of X-ALD

The occurrence of X-ALD in the population is a problematic field of research, particularly because the definition of what X-ALD really is, which subjects should be included in the diagnosis, has evolved throughout the last half century during which most epidemiological surveys have been performed. As noted above, X-ALD was first seen as a disease of boys only, until it was realized that AMN affecting adult males was actually part of the X-ALD spectrum. A few reports initially described an AMN-like picture in some heterozygotes, and recently, it has been shown that the majority (possibly all) of females will eventually develop symptoms from X-ALD. Thus, the patient population to be used as the numerator of the calculation has been successively expanded, from small boys only to all

subjects harboring the disease-causing mutation. This evolution is reflected in the epidemiological surveys performed in several populations throughout the decades; from France [72], the Netherlands [30], Germany [73], Australasia [74], Italy [75], USA [76], Japan [77] and South Brazil [78] (see Table 2). Our Paper 1 [35] is the latest offshoot of this expanding scope of X-ALD epidemiology, using the widest inclusion criteria yet.

Table 2. Epidemiological studies of X-ALD published 1993-2013. Based on Table 1 in Horn et al, Pediatr Neurol 2013;48:212-219.

Year (reference)	**Area studied**	**Sex incl.**	***n* subjects/ kindreds**	**Main finding**
1993 (72)	France	Males	129/79	Incidence 1:100,000 males
1994 (30)	The Netherlands	Males	81/31	Incidence 1:100,000 males Prevalence 0.5:100,000 males
1997 (73)	Germany	Both *	258/–	Incidence 0.8:100,000
1998 (74)	Australasia	Both	222/61	Incidence 3.3:100,000 males ‡
1998 (75)	Italy	Males	117/–	Incidence 3.6:100,000 males
2001 (76)	USA	Both †	–/616	Incidence 6:100,000
2002 (77)	Japan	Both *	286/144	Incidence 2-3.3:100,000 males
2005 (98)	Spain	Both *	160/62	Mutation study
2010 (78)	South Brazil	Males	152/21	Incidence 2.9:100,000 males
2013 (35)	Norway	Both	39/22	Incidence 1.6:100,000 Prevalence 0.8:100,000

* In these studies, males outnumber females.
† In this study, the frequency of heterozygotes was extrapolated from the number of identified hemizygotes.
‡ In Table 1 in Paper 1, the figure 1.6:100,000 is given; in the present table the denominator is male births only.

Even as the understanding of X-ALD has evolved, the awareness of the disorder and the diagnostic technology has also improved during this period, wherefore,

capture of affected subjects must be expected to have increased. This may be particularly true for affected females and adult males with AMN. On the other hand, the dramatic clinical picture of childhood CER, often seen in conjunction with Addisonism, may have been readily identified even early on. To some extent, the improved understanding of X-ALD has led to some conditions being chipped off as other distinct disease entities, as happened when the several disorders covered by the term "Schilder's disease" were elucidated [17]. However, this process of separation took place before the epidemiological studies were performed, from the 1990s onward.

Frequency of X-ALD. The frequency of X-ALD, almost universally cited in the literature, is based on the findings from the large American study performed by Bezman in 2001 [76]. This was based on X-ALD subjects identified at the Kennedy Krieger Institute in Baltimore from 1981 to 1998 and at the Mayo Clinic in Rochester from 1996 to 1998. The authors summated male cases of X-ALD identified at both laboratories during the years 1996-98 (the numerator), divided by the number of live births in the USA during the same time period (the denominator). The resulting figure of 1:42,000 was seen as the "minimum frequency of X-ALD hemizygotes" in the American population.

Only male subjects were used in this calculation, although the authors also identified a large number of heterozygotes by way of extended family screening (a total of 1,194 hemizygotes and 1,286 heterozygotes were identified at the Kennedy Krieger Institute from 1981 to 1998). For estimating the frequency of heterozygotes, the authors assumed that the ratio of heterozygotes:hemizygotes was 1.5. Thus, the combined frequency of hemi- and heterozygotes with the genetic disorder of X-ALD was estimated to be 1:16,800 (6:100,000).

The exact meaning of the term "frequency" used here is unclear: in articles on X-ALD it is often used to indicate the number of X-ALD subjects in relation to the population size (as a kind of prevalence), whereas the actual basis for the figure is the number of new cases (a kind of incidence) in relation to the birth numbers in

the populations (incidence at birth). In other articles [2] and at the www.x-ald.no website, the term is used as the incidence among newborns.

In conclusion, the most common interpretation seems to be that X-ALD affects one in about 17,000 when both genders are considered, or one male in 21,000 males. These are claimed to be minimum estimates (due to perceived incomplete capture in population surveys), and the frequency of X-ALD is believed to be similar around the world.

Incidence of X-ALD. According to Bezman [76], Aubourg estimated the incidence of X-ALD in France to be 1:20,000, although with no reference for this assertion. However, in a review from 2007, Moser [69] repeats the statement, referring to the 1993 article by Mosser [32], in which the *ABCD1* gene was identified. Strangely, that article provides no foundation for the assertion that X-ALD affects one in 20,000 males. A French survey from the same year [72] found the incidence at birth of X-ALD to be 1:100,000. The basis for the French incidence data therefore remains unclear.
A German study [73] found the incidence at birth of X-ALD to be 0.8:100,000, and in the Netherlands in 1994 [30], the incidence of X-ALD in the male half of the population was 1:100,000. The prevalence of living X-ALD hemizygotes in the male population was 0.5:100,000. Several other studies [74, 75, 77, 78] have used a method similar to the American, finding incidence figures of 1.6-3.6:100,000 (see Table 2, page 24). However, the epidemiological basis for these calculations is poorly defined.

A detailed critique of the American method of frequency estimates is provided in sections 3.3 (Incidence calculations) and 5.1.2. In short, we believe these estimates to be artificially high. These uncertainties regarding the true frequency with which X-ALD occurs in the population (internationally, and here in Norway), were the prime motivation behind the project presented in this thesis. The obvious solution to the enigma, however, would be actually screening newborns for X-ALD.

1.4 Newborn screening

Early diagnosis, like newborn screening, is essential in order to identify boys at risk of developing PAI, sometimes presenting as a life-threatening Addison crisis [46, 47], and to provide follow-up and timely treatment for CER. However, not all boys with X-ALD may be identified in the presymptomatic stage through family screening, particularly not in the case of a *de novo* mutation [79]. Additionally, a sad fact is that the usual delay between birth and onset of symptoms (typically between four and eight years of age in CCER) means that parents may give birth to another affected son before the diagnosis is revealed in the older brother. In the Norwegian cohort, four families lost two sons to CER. Out of 17 boys who died from CCER or Adol-CER, four (24 %) had an older brother who also died from X-ALD, but in whom onset of disease was too late to allow the parents to make reproductive decisions (unpublished data).

A series of methodological improvements researched by the Kennedy Krieger Institute and the Mayo Clinic in the USA has made newborn screening feasible [80-83]. These methods make use of dried blood spots obtained as part of the ordinary newborn screening program. VLCFAs cannot be directly analyzed by this method, so a high-throughput method for quantification of lysophosphatidylcholine (lyso-PC) species containing C24:0 and C26:0 using flow-injection tandem mass spectrometry (FIA-MS/MS) has been developed [83]. Newborn screening is now being implemented in the states of New York and Connecticut, USA, and several other states may follow.

Several issues, practical, economical and ethical, needs to be evaluated in this process [84]. Firstly, determination of levels of VLCFAs in newborns will identify not only subjects with X-ALD (for whom therapy is available for PAI and CER), but also subjects with other peroxisomal disorders, as the Zellweger Spectrum Disorders [25] (for whom only supportive therapy is available). Secondly, the question remains whether to include girls in the newborn screening program. Given current knowledge, girls < 16 years of age will not profit from early

diagnosis as no therapy is needed or available. Also, newborn screening of girls suffer from the same problem as for standard VLCFA analysis; sensitivity of lyso-PC measurements is less than 100 % in heterozygotes [83], so some girls will be missed. Finally, systematically screening newborns for X-ALD may reveal X-ALD in related family members who do not desire this knowledge. This issue is, perhaps, made more complicated in light of recent research, painting a more somber prognosis for both male and female X-ALD subjects.

During the latest revision of the Norwegian newborn screening program in 2012, X-ALD was not included among the 21 disorders added to those initially screened for (phenylketonuria and congenital hypothyreosis). The technology available at that time did not allow high-throughput screening for X-ALD; as described above, these methods are now being improved. Furthermore, there was at that time uncertainty regarding the therapeutic options in X-ALD (Rolf Dagfinn Pettersen, e-mail correspondence March 24th, 2009). However, since then, HCST (first performed in 2011) has become established therapy for CER in Norway.

1.5 Pathophysiology

Schaumburg [21, 29] and Powers [54, 85, 86] did the major work describing the pathological lesions in the brain and spinal cord in X-ALD, reviewed by Powers [87] and Ferrer [88]. There are two distinct types of pathology: In AMN a slowly progressive, degenerative dying-back axonopathy of the long tracts in the spinal cord is seen, with no evidence of inflammation [86]. In CER there are enlarging, confluent lesions of demyelination, inflammation and gliosis [54, 87, 88].

Pathologically, the long tract degeneration of the cord is most apparent in the corticospinal and dorsal column tracts [86]. This may also be traced on MRI studies of the cord and brainstem [58, 89], and reflected in motor and sensory evoked potential studies [8, 9, 90]. Peripherally, there is also pathological evidence of primary axonal damage [86]. This is supported by neurophysiologic studies [65],

although some reports have described demyelinating changes in peripheral nerves as well [66, 91].

The axonopathy may be related to chronic toxicity from the high level of VLCFAs [4, 92]. The *Abcd1* knockout mouse model [42-44], while a poor model for CER, develops a clinical picture resembling AMN [45]. Evidence from this model indicates that oxidative stress and mitochondrial dysfunction may partake in the pathophysiologic process [93], and that antioxidant therapy might be able to halt the axonal degeneration [94].

The cerebral lesions consist of at least three distinctive zones, with the layer of demyelination apparently being the leading edge, trailed by inflammation and gliosis [87]. One hypothesis is that the inflammatory response may be secondary rather than primary to myelin destruction. Furthermore, given that the basic pathology in X-ALD is believed to be the chronic axonopathy, the question remains whether demyelination in the brain is a primary, separate event, or whether it might be secondary to axonal damage. Microglial apoptosis, possibly due to the inability of microglia lacking ALDP to process VLCFAs, has been proposed as an early stage in the development of cerebral lesions [92]. This may also have bearings on our understanding of the mechanism of effect of HCST; possibly, one mechanism could be providing functioning microglia to the edges of the cerebral lesions [63].

The rapidly evolving lesions of the brain in CER are associated with intense inflammation, also evident as contrast enhancement on MRI [64]. Still, the exact role of inflammation in CER is unclear. Immunosuppressant therapies like steroids, immunoglobulins and cyclophosphamide have been ineffective [95], although trials with modern immunomodulatory drugs are lacking.

In sum, the pathogenesis of CER remains incompletely understood [4]. Future research will hopefully bring more insight into the relative contribution of the different pathological processes, and at which steps of the process therapeutic interventions might be targeted.

1.6 Genetic aspects

Only one gene is found to be directly involved in X-ALD [63], the *ABCD1* gene on chromosome Xq28 [32]. The 20 kb gene consists of ten exons, and encodes the ALD protein (ALDP) consisting of 745 amino acids. ALDP is a member of the ATP-binding cassette transporter superfamily, it is located in the peroxisomal membrane, and is involved in the transport of VLCFA-CoA esters into the peroxisome. Even though the enzymatic apparatus for β-oxidation of VLCFAs is intact in patients with X-ALD, absent or deficient ALDP hinders delivery of VLCFAs for breakdown, and causes accumulation of VLCFAs outside the peroxisome [63]. High levels of VLCFA-CoA in the cytosol may in turn increase levels of the longest VLCFAs by action of the ELOVL1 elongase [96]. As noted above, high levels of VLCFAs are thought to have a toxic effect on neural and other tissues; this is the suspected link between *ABCD1* mutations and pathogenesis in X-ALD.

About 700 unique mutations have so far been reported in the *ABCD1* gene (www.x-ald.nl), 51 % being missense mutations, 28 % frame shift mutations, 12 % nonsense mutations, 6 % amino acid insertions/deletions and 3 % larger deletions of one or more exons [63]. The c.1415delAG mutation is the most common, having been identified in about 10 % of X-ALD kindreds [33] (including one of the 23 Norwegian kindreds with an established mutation [35]). However, a general observation is that each family has its own, "private" mutation; each population studied has its own mix of mutations [35, 97, 98]. This makes mutational analysis in a subject dependent on whether the family mutation has been identified or not.

With such a plethora of unique mutations and lack of one or a few dominating mutations, one should expect a constant supply of new mutations [99]. However, previous studies on X-ALD have found a rather low frequency of *de novo* mutations. Among the 616 American kindreds presented by Bezman [76], only 5 % of male probands had *de novo* mutations. This was later reproduced in a study of 489 American families tested at the Kennedy Krieger Institute [79], where

4.1 % of probands had *de novo* mutations. A Spanish series of 35 kindreds found *de novo* mutations in 5.7 % [98]. The frequency of *de novo* mutations is important particularly in regard to the issue of newborn screening programs [81, 84]: if *de novo* mutations are frequent, extended family screening alone is unlikely to capture all affected subjects. Our Paper 1 presents the occurrence of *de novo* mutations in the Norwegian cohort [35].

In Norway, DNA analysis has been available in since 2001 (Lars Retterstøl, email correspondence, April 27th 2015), and is performed at the Department of Medical Genetics, OUS. Testing of a- or presymptomatic subjects requires genetic counselling, and is normally not allowed for females < 16 years of age. In the absence of a known family mutation, genetic testing starts with Sanger sequencing of the ten exons and exon-intron transitions of the *ABCD1* gene, followed by multiplex ligation-dependent probe amplification (MLPA) analysis (MLPA kit P049, MRC-Holland) to rule out duplications and large deletions. Mutational analysis is of particular importance in ruling out carriership in females, for whom sensitivity of VLCFA measurements in blood is only about 85 % [100, 101].

1.7 Follow-up and treatment of X-ALD

There are several reasons why subjects with X-ALD, regardless of age and gender, may benefit from systematic follow-up by specialists, general practioners and other health care workers (see Table 3). Recommendations in the following section are largely based on those developed as part of the newborn screening program for X-ALD being introduced in New York State, USA [84]. Suggestions for a Norwegian version are presented in the Appendix of this thesis, and could form the basis of a Norwegian or Nordic reference program.

Primary adrenal failure. All X-ALD males are at risk of developing symptomatic PAI during their lifetime. Although this most frequently occurs in childhood (often preceding neurological symptoms, as in the AO phenotype), it may also occur later in life [102]. Onset of PAI may be abrupt, in the form of an Addison crisis [46, 47],

sometimes with fatal outcome [51, 103]. Detection of latent PAI allows timely steroid replacement therapy and prevention of acute crises. Cortisol and adrenocorticotropic hormone (ACTH) levels in blood should be performed every six months in boys, and yearly from 18 years of age. Females have a minimal risk of developing PAI, but caution should be exercised when prescribing NSAIDs [11].

Table 3. Items for follow-up and treatment of X-ALD

- Identification and treatment of PAI, prevention of Addison crises
- Follow-up of testicular function in adolescent and adult males
- Dietary intervention, with or without use of Lorenzo's oil, aiming to prevent CER and possibly modifying the progression of myeloneuropathy
- Timely intervention with HSCT or gene therapy for CER in boys, as well as in adolescent and adult males
- Emerging therapies to slow axonal degeneration in AMN
- Symptomatic treatment for medical problems related to AMN (spasticity, impaired ambulation, neuropathic pain, sphincteric dysfunction)
- End-of-life care for subjects dying from CER
- Psychological and social support for patients with X-ALD and their families
- Genetic counselling and reproductive health care (preimplantation genetic diagnosis, assisted fertilization, prenatal diagnosis)

Testicular function. So far, clear recommendations regarding follow-up and therapy for testicular failure in males with X-ALD are lacking. In clinical practice, we have encountered situations where the question of delayed puberty is raised. Some adult males have experienced infertility due to azoospermia. Males who are about to undergo HCST are offered storage of semen; possibly, assisted fertilization based on previously stored semen could be part of the reproductive care offered to X-ALD males. Some males suffer from impotence; however, this may be multifactorial, and partly due to the myelopathy or small nerve fiber neuropathy [104]. Androgen replacement therapy might be beneficial for some males. At the very least, endocrinologists following X-ALD males need to be

aware that testicular dysfunction may be part of the phenotype [12, 55, 56], and offer monitoring and therapy as appropriate.

Dietary intervention and Lorenzo's oil. The ability of Lorenzo's oil (LO) (a 4:1 mixture of glycerol trioleate (GTO) and glycerol trierucate (GTE)) in combination with a low-fat diet to reduce VLCFA levels in blood is well established [105-108]. However, controversy remains as to whether the reduction or normalization of VLCFA levels in blood is effective in preventing outbreak of demyelination and inflammation in CER, or slowing the progression of axonal degeneration in AMN. In a single-arm, uncontrolled study by Moser [108], 89 asymptomatic boys were treated with LO and fat restriction. Achieving normalization of VLCFA levels was associated with a lower risk of developing cerebral MRI abnormalities. Other studies from Europe have found progression of X-ALD despite effective lowering of VLCFAs using LO and fat restriction [109, 110].

As a result, some authorities recommend or offer LO or other dietary intervention to X-ALD subjects regardless of age and gender [95], some do not [2], while some are uncertain of the efficacy [84, 111]. However, there seems to be a general understanding that elevated VLCFA levels in tissues constitutes a chronic toxic effect on nervous and other tissues [2, 63, 88, 95], and that reduction of VLCFA levels might reduce the damage to these tissues. At the Nordic Workshop in X-ALD in Oslo, November 2014, neurologist Wolfgang Köhler from Germany advocated interventions aimed at normalizing the biochemical disturbance in X-ALD, i.e. the VLCFA accumulation. According to Köhler, this may be achieved without the use of LO, by dietary changes alone (at least in females; in males, LO needs to be included in the diet) (Wolfgang Köhler, personal communication, November 14th 2014).

Controlled studies on the efficacy of LO or dietary modification to prevent CER in boys have been difficult to perform, because of ethical issues and the unwillingness of families to participate. As for the prevention of AMN, the main problem has been the slowly progressive natural course, the lack of useful

biomarkers of progression, and hence the challenges in performing controlled studies with long enough follow-up to be able to detect a clinical meaningful effect of the intervention. An American randomized controlled trial studying the efficacy of LO in adult male and females with AMN [111] was aborted due to problems with the placebo substance [2].
As a personal thought, we note that X-ALD heterozygotes generally have lower VLCFA levels than hemizygotes, and their clinical course (in terms of the age at onset and rate of progression of the myeloneuropathy) is markedly milder than in hemizygotes. Other factors (genetic, metabolic, hormonal or others) may contribute to this milder course in females; however, one may wonder whether this could be nature's own "experiment", demonstrating a beneficial effect of lower VLCFA levels.

Currently, at OUS we recommend LO and dietary modification to X-ALD boys < 12 years of age, if the intervention is deemed acceptable by the patient and the family and may be carried out without serious side effects (low platelet count being most prominent). Discussion is ongoing as to whether dietary modifications should be suggested to older males and to females.

HCST and gene therapy. HCST for CER was first reported by Moser [112] in 1984 in a failed attempt to save a 12 year old boy with symptomatic cerebral disease, inspired by experiences from HCST treatment of patients with lysosomal disorders. Aubourg [113] reported in 1990 the successful reversal of neurological and neuroradiological signs of CER in an eight year old boy, 18 months after treatment. Peters [114] reported the international experience of HCST for CER in 126 boys < 19 years of age from 43 centers during the period 1982-99 (five of these performed prior to 1990). Five-year survival was 92 % in boys with zero or one neurological deficits and Loes score < 9 [60]. Boys with more advanced disease had a worse outcome, reflected in an overall five-year survival in the entire material of 56 %. Shapiro [115] published 5-10 year follow-up of 12 boys aged 5-12, showing prolonged beneficial effect of HCST.

Miller [61] presented in 2011 the results of HCST for CER performed at the University of Minnesota, Minneapolis, USA. Sixty boys aged 4-23 had been transplanted between 2000 and 2009. Transplantation-related mortality at day 100 was 8 %. Five-year survival was 89 % for boys with Loes score < 10, and 60 % for those with Loes score ≥ 10. Absence of clinical cerebral disease (defined as zero points on the Neurologic Function Score, NFS [116], see Table 4) was associated with five-year survival of 91 %. The article suggests Loes score < 10, NFS < 1 and performance IQ ≥ 80 as predictors of favorable outcome after HCST.
Petryk [117] found no evidence that HCST reverses PAI due to X-ALD. van Geel [118] reported that HCST did not prevent development of myelopathy in boys treated for CER in childhood: 3/5 transplanted males showed myelopathy upon examination 15-19 years post-transplantation.

Table 4. The Neurologic Function Score used in evaluation for HCST. Points assigned are accumulative in sum score. Based on Moser et al, Adv Exp Med Biol 2003;544:369-387.

Hearing/auditory processing problems	1
Aphasia/apraxia	1
Loss of communication	3
Vision impairment/fields cut	1
Cortical blindness	2
Swallowing difficulty	2
Tube feeding	2
Running difficulties/hyperreflexia	1
Walking difficulties/spasticity/spastic gait (no assistance)	1
Spastic gait (needs assistance)	2
Wheelchair required	2
No voluntary movement	3
Episodes of urinary or fecal incontinence	1
Total urinary or fecal incontinency	2
Nonfebrile seizures	1
Possible total	**25**

In 2009, Cartier [34] reported the results of autologous HCST using stem cells genetically corrected with a lentiviral vector, in two patients with CER for whom no matching donor could be found. Based on the encouraging preliminary results, this method is currently being investigated. In clinical practice, boys with CER with contrast enhancing lesions and no related donor are now eligible for participation in clinical trials of gene therapy (Gerald Raymond, e-mail correspondence, August 22nd 2014).

Current evidence considers HCST as treatment for CER in children. However, HCST has also been offered to adult patients developing CER at French and German centers [4, 88]. With emerging evidence that the lifetime risk of CER is high in males [35-37], thorough data on efficacy, tolerability and long-term survival of HCST performed in adult males need to be obtained.

The first HCST procedure for CER in Norway was performed in 2011. Three males with Adol-CER (aged 14-18 years) have been transplanted at OUS so far, all of them are alive and without evident progression of cerebral disease 2-3 years post-transplantation (unpublished data). Clinical practice at OUS (see Table 5) is that HCST is offered to boys $\leq$ 21 years who develop MRI evidence of CER, and who fulfill the criteria for favorable outcome identified in the report from Minneapolis [61]. Experts abroad are consulted during decision-making. Boys < 12 years are followed with cerebral MRI every six months; for older males, a follow-up regime remains to be established. According to the protocol from New York [84], cerebral MRI without contrast should be performed every six months from age 36 months to 10 years, thereafter annually.

Table 5. Proposed criteria for HCST for X-ALD (based on Miller et al, Blood 2011;118:1971-1978, and clinical practice at OUS)

- Male aged $\leq$ 21 years with cerebral ALD on MRI
- Contrast enhancing and/or rapidly enlarging lesions
- Loes score < 10
- NFS < 1
- Performance IQ $\geq$ 80

Emerging therapies for AMN. As noted above, VLCFA lowering by way of LO and diet is not proven to ameliorate the course of AMN. Lovastatin was not found to have a significant *in vivo* effect on VLCFA levels, and is not recommend for AMN [119]. Other possible treatment strategies for AMN are reviewed by Berger [120]. Research based on the *Abcd1* knockout mouse, which develops an AMN-like syndrome [45], is underway. Pioglitazone [94] and activation of SIRT1 [121] have slowed axonal degeneration in the mouse model. Systematic follow-up of X-ALD subjects in the Norwegian cohort will allow inclusion in therapeutic trials and expedited dissemination of new treatment options.

Symptomatic treatment. Subjects with AMN may benefit from pharmacological treatment of symptoms related to their myeloneuropathy, like spasticity (baclofen), impaired ambulation (fampridin), neuropathic pain (antiepileptic drugs and tricyclic antidepressants) and sphincteric dysfunction (tolteridin and similar drugs). Physiotherapy and occupational therapy may be important to relieve symptoms and improve mastery of activities of daily living.

Of particular interest is the symptom of fecal incontinence, reported by 28 % of heterozygotes in the Dutch cohort [8] and also reported by some of our patients (unpublished data). This disabling, but previously unheeded problem may require a multidisciplinary approach [122].

End-of-life care and palliation. In the absence of effective newborn screening or other means to capture presymptomatic boys, there will unfortunately still be some boys who present with advanced CER, with such a burden of MRI lesions and neurological deficits that HCST will either be withheld, or will prove ineffective. These patients face the devastating course for which X-ALD has been known, with progressive, unrelenting deterioration, loss of all faculties and eventually death, usually within 2-4 years. Although rare (< 15 recorded cases in Norway during the last 50 years), these situations pose an immense challenge for both the families [123] and for the health care providers.

Psychological and social support. X-ALD is a difficult disease to live with, for both young and old, males and females. Young boys must go through frequent MRI examinations, while their parents each time must brace for the possible "verdict" that CER has started and HCST must be initiated. Even though this regimen is most intense during the childhood years, the recent observations imply that the risk of CER may never disappear. Rather, even into adulthood, male subjects will need continued follow-up in order to detect and treat emerging CER. Furthermore, as the boys get older, the issue arises of when to inform them of the high probability that AMN will appear, and bereave them of ambulation. Given current knowledge, it is unclear how to handle the possibility that head trauma may trigger CER [38]. Should boys be advised to stay away from football and other activities with risk of head trauma? Should they use helmets? The balance between prevention of disease and the need to live ordinary lives may be difficult to strike.

Females diagnosed early in life must now cope with a more somber prospect of the disease than previously envisioned, as recent research [8, 9, 35] indicates that the vast majority will develop some degree of neurological involvement during life. Moreover, while the options offered by reproductive care may help them avoid giving birth to severely affected boys, the women may face some hard choices. For girls diagnosed early in life (currently from age 16 and onwards, but newborn screening may change that), they must face the reality that they are carriers of a severe genetic disorder, making it more difficult to lead an "ordinary" sex life as other young people do. Furthermore, knowledge about the genetic diagnosis may feel like an obstacle in the process of entering relationships and making a family.

One important aspect of X-ALD as a genetic disorder is the tendency, seen in many kindreds, that once the diagnosis is made in one subject, it is subsequently disclosed in many unsuspecting relatives. This is utilized in pedigree screening, in order to provide early diagnosis and timely treatment to patients. However, it also means that family members may be forced to realize, even though they might wish to remain ignorant, that the pedigree points them out as certain or possible carriers

of the mutation. Given what we now know about the age-dependent penetrance of the disease, and the somber prognosis as subjects age, this may come as a shock. Family members may also be torn between the desire for privacy, and the need, or opportunity, for warning relatives that they too may harbor the mutated gene.

Genetic counselling and reproductive options. Given the unusual mode of inheritance (compared to "ordinary" autosomal dominant inheritance) of an X-linked disorder, genetic counselling is obviously of importance for subjects with or at risk of having X-ALD. However, counselling is complex, because of the highly variable spectrum of phenotypes, and the apparent lack of any genotype-phenotype correlation [33, 63]. Furthermore, our understanding of the natural history of X-ALD is changing; for instance, we have just now realized that females are not simply carriers, but face a high risk of neurological symptoms and disability as they get older. Schaller [124] investigated in 2006 the attitudes of American X-ALD families towards prenatal diagnosis, presymptomatic testing, carrier testing and newborn screening. Respondents were largely positive to genetic diagnosis. Still, one must bear in mind that the response rate was only 39 %, and that when this study was performed, the prognosis of X-ALD for males and females was less bleak than it may appear today. On the other hand, future development of effective therapies for CER or AMN may obviously influence the attitude to genetic testing.

The options for prenatal diagnosis [125] and preimplantation genetic diagnosis (PGD) [126] are reviewed by Kemp [63]. In Norway, PGD is legal for severe X-linked disorders, and has been utilized by some X-ALD heterozygotes (carriers). Possibly, non-invasive prenatal testing (NIPT) [127] may become another option for parents harboring a mutation in the *ABCD1* gene, at least for sex determination. Interestingly, the often-used term "carrier" for X-ALD females echoes the era [5] when the main issue for heterozygotes was that of reproduction choices. However, the option of fetal sex determination (by NIPT or PGD) would allow even males with X-ALD to have reproductive choices: selecting a male fetus will guarantee that the child is free of X-ALD; a female fetus will be automatically affected.

2 Aims of the thesis

I. To determine the prevalence and incidence at birth of X-ALD in a well-defined population

- Including all genetically affected subjects, both males and females

- Including all phenotype categories, presymptomatic subjects included

- Using modern diagnostic methods for case ascertainment

II. Investigate the clinical manifestations of X-ALD in a population-based setting

- Challenging the concept of static phenotype categories

- Classifying subjects according to age

- With special emphasis on female phenotypes, previously overlooked

3 Methodological considerations

This has been a project in the field of clinical epidemiology. We have looked at X-ALD from the point of view of the clinical neurologist: How many patients are there, how is the spectrum of their phenotypes, are they misdiagnosed as other disorders? However, methods such as biochemistry, genetics and neurophysiology have also been used. This section discusses the main methodological issues involved in this thesis.

3.1 Study design

This project is based on the cross-sectional study of Norwegian subjects with X-ALD (Paper 1). The other studies are observational studies as well, using subjects with X-ALD (Paper 2), non-autoimmune adrenal failure (Paper 3) and primary progressive multiple sclerosis (PPMS) (Paper 4) as the study population. Paper 5 is a case report. We have attempted to report our findings in accordance with the STROBE statement [128].

3.2 Clinical examination, case ascertainment and phenotype assignment

We established a database of Norwegian subjects with ascertained X-ALD, classified according to established phenotype categories. With a few exceptions (subjects unavailable for clinical consultation), all live X-ALD subjects in this project underwent ordinary neurological examination by the project leader (Morten Horn).

Case ascertainment. Subjects encountered during the inclusion process were offered a short talk with the project leader, with basic questions to disclose symptoms of myeloneuropathy, Addisonism and/or encephalopathy. Subjects with symptoms suggesting X-ALD were offered routine clinical work-up to establish the diagnosis. Those who seemed to be asymptomatic were offered referral for genetic counseling and mutational analysis of the family mutation.

The diagnostic criteria for X-ALD used in this thesis are listed in Table 6. Importantly; X-ALD is used in this thesis as the genetic condition, regardless of gender, age or clinical symptoms. In contrast, several previous studies have included only symptomatic subjects, or only males (see Table 2, page 24).

Table 6. Diagnostic criteria for X-ALD used in this thesis (one is sufficient for diagnosis)

- Disease-causing mutation in the *ABCD1* gene, regardless of gender, age or symptoms
- Clinical picture consistent with X-ALD (one of its forms), typical elevation of VLCFAs in blood or other specimen, regardless of age or gender
- Clinical picture typical of X-ALD in the absence of laboratory confirmation, with position in the pedigree allowing X-ALD as diagnosis (used for deceased subjects)
- Obligatory carrier by way of pedigree (having both parents and offspring with ascertained X-ALD), regardless of symptoms and in the absence of laboratory confirmation (used for deceased subjects)

Phenotype assignment. We utilized the phenotype categories drawn up by Hugo Moser [1, 7, 69], used also by the Dutch group [2, 33]. This classification (see Table 1, page 17) groups male X-ALD subjects according to the presence and age at onset of cerebral demyelination (CCER, Adol-CER and Adult-CER), presence of adrenomyeloneuropathy (AMN), presence of Addisonism in a neurologically intact subject (AO), and the completely asymptomatic state (ASYMP) found in some subjects diagnosed by pedigree screening. In this thesis, CER is used to designate cerebral demyelination, otherwise often labelled “cerebral ALD”.

This classification of phenotypes is challenging [4], as a subject may move from one phenotype category to another with rising age and progression of the disease. The ASYMP and AO categories are believed to be transitional [69]. Recent research [37, 109] has shown that in males, the AMN phenotype will frequently evolve to a state of cerebral demyelination (AMN-CER). The cerebral phenotype of young boys may, if successfully treated with HSCT, evolve into AMN as the boy gets older [118]. How individual subjects are allocated to the different phenotype categories is therefore age-dependent. Hence, the age composition of the studied cohort becomes an important factor.

Female phenotypes. For females, the categories established by Moser are simply asymptomatic or symptomatic, the latter referring to a slowly progressive myeloneuropathy without clinical addisonism, resembling the AMN phenotype seen in males. Females in X-ALD kindreds are often labelled as "carriers" [84, 90, 100], that is carriers of a mutation that may cause disease in male offspring. However, it is increasingly realized [5] that heterozygotes may themselves develop neurological symptoms, while clinically significant endocrinologic disturbances are rare [11]. The label "carrier", therefore, seems inappropriate at least for the symptomatic heterozygotes; these females are patients in their own right.

At the start of the inclusion process, it soon became apparent that some heterozygotes were subjectively asymptomatic (even when asked specifically about typical symptoms), but showed upon clinical examination clear-cut evidence of an emerging myeloneuropathy – such as hyperreflexia and loss of vibration sensation in the lower extremities, and positive Babinski signs. These females were able to walk and run without any difficulty, and classifying them as "symptomatic" or as having AMN seemed misleading. Therefore, a phenotype category was added to the structure set up by Moser [7]: The SIGNS category, used for subjects with no subjective symptoms, in whom neurological signs of myeloneuropathy were found (see Table 7).

Allocation to the SIGNS category relied, by definition, upon neurological examination. Hence, if a female who had not been seen by a neurologist reported being healthy, it was impossible to assign her to either the ASYMP or the SIGNS category. However, she decidedly did not have AMN, in this classification system. This became an issue in Paper 1, where four (15 %) of the included females were unavailable for clinical examination (being symptom-free they might be either ASYMP or SIGNS, but they had probably not AMN).

The system of classification of females is important when comparing our results to other studies on female phenotypes in X-ALD. For instance, in a paper by Moser [7], 16 % of heterozygotes were reported to have "contacted their physicians

because of neurological symptoms". On the other hand, as many as 53 % of 60 female heterozygotes who underwent neurological examination by Dr. Sakkubai Naidu had "neurological signs or symptoms", ranging from mild hyperreflexia and vibratory sense impairment (as in our SIGNS category) to being wheelchair-bound (as in our AMN category).

Table 7. Revised phenotype classification system used in this thesis. Modified from Horn et al, Pediatr Neurol 2013;48:212-219. See list of abbreviations (page 10) for clarification.

Phenotype	Description	Onset *
Hemizygotes		
CCER	Progressive behavioral and cognitive neurological deficits, inflammatory brain demyelination. Total disability often within 3 years.	<10 years of age
Adol-CER	Like childhood cerebral, but onset in adolescence	10 to < 21 years of age
Adult-CER	Rapid inflammatory cerebral demyelination resembling the childhood form, without preceding AMN	21 years of age or older
AMN	Paraparesis progressive over decades, distal axonopathy, inflammation mild or absent, mainly spinal cord involvement	At 28±9 years
AMN-CER	Onset like AMN, subsequent development of cerebral demyelination resembling the childhood form	Any
ADD	Primary adrenocortical failure without neurological abnormalities	Typically boys
SIGNS	Without subjective symptoms, but neurological signs of long tract involvement evident on examination	?
ASYMP	Harboring the genetic condition of X-ALD without neurological or endocrinological abnormalities	Usually seen in boys
Heterozygotes		
ASYMP	Like in males, but usually without endocrinological abnormalities	At 37±15 years
SIGNS	Without subjective symptoms, but neurological signs of long tract involvement evident on examination	Any
AMN	Harboring the genetic condition of X-ALD without neurological or endocrinological abnormalities	Any

* For the cerebral phenotypes in males (CCER, Adol-CER and Adult-CER), Onset is used to assign subjects to the different categories. For the other phenotypes, Onset denotes the age at which this phenotype is typically seen.

In the study of Australasian X-ALD families, Kirk [74] found that only 7 % of 118 heterozygotes had "neurological symptoms which could be attributed to ALD" (these were probably subjects with AMN). This classification was based not on systematic neurological examination of all females, but rather on the information provided by referring physicians. When comparing our results with those of others, one should consider whether the classification as being symptomatic is comprehensive [9] (all females with any sign of neurological involvement are classified as "symptomatic") or restrictive [74] (only females with subjective symptoms and/or disability due to myeloneuropathy are classified as "symptomatic").

Laboratory methods in phenotype assignment. Phenotype assignment relied on clinical, not neurophysiologic, findings. Previously, Restuccia [90] found abnormal motor (MEP) and/or sensory (SEP) evoked potentials in 7/14 clinically asymptomatic X-ALD heterozygotes. The same phenomenon was seen in our own cohort [104]. If we had used neurophysiological studies for classification, the SIGNS category might have expanded, possibly eliminating the truly "neurologically intact" (i.e. the ASYMP) category.

Similarly, MRI was not used routinely in phenotype assignment. Among boys with X-ALD, cerebral MRI is performed at regular intervals. If cerebral demyelination is detected on MRI, the boy is moved from the ASYMP or AO phenotype to the CCER category (or Adol-CER or Adult-CER, depending on the age at conversion). In principle, the same conversion may occur in an adult male with AMN, in whom MRI demonstrates emerging cerebral demyelination, as yet in the absence of clinical symptoms thereof. In theory, updated MRI studies in all included male subjects might in some have revealed imminent, but as yet clinically undetectable, cerebral demyelination. In practice, however, this period of presymptomatic MRI changes in the brain is usually short. Therefore, MRI was used for detection of CER only in boys who were followed with frequent MRIs as part of clinical routine.

Among females, MRI studies have been difficult to utilize in the evaluation of cerebral involvement. Fatemi [129] found that among 76 heterozygotes (89 % of whom had "various degrees of neurologic abnormalities"), 11 had abnormal cerebral MRI. However, in eight of these, the MRI changes were attributed to causes other than X-ALD. In that study, X-ALD heterozygotes had on MR spectroscopy reduced N-acetylaspartate/choline (NAA/Cho) ratios compared to healthy controls, but with no clear correlation to clinical severity. Kumar [58] found diffuse medullar atrophy on MRI of the thoracic spine in 8/10 heterozygotes. As yet, there is insufficient evidence that MRI is useful in the subclassification of X-ALD heterozygotes.

Disability scoring. All subjects were scored according to the Adult Adrenoleukodystrophy Disability Severity scale (AADS) [130]. This is a 24-point scale based on motor (0-6 points), sensory (0-3 points), bladder (0-3 points) and cerebral symptoms (0-12 points). Zero points indicates a completely healthy subject, 24 points indicates a subject that is bedridden due to paralysis, has lost all sensation below the head, has no control of bladder function and is demented with loss of all intellectual functions, requiring constant assistance. The scale was developed in 1999 by Köhler and Sokolowski for use in X-ALD studies. It has, however, been used sparingly afterwards, and based on feedback from the referees we decided not to include it in the published Paper 1.

In Paper 2, we scored neurological function using the Expanded Disability Status Scale (EDSS) [131]. Although originally constructed for use in MS and not ideal for X-ALD, the EDSS scale has been used in other studies on X-ALD [8, 129], to provide some indication as to the severity of disease in the included subjects. A Brazilian study [9] used the Japanese Orthopedic Association (JOA) scale [132] and a newly composed Severity Score System for Progressive Myelopathy (SSPROM) [133] for determining neurological function in women with AMN. However, the JOA scale is designed for patients with myelopathy due to cervical vertebrogenic cord compression, and therefore not ideal for degenerative cord

disease like in AMN. The SSPROM scale has so far not been used in X-ALD studies outside Brazil, which limits the ability to compare results between studies. In Paper 5, we made use of the Spastic Paraplegia Rating Scale (SPRS) [134], which in our opinion is more suitable for scoring subjects with non-compressive myelopathy.

3.3 Epidemiological analyses

The main goal of this project was to establish the frequency with which X-ALD occurred in the Norwegian population (the source population). Several methodological considerations were relevant to reach this goal.

Inclusion criteria and study parameters. The occurrence of a genetic disorder in a population may be described by the frequency of the mutation, by prevalence, by incidence of new cases diagnosed in a period of time, or by incidence of new subjects born with the disorder. For X-linked disorders one may look at the occurrence of male subjects only (usually more severely affected) or both genders. Since subjects with X-ALD are asymptomatic at birth, and only at some later stage develop neurological and endocrine involvement, one must decide whether to include all subjects with the disease-causing mutation, or only those who manifest clinical symptoms.

Prior to this project, there was clear evidence [5, 7] that at least some females develop neurological symptoms due to X-ALD. In theory, any female with a disease-causing mutation might end up developing symptomatic X-ALD. Regardless of subjective symptoms, all females might need professional care and genetic counselling in relation to reproduction. Therefore, both female and male subjects were included in this study. This is an important methodological difference between our Paper 1 and several of the previous studies [30, 72, 75, 77, 78], which look at males only (see Table 2, page 24). The most widely cited study, from the USA [76], used extrapolation of the results from the male population to estimate the frequency among females. Only the German [73] and Australasian [74]

studies systematically included female subjects, though in the German study the number of included females was only 27 % of the total X-ALD population. The Australian study had no gender bias (104 males vs. 118 females); we aimed to achieve the same gender balance in our study.

A small number of asymptomatic males are typically included in surveys of X-ALD, whereas asymptomatic females have typically been overlooked [5]. Although symptomatic subjects are more easily captured in a survey, restricting the study to symptomatic subjects only seemed inappropriate. It is common knowledge that for some X-ALD subjects, the asymptomatic state is purely transitional, more correctly termed presymptomatic. Furthermore, even presymptomatic subjects may need extensive health care services. Therefore, all genetically affected subjects were included in our analysis, regardless of clinical symptoms. This more comprehensive criterion must be kept in mind when comparing our findings to other studies, although the effect is largest in terms of inclusion of females.

As seen from the diagnostic criteria for X-ALD used in this thesis (Table 6, page 42), mutational analysis was necessary for the diagnosis in the absence of clinical evidence of X-ALD or obligatory carriership based on pedigree information. Heterozygous X-ALD girls < 16 years of age are almost universally asymptomatic, and VLCFA analysis cannot reliably exclude carriership [8, 100]. According to Norwegian law, genetic analyses cannot be performed in asymptomatic subjects in whom the diagnostic process has no therapeutic consequences. Thus, we omitted inclusion of female subjects < 16 years, thereby introducing a systematic error perhaps leading to underestimation of the female patient population. However, these subjects were included in the *at risk* population (defined as first degree relatives of an ascertained X-ALD subjects having a risk (in most cases 50 %) of having inherited the gene), which we tried to take into consideration in Paper 1.

Incidence calculations. The method of incidence[1] calculations used requires special attention. Two principally different methods have been used in the X-ALD literature (see Table 2, page 24). Method 1, used in the widely cited American [76] and most other population surveys [74, 75, 77, 78] compares [number of new identified cases] per [number of live births] during a given time period. Method 2, used in the French [72] and German [73] surveys, compared [number of subjects found to have X-ALD] per [number of live births in the period these subjects were born].

We saw Method 1 as problematic for three reasons (see section 5.1.2 where the Australasian study [74] is used to exemplify this).

1. The mode of onset of X-ALD is heterogeneous. It may present acutely, with dramatic cerebral symptoms as in CCER, or it may develop insidiously, with slowly progressive myeloneuropathy as in AMN. The onset of CCER – and hence its incidence in the population – may be clearly defined. It is far more difficult to determine when AMN actually manifested in a male or female subject.
2. X-ALD is often diagnosed in a subject not because of clinical symptoms in that subject, but rather because the diagnosis has been made in a relative. As a result, a diagnosis of X-ALD (for instance spurred by an Addison crisis, or a case of CCER) is typically followed by a rush of new diagnoses in family members: both a- or presymptomatic subjects, and subjects in whom it is now revealed that long-standing symptoms are actually due to X-ALD. Therefore, [new diagnoses per time period] will not necessarily equal [new manifesting subjects per time period]; rather, it reflects [new recognized cases per time period].
3. The medical understanding and recognition of X-ALD has evolved, particularly during the last half of the 20th century. AMN has been recognized as a phenotype of X-ALD [6], perhaps the most common form of the disorder [30]). Technical

[1] The term incidence is frequently used in the X-ALD literature, although with various meanings. The cumulative incidence is the number of new cases within a specified time period divided by the size of the population initially at risk. The incidence rate is the number of new cases per population at risk in a given time period. The most common usage in the X-ALD literature appears to be cumulative incidence, with all newborns as the population at risk.

improvements in the biochemical and genetic diagnosis and a greater awareness of the disease have led to a large number of patients being diagnosed. The [number of new identified cases] from this era may have been artificially high: it may have included cases with long-standing AMN that were finally diagnosed, whereas a rush of presymptomatic subjects may have been diagnosed through systematic pedigree screening, often in the setting of concerted research efforts.

Method 2 calculates how many subjects with X-ALD that are born during a certain time period, compared to the birth numbers of the same period. This can most precisely be done prospectively by way of newborn screening [81], if practically feasible and with acceptable sensitivity [83]. However, this method is only now being implemented in some American states [84], and was not available to us (see section 1.4).
The other option, chosen by us, was to study incidence at birth retrospectively as done in the French [72] and German studies [73]. The validity of this approach hinges on the ability to capture all subjects born with X-ALD, retrospectively. A detailed criticism of this method, and comparison with Method 1, is provided in section 5.1.2.

Prevalence calculations. The point prevalence[2] of X-ALD is not necessarily a good measure of its impact on the families and on the health care services: the most severe cases, boys with devastating CCER, usually die rapidly and contribute little to the prevalence, but still they pose the greatest challenge from X-ALD in terms of human suffering and resource needs. However, for determining the size of the X-ALD population in a country, particularly presymptomatic or undiagnosed females with AMN, prevalence is more informative than incidence. For this kind of research, the Norwegian social security number system provides an excellent way of keeping track of subjects, to ensure that they are alive on the prevalence day. The source population was the inhabitants of Norway on July 1st 2011, based on figures from the Central Bureau of Statistics (www.ssb.no).

[2] Point prevalence defined as the proportion of a population having a condition at a specific point in time.

3.4 Inclusion strategies

The key to determining prevalence and incidence of X-ALD in Norway was to capture as many subjects with X-ALD as possible. We used a multi-staged inclusion strategy: 1. Identifying probands. 2. Exploring kindreds extensively. 3. Screening patient groups with diagnoses that might mimic or hide subjects with X-ALD, to capture subjects missed in the two first steps (see Figure 3).

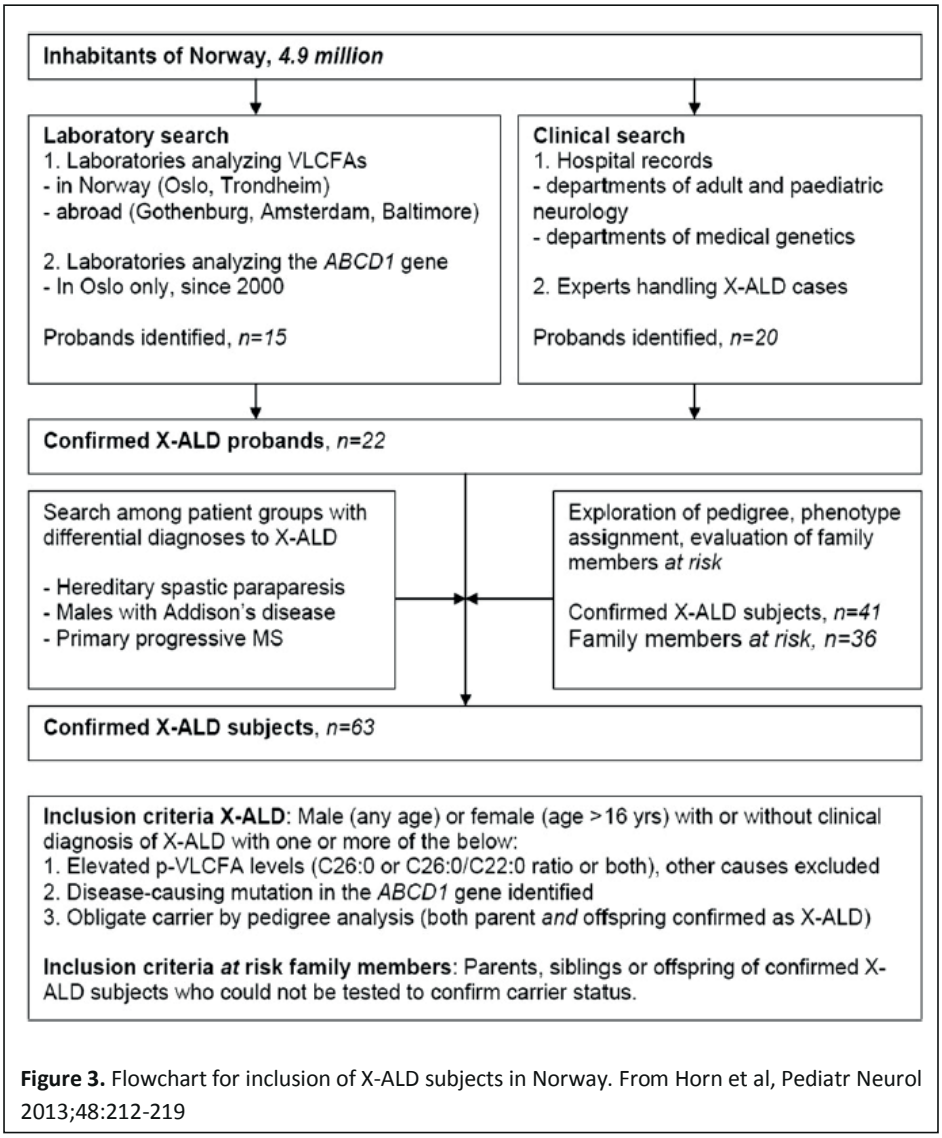


Figure 3. Flowchart for inclusion of X-ALD subjects in Norway. From Horn et al, Pediatr Neurol 2013;48:212-219

Proband identification. Stage 1 started with including patients with X-ALD already known to us, namely boys with AO or CCER followed at the Department of Neuropediatrics OUS, and adults with AMN followed by professor Chantal Tallaksen at the Department of Neurology, OUS. This patient material was supplemented by searching the records of the laboratories performing diagnostic VLCFA analyses. In Norway, this has mainly been done at the Section for Inborn Errors of Metabolism, Department of Medical Biochemistry, OUS-RH (contact person: Berit Woldseth). However, some samples were also analyzed at the laboratory of the Department of Medical Biochemistry, St. Olav University Hospital, Trondheim (contact person: Kristian Bjerve). Furthermore, we knew that some physicians sent samples abroad, to the Laboratory for Neurochemistry at the Sahlgrenska University Hospital, Gothenburg, Sweden (contact person: Jan-Eric Månsson), to the Laboratory for Genetic Metabolic Diseases, Department of Clinical Chemistry, Academic Medical Centre, Amsterdam, the Netherlands (contact person: Marinus Duran) and to the Genetics Laboratory, Kennedy Krieger Institute, Baltimore, USA (contact person: Richard Jones). With the aid of our contacts, these records were searched for Norwegian X-ALD subjects (this search actually yielded one pedigree, otherwise lost to us, from each laboratory).

We sent letters to the heads of the departments of neurology, pediatrics, and medical genetics at all relevant hospitals in Norway (including the Frambu center for rare diseases), inquiring whether they had any patients with X-ALD. In two hospitals (OUS and St. Olav University Hospital in Trondheim), we searched the patient administrative systems for patients with the ICD-9 diagnoses 330, 330.0 and the ICD-10 diagnosis E71.3 for the time period 1988-2010, reviewing the patients' records looking for information indicating X-ALD.

Finally, we contacted directly known experts (included retired ones) in the field of pediatric and adult neurology and medical genetics, asking them if they could remember patients with X-ALD from long ago (this method actually produced two

kindreds otherwise missed by us). We also established contact with the person in charge of the peer support website http://ald-minnefond.com/index.html.

Pedigree exploration. At Stage 2, probands consenting to inclusion in the study provided information regarding their pedigree. The probands were asked to consider inviting those of their relatives who, based on the pedigree, were possible carriers of the family mutation. The probands themselves decided whether to inform family members of the study.

Family members interested in participation contacted the project leader. Subjects who reported symptoms suggestive of X-ALD were offered neurological consultation and investigation for X-ALD. Asymptomatic subjects were offered referral to routine genetic counseling and molecular diagnostics. If new affected subjects were identified in this way, the pedigree screening was extended accordingly.

The pedigree was explored as extensively as possible, based on the willingness of probands and family members to participate. If the pedigree exploration left "loose ends", subjects who might be genetically affected, but who could not be diagnostically clarified (due to death, unwillingness to participate, age below 16 years (for girls) or other causes), we sought to obtain information that might hint about the likelihood of that subject being affected, and the size of that branch of the pedigree.

Family members who had a 50 % risk of carrying the family mutation (that is, sons and daughters of an affected female subject) but who could not be ascertained were considered to be *at risk* of having the mutation. Family members further out in the pedigree were also at risk of having the mutation, however, typically with a diminished likelihood. For instance, the daughter of a daughter of a female with verified X-ALD would have a statistical 25 % risk of harboring the family mutation.

Daughters of male patients were assumed to have a 100 % risk of inheriting the father's mutation; however, due to the possibility of alternative paternity, these daughters were not considered to be carriers unless this was ascertained by either 1) mutational analysis (if VLCFA elevation did not establish the diagnosis) or 2) having affected offspring. For the same reason, fathers of affected females were not considered to be affected unless proven so by way of VLCFA analysis or clinical information indicating X-ALD. Sons of male subjects were considered to have a 0 % risk of inheriting the mutation. Due to the possibility of *de novo* mutations [79], mothers of affected subjects were not classified as obligatory carriers unless this was ascertained by VLCFA and/or mutational analysis.

Screening differential diagnoses for X-ALD. At Stage 3, we screened relevant differential diagnoses looking for misdiagnosed subjects with X-ALD. It is well known that the diagnosis of X-ALD may be delayed despite typical neurological symptoms [135]. We were most concerned with diagnoses producing a slowly progressive myelopathy in adults, like hereditary spastic paraplegia (HSP) and PPMS. Furthermore, since it was unknown whether the AO phenotype might persist into adulthood (with the slowly progressive myelopathy not emerging or going unrecognized), we decided to screen the population of adult males with Addison's disease.

A complete survey of all Norwegian patients with these differential diagnoses was not possible. However, we aimed to at least test the magnitude of the problem of missed subjects due to mis- or delayed diagnosis of X-ALD, by screening populations in representative samples from Norway. To our knowledge, such efforts have not been made in relation to previous population surveys of X-ALD.

a. Prior to this project, Chantal Tallaksen's group made a survey of all identified subjects with HSP in South East Norway. As part of the project, subjects with other causes of spastic paraplegia were excluded, with testing that included VLCFA analysis [136]. This process yielded two patients with previously

unrecognized X-ALD (notably, the problem of incomplete sensitivity of VLCFA assays for female subjects, discussed in section 3.5 below, applies to this study).

b. In cooperation with Martina Moter Erichsen and her colleagues, we screened adult male patients with non-autoimmune Addison's disease included in the National Addison's Disease registry (abbreviated here as NADR), located at the Registry of Organ-specific Autoimmune Diseases (ROAS) at Haukeland University Hospital, Bergen, Norway (see section 3.4.1 below).

c. In cooperation with Elisabeth Gulowsen Celius, we recruited patients with PPMS from the Oslo MS Registry (abbreviated here as OMSR), to be screened for X-ALD by VLCFA analysis. The registry comprises all identified and consenting MS patients located in Oslo, Norway (see section 3.4.2 below).

Registries such as these are good sources of verified and well-described patient materials. However, the quality of a registry study depends on whether the included patients are representative of the "real" patient population. Two important factors determine this: the degree to which the registry has achieved coverage of all eligible patients, and the narrowness of the eligibility criteria. If a substantial portion of patients are missed by the registry, or if strict inclusion criteria fence out patients, the registry may not adequately mirror the patient population.

3.4.1 Screening adult males with non-autoimmune Addison's disease

In Paper 3 (see Figure 4 for a flowchart), we utilized the NADR as a source of adult male subjects with PAI. The NADR has been described elsewhere [137]. It consists of > 600 patients with PAI, and was to our knowledge the largest and most complete nation-based collection of PAI patients worldwide. During construction of the registry, all known cases of PAI in Norway were identified. However, about 25 % of eligible patients did not consent to registration. Although the registry focuses on autoimmune causes of PAI, the actual testing to detect autoimmunity is done as part of registry inclusion. Hence, even subjects with non-autoimmune PAI are referred to the registry. Still, we cannot rule out that

endocrinologists may have abstained from referring PAI patients who already had an established non-autoimmune cause for PAI.

We excluded females with PAI from the study, because previous research has shown that clinically significant adrenal insufficiency is uncommon among females with X-ALD, seen in only 1.4 % [11].

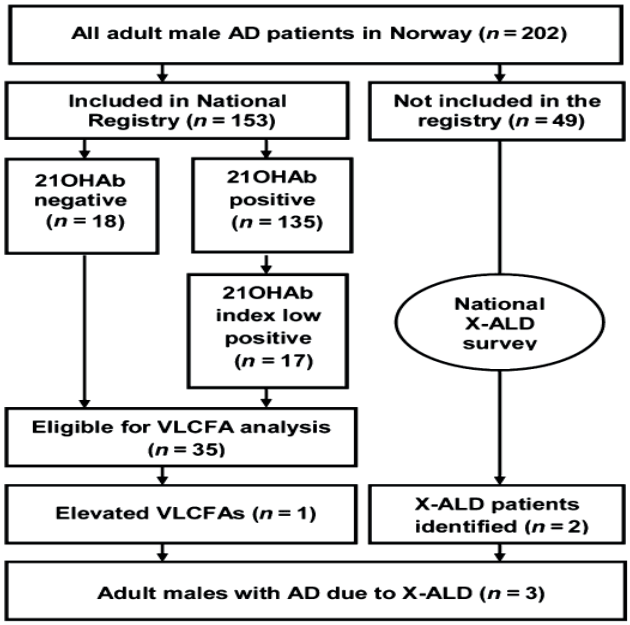


Fig. 1 Flow chart showing patient inclusion and results. For details refer to text.
AD, Addison's disease; 21OHAb, 21-hydroxylase autoantibodies; X-ALD, X-linked adrenoleukodystrophy; VLCFAs, very long-chain fatty acids.

Figure 4. Flowchart for inclusion of subjects in Paper 3. From Horn et al, Clin Endocrinol 2013;79:316-320

Among males > 18 years of age in the NADR we selected subjects with negative 21OHAb indices (< 48, arbitrary unit, cut-off based on the mean results for 50 healthy blood donors + 3 standard deviations) for our study. We also included subjects with weakly positive 21OHAb indices (arbitrarily defined as 48-200, since subjects with autoimmune PAI typically have indices of 200-1000 with this methodology).

Previous research has not found 21OHAb among male X-ALD patients with PAI as part of their phenotype [138, 139], and pathological 21OHAb levels are uncommon as an incidental finding [140]. Furthermore, pathological studies of the adrenal glands of X-ALD patients have found little evidence of inflammation [86]. We therefore assumed that presence of 21OHAb in high levels made autoimmune cause of PAI highly likely, and X-ALD highly unlikely. Those with weakly positive 21OHAb indices were included in case they had been wrongly classified as autoimmune.

At inclusion in NADR, all subjects had given blood for research purposes, which was stored at the registry. Frozen serum blood samples were retrieved and shipped to the Laboratory of Neurochemistry at Sahlgrenska University Hospital, Gothenburg (contact person: Jan-Eric Månsson). Samples were analyzed for VLCFA levels using clinical routine measurements [141]. Cut-off for pathological VLCFA levels was set according to the normal values for the laboratory.

To supplement our search of non-autoimmune PAI patients recruited through the NADR, we reviewed the adult male X-ALD patients identified through our own survey (Paper 1). The purpose was to identify adult male patients with PAI due to X-ALD, who might have been missed by the NADR.

3.4.2 Screening patients with primary progressive multiple sclerosis

In Paper 4 (see Figure 5 for a flowchart), we made use of the geographically, demographically and clinically well-defined MS population of the OMSR, described in previous publications [142]. This registry was established at OUS-Ullevål hospital in 1992 by Professor Bodvar Vandvik, and maintained and updated by Drs. Elisabeth Gulowsen Celius and Cathrine Smestad. It aims at including all MS patients living in the county of Oslo, capital of Norway (2010 population: 587,000, www.ssb.no). As part of registry maintenance, the MS doctors have contacted neurologists in private practice and at adjoining hospitals, in order to achieve maximum capture of MS patients. At entry, registered patients are scrutinized and classified according to MS phenotype. The OSMR has no

established research biobank containing serum samples; therefore, participating subjects had to deliver blood samples solely for the purpose of this study. This may have presented an obstacle for the patients' willingness to participate in this particular study.

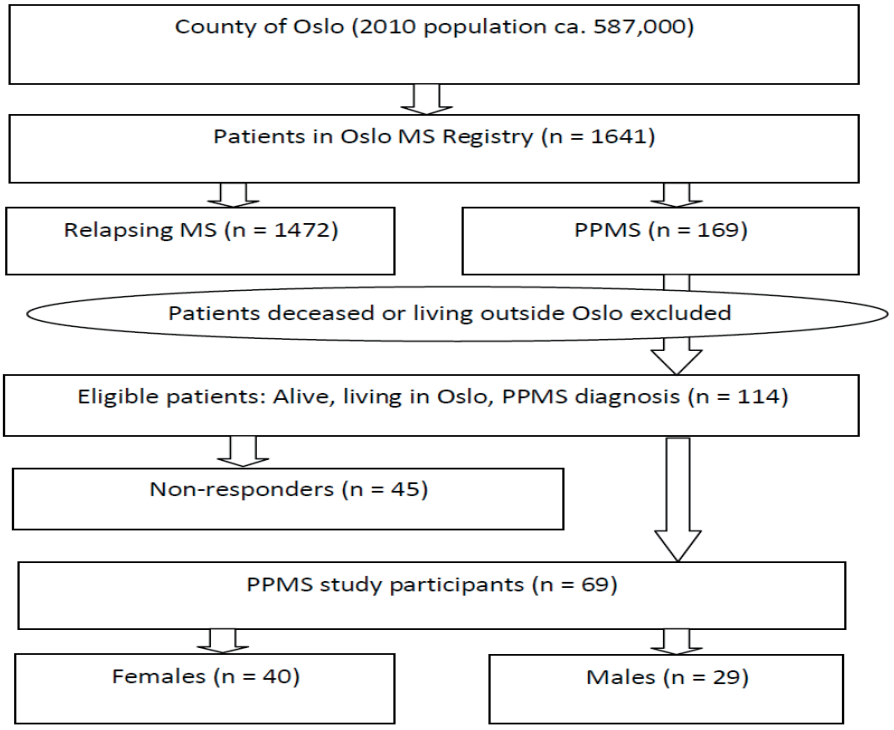


Figure 5. Flowchart for inclusion of patients in Paper 4. Relapsing MS includes both relapsing-remitting and secondary progressive forms.

Our source population was patients with PPMS living within Oslo in 2010. PPMS patients were chosen, as the slowly progressive and usually non-relapsing course seen in PPMS may closely resemble the equally slowly progressive AMN phenotype, dominated by myelopathy with spastic gait and sphincter disturbances. Indeed, several reports have described X-ALD patients initially misdiagnosed with MS [135, 143-146].

One other study, from Wales [147], had tested MS patients systematically for X-ALD by way of VLCFA analysis. This study recruited MS patients from the

University Hospital of Wales and outpatient clinics across South East Wales. MS patients were included if they either 1) had a first degree relative with MS or 2) had the primary progressive form.

The authors thought that the former inclusion criterion might increase the likelihood of capturing misdiagnosed X-ALD patients, since they will typically have affected family members. However, while subjects with X-ALD will often have an affected relative, the likelihood of this relative also being misdiagnosed as MS seems small. On the contrary – X-ALD in males is normally rather easily diagnosed if PAI is present, or if the severe, usually fatal CER of childhood occurs in the kindred. Actually, this criterion – having a first degree relative with an apparent MS diagnosis – might reduce, rather than increase, the possibility that the case at hand is really a misdiagnosed X-ALD patient. For this reason, family history was not used as an inclusion criterion in our study (it was, however, one of the parameters extracted from the OMSR database for each patient).

The decision to include both males and females was based on observations that females in X-ALD pedigrees frequently develop AMN-like (and thus PPMS-like) neurological symptoms. However, this created a problem due to the unsatisfactory sensitivity of VLCFA analyses in females (see section 3.5 below). Adding fibroblast studies or extensive DNA analysis would have improved sensitivity, however, for practical and economic reasons, these techniques were not feasible in the context of this study. By relying solely on VLCFA analysis in blood, we were unable to entirely rule out X-ALD among female participants. On the other hand, we might expect to capture at least 2 out of 3 of any females with X-ALD misdiagnosed as PPMS in Oslo.

3.5 Biochemical analyses

VLCFA studies in males. The workhorse of X-ALD diagnostics remains analysis of VLCFA in blood, using gas chromatography-mass spectroscopy or related technology [22, 82, 83, 148]. In a major paper from 2001 [101], Ann Moser and

coworkers from the Kennedy Krieger Institute in Baltimore presented the results from VLCFA testing in 3000 peroxisome disease patients and 29,000 controls. Their findings indicate that almost all males with X-ALD have abnormally elevated plasma levels of VLCFA, when the results of C26:0 absolute levels and the ratios C26:0/C22:0 and C24:0/C22:0 are considered together. There may in some instances be overlap between findings in controls and in X-ALD males, a theoretical possibility for either false positives or false negatives. However, this overlap may be eliminated by using a "discriminant function", which incorporates the absolute figures (C26:0 levels, C26:0/C22:0 and C24:0/C22:0 ratios) into a formula that enhances separation between normal and pathological findings. At present, the Kennedy Krieger Institute is not using the discriminant function in the analyses of male subjects investigated for possible X-ALD (Richard Jones, e-mail correspondence, April 22nd 2015).

Lorenzo's oil [107, 111, 149] is effective in lowering VLCFA levels in the blood of X-ALD subjects, and a diet rich in erucic acid (from rapeseed and mustard oil, in particular) may lower VLCFA in a similar fashion. In one case described by Ann Moser [101], a 10 year old Indian boy had normal VLCFA levels in plasma (abnormal in skin fibroblasts), attributed to a very high dietary intake of mustard oil. Some very few other examples of hemizygotes with false negative plasma VLCFA samples have been reported [150, 151]. However, the general assumption is that all male subjects with X-ALD will have abnormal VLCFA levels, seen even on the day of birth and unchanged as the subject gets older [101].

There are, however, examples of technical errors leading to false negative or ambiguous results, in particular if the laboratory has little experience with VLCFA analysis. Indeed, during our search for X-ALD patients in Norway, we came across one male subject in whom X-ALD had been ruled out due to VLCFA analysis several at another hospital, interpreted as "negative". However, the clinical symptoms were typical for X-ALD, and retrieval of frozen fibroblast cultures disclosed a disease-causing mutation in the *ABCD1* gene, confirming the

diagnosis. We cannot exclude that there have been other false negative results, although none have so far been observed for males analyzed at OUS.
The hereditary nature of X-ALD would allow kindreds a "second chance" to have X-ALD correctly diagnosed, as other affected family members appear. This would not help finding single-member families with *de novo* mutations; however, as discussed in section 5.3, our material is actually rich in such kindreds compared to other surveys. We can see no particular reason to suspect that the diagnostic sensitivity of VLCFA analyses has been significantly worse at OUS compared to other laboratories.

VLCFA studies in females. A small proportion of females heterozygous for an *ABCD1* mutation have normal VLCFA levels in blood. This was first reported by Moser in 1983 [100], and has subsequently been reproduced [101]. Generally, about 15 % of heterozygotes are thought to be missed by VLCFA analysis of blood, although the sensitivity may be increased to 93 % if VLCFA measurements in cultured skin fibroblasts are performed. Interestingly, in the latest report from the Netherlands [8], as many as 31 % of genetically ascertained heterozygotes had normal plasma C26:0 levels. This underscores that normal levels of VLCFA in blood cannot rule out carriership in female subjects. Application of the discriminant function, as shown in studies from the Kennedy Krieger Institute [100, 101], improved the sensitivity of VLCFA analysis in blood, but could not eliminate the problem of false negatives among females. Adding fibroblast studies increased sensitivity to 95 % in the Dutch study [8]. However, such studies are time-consuming and require harvesting a skin biopsy, and still there remains a possibility of missing the diagnosis. Therefore, while VLCFA analysis has a good chance (at least 71 %) of capturing X-ALD heterozygotes, the method cannot be used to exclude the heterozygous state.

In the Norwegian population, 31 X-ALD heterozygotes have so far been identified (unpublished data), 24 of whom have been analyzed for VLCFA levels at different laboratories. Two females (8 %) of those tested had false negative VLCFA levels.

Among the nine heterozygotes tested at OUS, using two different methods, one (11 %) had false negative results. The other false negative sample, analyzed at the laboratory at Sahlgrenska University Hospital, was from a female with genetic mosaicism in leukocytes, who had a son hemizygous for her mutation.

Consequences of VLCFA sensitivity. These aspects of VLCFA sensitivity in blood samples have important consequences for the studies presented in this thesis. Paper 1 relied on our ability to capture X-ALD subjects in the population, and in Papers 3 and 4 we used VLCFA analyses to screen patient populations for X-ALD.

Samples were sent either to the laboratory at OUS-Rikshospitalet, or (to a large extent) to the laboratory at the Sahlgrenska University Hospital, Gothenburg. For females, VLCFA levels were analyzed as part of the diagnostic work-up, but exclusion of X-ALD was always based on DNA studies of the family mutation.

In Paper 3, only males were included, and we relied on VLCFA measurements performed at the laboratory at Sahlgrenska, according to previously published methodology [141]. Although we cannot exclude the possibility of false negative results, due to patient-specific factors or technical errors, there is no reason to believe that this was a significant factor in this study. Adding fibroblast studies, or mutational analysis, might have reinforced our conclusions. However, these supplementary studies are not believed to be necessary to rule out X-ALD in males, and they were not feasible due to practical and economic reasons.

In Paper 4, similar considerations are true for the male part of the patient material. These VLCFA studies were performed at the laboratory at OUS-Rikshospitalet, measuring both total levels of C26:0 and the C26:0/C22:0 and C24:0/C22:0 ratios [152]. As for the female participants, new evidence published after the study was initiated [8] confirmed and even reinforced earlier assertions that VLCFA analysis alone was not sufficient to rule out carriership of X-ALD. This is a major weakness of this study, worsened by the fact that females with AMN generally lack the tell-tale adrenal failure seen in most males [11]. Therefore, there is a real risk that cases of X-ALD may have been missed by this screening effort, although

the risk of false negative results seems to be only about 15-31 % per case based on previous publications [8, 100, 101]. Assertions by Stradomska [153] that VLCFA levels decline with rising age in females with X-ALD (this was used by Wilkins [147] as an argument for higher sensitivity of VLCFA analysis among younger females) were not reproduced in the Dutch cohort [8], nor was this seen among males [101], or among French patients (Patrick Aubourg, e-mail correspondence, April 7th 2010). In conclusion, accumulating evidence and expert advice convinced us of the serious shortcomings of VLCFA measurements in screening females for X-ALD.

To compensate for this weakness, we looked at the hospital records of all female PPMS patients eligible for the study, trying to spot clinical features making AMN a more likely diagnosis. As a result, one female with peripheral neuropathy in combination with PPMS-related myelopathy was further investigated with DNA studies to rule out X-ALD, despite normal VLCFA levels.

At the start of this study, it was not economically feasible to perform mutational analysis in all females. Harvesting skin biopsies for fibroblast studies was deemed impractical, and might be ethically challenging, given the low probability that such an invasive procedure would actually prove to be beneficial for the patient. However, DNA studies are rapidly becoming cheaper and more suited for mass screening. We believe that future screening efforts of female patient populations (with for instance MS or HSP) ought to proceed directly to mutational analysis.

3.6 Genetic studies

Genetic studies were performed as part of the routine clinical work-up of X-ALD subjects and their relatives seeking genetic counselling and diagnosis. DNA diagnosis of X-ALD is dependent on whether the family mutation is known or not. If unknown, mutational analysis at OUS starts with Sanger sequencing of the ten exons and exon-intron transitions, supplemented with MLPA analysis (MLPA kit P049, MRC-Holland) to rule out duplications and large deletions. Due to the large

number of disease-causing mutations in the *ABCD1* gene, mutational analysis in the absence of a defined family mutation is challenging. Technical problems or the issue of variants of uncertain significance [154] may in theory lead to disease-causing mutations being overlooked, particularly if the clinical suspicion of X-ALD is low. If uncertainty arises as to whether the DNA variant uncovered is pathogenic, prediction tools like SIFT[155] (http://sift.jcvi.org/) and Mutation Taster [156] (http://www.mutationtaster.org/) are used.

The technical quality of mutational analysis at our hospital may have improved during the period of about 15 years it has been performed at OUS (Lars Retterstøl, personal communication, May 18th 2015). We cannot rule out entirely the possibility of false negative reports, particularly in the early part of the period. However, no example of this has so far been observed in the Norwegian X-ALD pedigrees.

The family mutation was established in 21 of 22 included kindreds in Paper 1; the exception being a single-member kindred with only one deceased male, from whom no biological material was available for genetic diagnosis. Based on pedigree analysis and location, it seemed unlikely that this patient was related to any of the other X-ALD kindreds identified in Norway, however, this cannot be ruled out entirely.

De novo mutations were defined by Wang [79] as instances where the disease-causing mutation found in a male index patient with clinical X-ALD was not found in the maternal DNA. We used the same definition for definite *de novo* mutations in Paper 1.
We used the term "possible *de novo* mutation" for cases where the mother of an affected female did not have her daughter's mutation in DNA analysis of blood, whereas the alleged father was an apparent non-carrier. The latter assertion relied on the age of the father and his apparent (lack of) symptoms of AMN, based on the assumption that all elderly males with an *ABCD1* mutation will develop myeloneuropathy, and the majority will have signs or symptoms of adrenal failure.

However, as shown in our Paper 5, this assumption is not necessarily valid.
In our cases, the assumption that the alleged father was not a carrier of X-ALD was somewhat reinforced by other data, such as normal VLCFA measurements or absence of other affected offspring. Still, there remains a possibility that some of these elderly males were actually unrecognized mild cases of X-ALD.
Also, there is the possibility of alternative paternity, where in theory, the affected female might have inherited the mutation from her unknown, genetic father. However, based on the specific mutations found, and on the family backgrounds and locations, it seems unlikely that any of these females actually belong to another identified X-ALD kindred in Norway.

3.7 Neurophysiologic studies

As noted above, neurophysiologic studies were not routinely used for phenotype assignment in Paper 1. A numbers of studies [65, 90, 157] have demonstrated neurophysiologic abnormalities in subjects with X-ALD, with or without clinical correlates. As shown in our Paper 2, abnormal large and small nerve fiber findings may occur even in very young asymptomatic females. Other reports, like the Dutch [8] and Brazilian [9] studies of women with X-ALD, have utilized evoked potentials to study central conduction velocities. There is as yet no established way to translate these neurophysiologic abnormalities into clinically meaningful diagnostic or prognostic categories in X-ALD.

In Paper 2, the aim of the study was to investigate evidence of small nerve fiber involvement. To this end, we relied on the practice parameters from the American Academy of Neurology [158], recommending the usage of multiple diagnostic methods: Quantitative Sensory Testing (QST) [159], tests of autonomic function using heart rate and blood pressure variability and evoked sweat response (the Quantitative Sudomotor Axon Reflex Test, QSART), and determination of Intraepidermal Nerve Fiber Density (IENFD) in skin biopsy [160]. The results of autonomic function tests were integrated in the Composite Autonomic Scoring Scale (CASS) [161]. The different tests of small nerve fiber pathology were

performed by experts in the field of small fiber neuropathy from the University of Oslo (Ellen Jørum), the Norwegian University of Science and Technology, Trondheim (Kristian Bernhard Nilsen, also at OUS) and the Arctic University of Northern Norway, Tromsø (Svein Ivar Mellgren). A detailed description of the tests performed is given in Paper 2.

After data collection was completed, the investigators analyzed the data for each subject in a consensus meeting. We determined whether there was evidence of small nerve fiber involvement, and if so, whether the combination of small nerve fiber pathology was most likely due to damage to the spinal cord or to small fiber nerves in the periphery, or a combination of these.

We included 11 subjects with ascertained X-ALD diagnosis, intentionally selected in order to cover both genders, aiming at including both young and elderly, both asymptomatic and symptomatic subjects. Thus, inclusion was not random, not representative of the X-ALD population – but rather attempted to be representative of the different stages of X-ALD in both genders. We specifically did not consider symptoms of small fiber neuropathy in the selection process.

3.8 MRI studies

As noted above, MRI was not used routinely in the diagnostic work-up and classification of X-ALD subjects for the purpose of this study. This was mainly due to the lack of established criteria for how to classify X-ALD based on MRI findings, beyond the staging of cerebral ALD for which the Loes criteria [60] are used. Except for the determination of whether a male subject has converted to cerebral ALD, MRI is of little use for classification. This may change if modern MRI techniques will allow detection and quantification of axonal damage in the brain and the long tracts of the spinal cord, with such detail as to allow separation of subjects into meaningful categories.

In Paper 5, we performed detailed MRI studies of the brain and spinal cord to detect subtle evidence of demyelination in the pattern typical of cerebral ALD, or degeneration and atrophy of the long tracts as seen in AMN.

3.9 Ethical considerations

Both clinical care and research on disorders like X-ALD is ethically challenging. From other genetic disorders, it is well known that harboring a mutated gene, or giving birth to a child that is diseased or dies due to a gene inherited from you, can give rise to feelings of guilt and "why me?". The experience from predictive genetic testing for Huntington's disease has been particularly well studied, and illustrates that subjects at risk of harboring a severe genetic disorder will not necessarily perceive an opportunity to undergo diagnostic clarification as something straightforward and beneficial [162]. The attitudes among American families with X-ALD were studied by Schaller [124]; however, on the individual level, a wide range of reactions may be encountered when subjects are offered participation in surveys of genetic disorders.

3.9.1 Areas of particular concern for X-ALD

Loss and mourning. Families affected by X-ALD will often have lost one or more boys or older males due to CER. They may be in a state of acute or protracted mourning, which may affect family members in different ways. For some, the mourning for the lost child may be something belonging to the past, where either diagnostic approaches or research efforts may reopen old wounds. Other family members may not have experienced loss, but they may have witnessed the agony of their relatives, as a boy fell ill and died from CCER.

In addition to the feeling of guilt some subjects with genetic disorders may experience, the insidious and frequently unspecific symptoms of onset of CCER may often cause delay in diagnosis – whereas life-saving treatment relies on a prompt diagnosis being made. Therefore, a feeling of guilt for not having seen the signs early enough may add to the burden of these family members. Also, some families may be in the situation where one child is already lost due to CCER, while another child has just recently been diagnosed, creating a mixture of past and ongoing bereavement.

Dual roles and role conflicts. Secondly, subjects with X-ALD will often have dual roles, possibly conflicting. Typically, women who are mothers of a boy either affected by or deceased from CCER will also be genetically affected heterozygotes. Some may have reached the age where they develop symptoms of their own, or they may (based on new knowledge about female phenotypes) face the prospect of neurological deterioration in the future. Additionally, the diagnosis of X-ALD in one son may put them in the position where they must decisions regarding prenatal or presymptomatic diagnosis in their other children. The subjects may have to combine the need to ”be strong” in their role as mothers or fathers of affected children, with the need to pay attention to their own subjective symptoms, to allow themselves to ”be weak” and to be recipients of medical aid and comfort from others.

For most subjects affected by X-ALD, there is a potential conflict between the role as a private person, affected by disease, and the role as a potential link to other affected family members. The person may struggle with coping with the disease in him-/herself, both physical symptoms and the psychological impact of the disease, while at the same time feel a responsibility for informing other family members so that they may get the chance to seek diagnosis or take preventive measures. Some patients prefer privacy about their own illness, and may be reluctant to inform others about it. Others may worry about their relatives’ desire or readiness for being told about the possibility that they too may harbor this serious disease.

The uncertainty of the prognosis. The hallmark of X-ALD is the uncertainty regarding whether or when the subject will develop serious symptoms due to the genetic condition one was born with. About one third of males will develop CCER, usually fatal if not detected early enough for HCST to be effective. HCST is in itself a very challenging therapy, with significant mortality and therapy-related morbidity. Even though early diagnosis allows us to attempt to save the boy’s life, this comes at the cost of frequent and repeated MRIs and clinical consultations, each looming as a crossroads at which it will be revealed whether the boy has

turned onto the path of devastating disease or not. Even when handled with care and compassion, the underlying fact of these situations is difficult to erase: Each MRI may be felt experienced as a possible death sentence.
Previously, we believed the risk of CER to diminish radically after the age of about 12-16 years. However, recent research [35-37] has shown that the risk of CER continues into adolescence and adulthood. As HCST is now being offered, at least experimentally, even to adults with CER, there is a need for continued MRI follow-up – meaning that these males may never get "off the hook".

Furthermore, we now know that most or all males with X-ALD will probably develop AMN during adulthood, even those who are successfully transplanted [4, 118]. This creates a dilemma for both parents and health personnel caring for boys diagnosed with X-ALD in childhood: First, they must be followed with MRI to detect CER during the high-risk period of 4-8-12 years. Then, when they appear to have avoided that serious complication, there comes the need to prepare them for the onset of AMN. We have experienced ourselves the difficulties for both parents and physicians, particularly concerning how and when to inform these boys about the expected evolution of their disease. The development of effective therapies for either CER or AMN would of course be beneficial for these patients – but it would increase the need for follow-up and for explaining the affected boys about the severity of their disease.

For female subjects, the research performed by us (Paper 1 and 2) and others [8, 9] now paints a less hopeful prospect than what was previously envisioned. Based on the existing evidence, females may not realistically hope to go unaffected by neurological manifestations. At the same time, in order to receive the options of reproductive care and choices, they need to be diagnosed several years – even decades – before symptoms appear. If effective therapies to ameliorate or prevent myeloneuropathy are developed, this would increase the need for early diagnosis – but also entrench the women in the role of a patient awaiting future neurological

deterioration. For the time being, the lack of therapies proven to be efficacious in preventing AMN may make this waiting period harder to endure.

3.9.2 Strategies to meet ethical challenges

Avoiding intrusion. In our project, we tried to take care not to intrude on the families, despite our scientific ambition to explore pedigrees as completely as possible. Our method of inclusion was to directly approach subjects (or their closest relatives) only if the subject had an established X-ALD diagnosis. We allied with the physician in charge of patient care in order to make the first contact with the affected subject or the relatives, encouraging them to consider how the family would react to the invitation to participate in X-ALD research. Therefore, in these cases, the first contact versus the family was made not by the researchers, but rather by a physician they knew and presumably trusted. Then, in the next step, the subjects and family members were invited to contact (or accepted being contacted by) the researchers in order to participate in the research.

Subjects who were at risk of having X-ALD, but who had not themselves sought diagnostic clarification, were not contacted directly by us. In stead, we encouraged subjects already diagnosed to consider informing their potentially affected relatives, offering them the opportunity to contact the project leader if they desired to enter the diagnostic process. We took care discussing with the probands how this might affect their relatives, and encouraged them to consider both their own feelings and the mind-set of their relatives, in regard to screening for X-ALD.

Offering follow-up. During this process, both diagnosed and undiagnosed family members were given contact information (including cell phone number) to the project leader, to allow expedient handling of questions, fears and uncertainties that might arise in this situation. We also informed local physicians to be prepared, in case our pedigree screening should give rise to needs for consultations or other actions on the local level. Similarly, the results of diagnostic consultations were reported to the subjects' general practitioners (if desired by the subject), including information regarding X-ALD and the consequences for the patient.

All subjects diagnosed in this fashion were offered clinical consultation with the project leader, either at OUS Ullevål hospital, or at the local department of neurology (the project leader travelling to Bergen, Lillehammer, Kristiansand). All subjects were given the (unlimited) opportunity to contact the project leader directly, if they had questions regarding any aspect of X-ALD.

Subjects at risk of having X-ALD, but who described themselves as asymptomatic, were offered referral for ordinary genetic counselling and subsequent mutational analysis, if desirable. As noted, asymptomatic females < 16 years of age were informed that the law did not allow genetic diagnosis until they reached 16.

3.9.3 Ethical issues experienced during the study

Ethical issues were frequent. During the process of patient inclusion, we faced a variety of reactions and exemplifications of the ethical issues described here. Some examples are presented in Table 8.

Table 8. Ethical issues encountered during this study.

- relatives of boys who died from CCER, who were still in a process of mourning
- mothers of boys who died from CCER, who reported being uncertain what they would have done, if prenatal diagnosis or PGD had been available to them
- subjects at risk of having X-ALD, who declined the option of diagnostic clarification
- parents expressing stress and anxiety related to the repeated MRI follow-up of their sons
- parents of minor-aged girls at risk of having X-ALD who wanted to test their daughters
- X-ALD patients facing the challenge of contacting family members to bring ill tidings of X-ALD
- relatives of severely affected X-ALD patients suddenly realizing that they themselves were carriers of the same disease
- patients suddenly becoming aware that perhaps their established MS diagnosis was wrong, that they perhaps had an altogether different disease with consequences for their family
- parents being uncertain how and when to inform their sons about the severity of the genetic disorder, and the possibly fatal outcomes
- adult males being reluctant to undergo diagnostic investigations in order to detect early signs of progression, thereby denying themselves opportunities to treat these complications.

Faced with these challenges, our strategy relied primarily on making ourselves available for questions, providing consultation and information, and informing local caregivers already involved in the follow-up of the subject. It was our experience that the subjects generally felt comforted by this approach, although we know little about the reactions of those who declined further contact. However, it was also clear to us that a substantial portion of those touched by this project had preexisting worries related to the disease in their family, unrelated to our intervention.

We have no clear solution for how to handle these issues perfectly. The underlying fact is that X-ALD has several manifestations that may be fatal if untreated or if diagnosed too late (in particular Addison crises and cerebral demyelination in boys), and that genetic counselling provides females with real choices regarding prenatal diagnosis. Were we to choose not to offer pedigree screening to these families, several subjects would lose their chance for life-saving treatment and reproductive choices. Were we to pick a more aggressive, intrusive strategy, we probably would have caused some subjects unwanted suffering and dreadful knowledge that could not be erased. We therefore believe that our approach was the most rational one: to rely on the sensibility of affected and diagnosed family members, letting them decide how and whom to approach with information about this project, and offering information and support to those who desired it.

The right not to know.[3] An important principle associated with genetic diagnostics is the right not to know: to abstain from the option to diagnose genetic conditions that may cause you harm in the future. However, in dealing with these families, we have encountered an even more complicated issue: *The right not to know that there is something you might get to know.* When a subject, believing himself to be entirely healthy, is informed that there is a genetic disorder in his family and that he might himself be affected, that subject still has the right to decline diagnostic clarification. He may choose to remain ignorant of whether or

[3] Thanks to Arvid Heiberg and Ellen Økland Blinkenberg for interesting discussions and input on this topic.

not he carries the defective gene. However, he can no longer live on in blissful ignorance that there might be something wrong with him. His right to naïvely believing himself to be healthy has been violated.

The right to live in blissful ignorance may be at the heart of the concept of "the right not to know". If someone is told that "you may harbor a deadly disease, and there is a way to settle the issue", that may create an enormous pressure on that subject, where the only real solution may be for the subject to give in to the pressure, take the test and find out for sure whether he is affected or not.

This issue is relevant for our survey of Norwegian kindreds with X-ALD, and for the follow-up of these families in clinical routine. Simply by offering diagnostic clarification, we place the family members in the position where they must make a choice – without them making the decision that they wanted to be in that position in the first place. On the other hand, by refraining from offering diagnosis, we withhold from them the option to receive diagnosis, prevention and treatment.

Crucial to this dilemma is whether there is available therapy that may effectively alter the natural course of the genetic disease, provided that a diagnosis is made. For X-ALD, at least two such therapies are available; cortisol substitution therapy for PAI and HCST for CER. The need to make these therapies available for the male subject may justify denying him the right of complete ignorance. For females, no therapy has so far been proven to prevent development of myeloneuropathy. However, fertile females may make use of prenatal diagnostics, which may heavily influence their life courses. We believe that for X-ALD, protecting the right not to know may be superseded by the right to be informed in due time about serious, treatable or preventable disease. Still, our experience with our patients has shown us that this is an area where patients may have different attitudes and values than medical professionals have. Caution is advisable when handling these situations.

4 Summary of results

Paper 1. Adrenoleukodystrophy in Norway: High rate of *de novo* mutations and age-dependent penetrance

The frequency of X-ALD in Norway was unknown, but appeared to be lower than estimates from the literature indicated. We assembled all Norwegian subjects diagnosed with X-ALD, using a broad search strategy. Pedigrees were explored as far as possible. Differential diagnoses were screened for misdiagnosed cases. Females < 16 years of age were excluded.

We identified 63 subjects (34 males, 29 females) from 22 kindreds with X-ALD in Norway. Of these, 39 (13 males, 26 females) were alive on the prevalence day. In 2011, the prevalence of X-ALD in Norway was 0.8:100,000 (both genders). The incidence at birth for the period 1959-1995 was retrospectively calculated to be 1.6:100,000 (both genders).

The phenotype distribution among males was comparable to the literature. However, we observed that no male patient that had been identified with X-ALD in Norway had yet died without developing cerebral demyelination – the exception being a man who died from cancer at age 41.

Among females, we observed a pattern of age-dependent penetrance. Neurologically intact and asymptomatic females were all < 50 years of age, whereas those with symptomatic AMN were all > 50 years of age. Those who were subjectively asymptomatic, but who displayed subtle neurological signs of long tract involvement (the SIGNS category) constituted a middle group, age-wise.

We identified 16 mutations in the *ABCD1* gene in the 21 kindreds that were analyzed; six of these were not previously reported. Four (19 %) probands had definite *de novo* mutations, while a *de novo* mutation was possible in another four families. In all, X-ALD appeared in a previously healthy family in 38 % of kindreds; underscoring the need for screening (like newborn screening) if these families are to be detected. This is necessary in order to provide timely treatment for adrenal failure and cerebral demyelination, and genetic counselling to presymptomatic females.

Paper 2. Small nerve fiber involvement is frequent in X-linked adrenoleukodystrophy

AMN is characterized as by a dying-back axonopathy of the spinal cord, and the peripheral findings are mainly axonal. Previous studies have described involvement mainly of the corticospinal and dorsal column tracts, and of the large nerve fibers of peripheral nerves.

Small nerve fiber involvement has not been described in X-ALD, however, complaints of pain and sphincteric disturbances are common. Spurred by the clinical observation of small nerve fiber involvement in one AMN patient, we screened 11 subjects (3 males, 8 females) with various X-ALD phenotypes for laboratory evidence of small nerve fiber involvement.

We performed clinical evaluation, large nerve fiber testing, tests of autonomic function (heart rate and blood pressure variability and quantitative sudomotor axon reflex test), quantitative sensory testing of thermal thresholds, and quantification of intraepidermal nerve fiber densities.

In 10/11 subjects we found evidence of small nerve fiber involvement. IENFD was reduced in 5/11 (and borderline low in 3/11), being evidence of loss of peripheral small nerve fibers. Thermal sensitivity was reduced in 8/10. Autonomic function was moderately impaired in 2/11.

The abnormalities were more severe with rising age, and in subjects with more severe AMN, yet even in young, asymptomatic females pathological findings were made.

This study demonstrates that small nerve fiber involvement is frequent in subjects with X-ALD, more pronounced with rising age, but present even in young, asymptomatic females. This indicates that most or all subjects with X-ALD will develop some degree of neurological involvement. Involvement of small nerve fibers with no or scanty myelin sheaths support the hypothesis of primary axonal damage in AMN. The widespread findings of small nerve fiber dysfunction may shed light on the pain syndromes and sphincteric disturbances frequently reported by X-ALD subjects, females in particular.

Paper 3. Screening for X-linked adrenoleukodystrophy among adult men with Addison's disease

X-ALD is established as an important cause for PAI in young boys, and the AO phenotype is a common presentation at onset of X-ALD. However, it is unknown to which degree the AO phenotype may persist into adulthood, as most X-ALD males are expected to develop neurological symptoms either in childhood (CCER) or in early adulthood (AMN).

We made use of the National Addison's Disease Registry, part of the Registry for Organ-specific Autoimmune Diseases (ROAS) at Haukeland University Hospital, to screen adult males with non-autoimmune PAI for X-ALD.

The registry covers about 75 % of Norwegian PAI subjects. From 153 adult males with PAI, we included the 18 males negative for 21-hydroxylase autoantibodies (21OHAb), indicating a non-autoimmune cause for PAI. Furthermore, we included 17 adult males with weakly positive 21OHAb indices, in case any of these had been misclassified as autoimmune due to low antibody indices.

Blood samples were analyzed for levels of VLCFAs, which are almost 100 % sensitive for X-ALD in males. Only one subject, from the 21OHAb negative group, had abnormal VLCFA levels. This subject had already been diagnosed with X-ALD, due to preexisting spastic paraparesis. However, his diagnosis had been delayed, probably due to lack of awareness of X-ALD.

Two more adult male subjects with PAI due to X-ALD had been identified through the X-ALD survey described in Paper 1. Another 13 subjects with PAI due to X-ALD were deceased. Ten were boys with PAI as part of childhood or adolescent cerebral ALD. Three had adult onset of PAI and neurological involvement.

Our study found X-ALD to be the cause in 15 % of adult males with non-autoimmune PAI; of all adult males with PAI, X-ALD was the cause in only 1.5 %. We recommend screening for X-ALD in adult males with absence of 21OHAb or other evidence of autoimmunity.

Paper 4. X-linked adrenoleukodystrophy as differential diagnosis of primary progressive multiple sclerosis

Several reports describe X-ALD patients initially diagnosed with MS. The form of MS most closely resembling the slowly progressive AMN phenotype seen in adult males and females with X-ALD, is PPMS.

We included subjects with PPMS recruited through the Oslo MS registry, which includes almost all subjects diagnosed with MS in the city of Oslo, capital of Norway (2010 population: 587,000). Out of 114 eligible subjects, 69 (61 %) consented to participation. Forty subjects (58 %) were female, 29 (42 %) were male. Non-participants had significantly earlier onset of disease, but were otherwise similar to the study populations. Participants were screened for X-ALD by VLCFA analysis in serum.

No subjects had VLCFA levels indicating X-ALD. Among male participants, this ruled out X-ALD. Among female participants, the lack of sensitivity of VLCFA analysis in blood means that the likelihood of capturing a female subject with X-ALD was only 69-89 %. X-ALD cannot be excluded by way of VLCFA analysis in this group.

We cannot rule out that some PPMS patients in Oslo are actually misdiagnosed cases of X-ALD. However, our findings indicate that this is not a frequent phenomenon.

We recommend that neurologist consider screening patients with PPMS for X-ALD, particularly in the presence of "red flags" provided in the article, which may increase the likelihood of X-ALD. For female PPMS patients, mutational analysis of the *ABCD1* gene is necessary to rule out X-ALD.

Paper 5. Mild phenotype in an adult male with X-linked adrenoleukodystrophy – case report

Recent research has indicated a more severe course of X-ALD among males than previously described, and a pattern of age-dependent penetrance.

However, we encountered a 61 years old male in whom X-ALD had recently been diagnosed through VLCFA analysis, performed because of subtle signs of myelopathy. The patient also had Parkinson's disease, which overshadowed his myelopathy symptoms. When Parkinsonism was treated, he appeared to be almost asymptomatic with regards to the myelopathy, although there remained neurological signs of long tract involvement.

The patient underwent complete diagnostic work-up. VLCFAs were pathological in blood and fibroblasts, and genetic studies disclosed a previously unreported mutation in the *ABCD1* gene (c.1205T>A). Elevated VLCFAs and the patient's mutation were found in his asymptomatic daughter. MRI studies of the brain and spinal cord were normal. Neurophysiologic studies were mostly normal, however, skin biopsy revealed a loss of intraepidermal small nerve fibers. There was no evidence of addisonism or hypogonadism.

The patient had long-standing symptoms of fasciculations starting in the legs, and spreading to the arms and abdomen. No cause for these symptoms was found, possibly, they may have been related to his mild myeloneuropathy.

This study verifies that X-ALD may be genetically, biochemically and clinically present in a 61 years old male who, based on appearances, seemed to be healthy, with only subtle signs of myeloneuropathy and no signs of addisonism. The lesson learned is that X-ALD should not be ruled out, even in elderly, healthy males, based on clinical condition only. The case report highlights the need for extensive studies to determine whether genetic sequence variants of uncertain significance are pathogenic.

5 General discussion

5.1 Epidemiological studies

Prior to the works presented in this thesis, we had no systematic knowledge about the Norwegian X-ALD population. There was a disturbing discrepancy between the number of cases known to us, as experts in the field, and the number of subjects that could be estimated from the frequency figures universally cited in the literature – generally given at 1:20,000 based on American reports [76]. If these figures were to be applied (in a very loose fashion) to Norway, there might be in the vicinity of 150-200 X-ALD subjects (of both genders) living in Norway. We knew of only a handful.

However, even in the international literature, the epidemiology of X-ALD was poorly described (Table 2, page 24). The main aim of this work was to test and challenge the frequency estimates for X-ALD, in order to either improve our knowledge of our own population, or to provide more accurate estimates.

The epidemiological findings for X-ALD in Norway are summarized in Table 9. The following sections will discuss the prevalence and incidence findings in comparison with the literature, and strengths and weaknesses of the Norwegian estimates.

Table 9. Key epidemiological findings for X-ALD in Norway (Based on Paper 1)

2011 prevalence:
- 0.8:100,000 both genders
- 0.5:100,000 males only
- 1.0:100,000 females only
- 1.0:100,000 with estimated contribution of *at risk* population
- 0.9:100,000 corrected 2015 prevalence when two more kindreds have been identified
- 1.1:100,000 when both new kindreds and at risk subjects are added.

Incidence at birth 1956-1995:
- 1.6:100,000 both genders

Kindreds relative to population:
- 4.5 per million

5.1.1 Prevalence studies

X-ALD Males. There existed only one prevalence study, performed in the Netherlands in 1994 prior to the discovery of the *ABCD1* gene and including males only [30]. Interestingly, the Dutch study found the same prevalence of X-ALD males (0.5:100,000) as in our study. The search for Dutch male X-ALD patients appears to have been thorough and efficient. Notably, it was performed while the film "Lorenzo's Oil" lifted X-ALD to fame in the Western world; the authors state that 7/33 identified kindreds contacted the researchers because of that film. The lack of access to genetic testing may have been less damaging for capturing males than it is for females, since all males can be diagnosed by VLCFA measurements [101]. Compared to other reports [68], the Dutch study managed to capture a higher proportion of the somewhat more elusive adult form of X-ALD: AMN was the predominant (46 %) phenotype in that study.

Comparison with other national surveys of X-ALD is difficult. No other report provides information as to whether the male patients included are actually dead or alive; since most males with CCER die within a few years, and CCER is the dominating phenotype in most other studies, one must suspect that most of these include a mix of dead and living X-ALD males. Prevalence estimates are, therefore, not possible.

However, during the preparation of Paper 1, we scrutinized the previous nation-based surveys of X-ALD [68, 73-75, 77, 78, 163] that included [number of X-ALD patients] and [identifiable source population], allowing rough estimates for comparison. We found no report in which the numbers of diagnosed X-ALD males, relative to the population size, exceeded ours (the Dutch study was ranked 2nd). For instance, in 2011, 34 Norwegian males (dead and alive) had been diagnosed and identified with X-ALD, relative to a population of about 5 million; that is 0.69:100,000. In the USA, there had by 1998 been identified 1,194 X-ALD males, relative to a population of 275 million; that is 0.43:100,000. This is also reflected in the number of reported kindreds relative to population size: In Norway, 4.5

kindreds per million inhabitants were identified, whereas the corresponding figure from the other national surveys was typically 1-3 (for the USA, the figure was 2.2).

Such calculations are admittedly un-scientific, as the other surveys were not designed to estimate prevalence, nor did they have a clearly defined source population. However, they illustrate that there is no apparent shortage of Norwegian X-ALD males, relative to the small size of our population. On the contrary, our multi-staged strategy for proband identification, pedigree exploration and screening of differential diagnoses appears to have been effective in identifying a large part of the Norwegian X-ALD population – or at least as effective as previous efforts to describe national cohorts of X-ALD subjects.

X-ALD females. Our study was the first to report the prevalence of females with X-ALD. The prevalence of X-ALD among males is not necessarily a good measure of the impact of X-ALD on families and on the health care services: the most severe cases, boys with devastating CCER, usually die rapidly and contribute little to the prevalence. Still, they pose the greatest challenge from X-ALD in terms of human suffering and resource needs. For males, incidence (of CER) may be more informative than prevalence. The opposite is true, however, when it comes to female subjects. They live normal lifespans, do rarely develop CER, but may need long-term specialized care both for reproductive services and for symptoms of myeloneuropathy (see section 1.7).

In Norway, the prevalence among females was twice that of males, reflecting that more than half of the male cohort had died from CER, whereas most of the females were still alive. When live and deceased subjects were taken together, the gender balance was almost 1:1, particularly when taking into account that some of the 12 girls < 16 years identified as having a 50 % risk of inheritance will probably turn out to have X-ALD. The gender balance in Norway, then, is similar to the Australasian study [74].

However, the prevalence figure found in Norway in 2011 (see Table 9, page 79) is a minimum estimate. Both because girls < 16 years were intentionally left out of

the survey (they are, however, included in the *at risk*-estimate) and because such surveys will probably never achieve complete capture. For instance, after completion of our study, two more X-ALD kindreds with a total of five affected subjects have been identified in Norway. Adding these to the published estimates, the minimum prevalence of X-ALD in Norway may approach 1.1:100,000 if *at risk* subjects are added as well. We suggest that the minimum prevalence of X-ALD (both genders) should be reported in the literature as approximately 1:100,000.

5.1.2 Incidence studies

Incidence-prevalence mismatch. Most population surveys of X-ALD [72-78] (see Table 2, page 24) make use of some kind of incidence estimates. The exact methodology used in these calculations is often difficult to understand, for instance, the precise size of the source population, or of birth numbers, are frequently omitted. Still, most of these studies purport to estimate the frequency with which subjects with X-ALD are born; that is, the frequency among newborns, or incidence at birth. Most are based on male cohorts only; the exceptions being the German [73] and the Australasian [74] surveys. The American study [76] provided incidence estimates for females as well as for males, however, the female estimates were based on extrapolation of the male results. In the remaining studies, females were left out or were grossly under-represented.

The two methods most commonly used for calculating incidence are discussed in section 3.3 (Incidence calculations). Readers must keep in mind which method was used, as the results are not readily comparable. What we called “Method 1”, used in the American survey [76] is prone to over-estimating the frequency, whereas “Method 2”, used in our Paper 1, may be criticized for under-estimating it. This section will discuss in greater detail the strengths and weaknesses of each method, with regards to the results they have produced in the populations surveys in the literature.

The different population surveys of X-ALD were presented in Table 2. This reveals a puzzling phenomenon: several of the publications [75, 77, 78] provide incidence at birth figures much higher than what we found in Norway, generally 2-3.6:100,000. Most strikingly, the American study [76] reports an incidence of 6:100,000, 3-4 times what we found in Norway (1.6:100,000). On the other hand, we showed in section 5.1.1 that the number of identified cases in Norway, relative to population size, far superseded that of the USA and any other population survey. Notably, even the Dutch study [30], which reported a minimum incidence of X-ALD males of only 1:100,000, had identified a higher number of X-ALD males relative to population size than the American study (0.51 vs. 0.46 per 100,000). The prevalence of a disease is dependent on the incidence and on the duration of the disease (for lifelong disorders: the lifespan of the patients) – so how can there be this mismatch between incidence and prevalence/reported cases, in the American compared to the Dutch and Norwegian populations?

We believe the cause for this discrepancy is more fundamental than simply a difference in calculations. The underlying problem is that the American figures – which form the basis of the nigh-universally cited statement that the frequency of X-ALD is around 1:20,000 worldwide – are erroneous. Method 1 is invalid, because it inflates incidence numbers and creates an image that X-ALD is more frequent than it actually is. We will illustrate this by looking at the Australasian study, which used Method 1 for incidence calculations, but provided more detail than the American study did.

The Australasian study. The Australasian study from 1998 [74] had many similarities to our own survey from Norway, in terms of scope and search strategy. Table II of that paper presents the relative frequency of X-ALD phenotypes among the 95 males diagnosed in Australasia from before 1981 to 1996 (nine patients could not be classified). There is a steady increase in the number of new diagnosed patients for each 5-6-year time period from 1981 to 1996 (see Table 10). At the same time, the *proportion* of boys with CCER or Adol-CER drops, while the

percentage of AO and ASYMP boys, and adult males with AMN, rises. However, the *absolute* number of cases of CCER/Adol-CER remains stable during this period – what changes is the absolute number of AMN and AO/ASYMP cases. Notably, these are the patients with the least well-defined time of onset; the ones least suited for incidence studies.

Table 10. Data from the Australasian X-ALD study. Absolute numbers of subjects derived from the percentages given in the article (Based on Kirk et al, Am J Med Genet 1998;76:420-423)

	1981-1985	1986-1990	1991-1996
New diagnosed cases	15	25	36
New CCER/Adol-CER cases	10	12	8
New AMN and Adult-CER cases	5	8	13
New AO and ASYMP cases	0	5	15
New estimated cases (all phenotypes) based on CCER numbers (3x CCER)	29	35	23

Given the slow evolution of AMN, some of the "new cases" of AMN diagnosed 1991-1996 will probably have had onset of symptoms far earlier. Had there been awareness of AMN and access to specialized health services, they "should really" have been placed in one of the earlier time periods, when the number of AMN subjects diagnosed was conspicuously low. However, since Method 1 was used, these AMN males are entered into the incidence estimates for the 1991-1996 period, thus creating the very high incidence figure of 4:100,000 males.
The authors interpret this incidence estimate to be the most accurate, being based on the most complete ascertainment during that period. However, this high estimate was reached by diagnosing a lot of patients at the same time – it was not the result of many patients suddenly manifesting X-ALD. Rather, it was the result of a concerted effort by the researchers to diagnose as many X-ALD patients as possible during a short study period. Also, it was probably influenced by increased

awareness of X-ALD both among physicians and in the general population (cf. the Dutch study [30], which claimed that 21 % of their kindreds were captured as a consequence of the 1992 film “Lorenzo’s Oil”), and by the increasing recognition of AMN as a phenotype of X-ALD.

Method 1 might be valid in a population where the concept of the disease, diagnostic criteria, public and professional awareness of the disease and diagnostic tools have reached a kind of a steady state: where new cases of both childhood and adult cases are diagnosed as they manifest, where extended families are routinely screened, where there is a constant trickle of new diagnosed cases and rather constant birth numbers. However, these factors were not in place when the American, Australasian and the other studies were performed. On the contrary, the concept of X-ALD was expanding to include adult cases, VLCFA analysis was being improved and genetic testing was introduced, public awareness was bumped by the film, and there were concerted research efforts. The result is a frequency estimate for X-ALD which has not, as far as we know, actually been observed in any country, and which failed to predict the number of X-ALD subjects in our own survey from Norway.

Criticism of Method 2. In comparison, our own chosen method of incidence calculations may be rightfully criticized for underestimating the incidence [164]. This is due to two factors:
1) For subjects born early in the period (stretching back to 1959 in our study), lack of awareness, different diagnostic criteria (the conundrum of "Schilder's sclerosis" was yet unresolved, AMN wasn't even "discovered") and subjects being forgotten or lost to follow-up, means that some of those who became ill during that period might have been missed – either by contemporary physicians, or by our retrospective study.
2) For those born during the latest part of the study period (terminating in 1995), diagnostic criteria, the quality of the health care services and our ability to remember or locate subjects with X-ALD had probably improved – one can

assume that more subjects from this period are correctly diagnosed and retrieved by our survey. However, some of these younger subjects may not yet have had time to develop symptoms of X-ALD, or the diagnosis (for instance of subclinical adrenal failure or mild myelopathy) may have been delayed. Therefore, Method 2 risks missing patients from both the early and the late study period, resulting in spuriously low incidence figures for the combined period.

However, this criticism – although serious – may partly be rebutted: Firstly, the hereditary nature of X-ALD means that many subjects may be diagnosed through family screening, even though they themselves have not yet developed symptoms. Indeed, some of the Norwegian male subjects included in our incidence estimate belong to this category. Furthermore, a large proportion of X-ALD males will develop PAI very early in childhood [10], even though neurological involvement may first appear as AMN in adulthood. If PAI develops in boys, X-ALD is well-known to be an important etiological diagnosis [49-53], and may be correctly diagnosed. Lastly, even though males who died long ago from X-ALD may be "forgotten" by the health care system, they will often be the uncles or cousins of today's patients. Therefore, thorough family screening may capture them in retrospect, some times even revealing the true diagnosis for a fatal or debilitating condition that was not understood or recognized in its day.

Cases and kindreds of X-ALD that have been missed by our incidence estimates must have been either kindreds entirely separate from those identified in Norway, or only distantly related to the existing ones. If single cases have been missed, there is a good chance that they may have had *de novo* mutations. However, one must bear in mind that the number of kindreds relative to population size, and the frequency of *de novo* mutations, is already higher in Norway than in any other country. Therefore, even though we acknowledge that our method of incidence calculation may have missed some subjects or kindreds, the magnitude of this problem may not be that large. Still, it must be clear that the Norwegian incidence estimate of 1.6:100,000 is a minimum estimate. We eagerly await the results of

newborn screening from the USA, which may show us the true incidence at birth of X-ALD.

5.1.3 Can the Norwegian figures be trusted?

When presenting our epidemiological findings to international colleagues, we have been met with suggestions that the low incidence figures may be due to incomplete capture or case ascertainment; in other words – we haven't found all the patients after all [164]. Therefore, there is a need to examine the quality of our inclusion strategy.

At the outset, there remains the rebuttal discussed in section 5.1.1 – that we actually appear to have captured more subjects, relative to population size, than any other population survey of X-ALD. However, as we identified two kindreds shortly after Paper 1 was published, our inclusion strategy was obviously not 100 % effective. The true incidence and prevalence of X-ALD may be higher than our findings indicate: Either because X-ALD subjects are underdiagnosed in Norway, or because we failed to identify and include them.

Diagnosing X-ALD. Reaching the diagnosis of X-ALD relies on several factors (see Table 11). In Norway, awareness of X-ALD has probably not been especially high, although this is difficult to measure and to compare with other countries. Before this work was done, there was no centralized program for diagnosis and follow-up of X-ALD in Norway. Furthermore, although VLCFA analysis (paramount in detecting X-ALD in males, and useful even in females) has been available for decades[4], the analysis has been performed in a specialized laboratory, using a special form without a separate, named box to tick for VLCFA analyses. Factors such as these may, in some cases, have been an obstacle to reaching the correct diagnosis: the distance from the patient to a facility knowledgeable about the disease and how to diagnose it may simply have been too long. The law

[4] Introduced at OUS-Rikshospitalet in 1990 by a research group, from 1999 performed at the Section for inborn errors of metabolism, Department of Medical Biochemistry. The current methodology was introduced in 2009 (Berit Woldseth, personal communication, April 28th 2015).

forbidding DNA studies in presymptomatic minors may have been another obstacle in some cases. A visible, accessible and structured program for resolving the diagnosis of X-ALD might have helped physicians who were wondering whether or not to embark on the diagnostic process. Conceivably, other countries like the USA, France and the Netherlands have a longer and stronger tradition for diagnosis and research on X-ALD.

Table 11. Factors important for diagnosing X-ALD.

Awareness
- of the disease entity
- of the diagnostic clues (addisonism, "ADHD-like", spastic paraplegia, polyneuropathy)
- that both males (boys and adults) and females may be affected

Access to diagnostic measures
- pediatric and adult neurologists (good access in Norway, free/public health care)
- MRI machines – available in Norway from 1985, now good access across the country
- VLCFA analysis – available in Norway since 1990, one laboratory only
- mutational analysis – available in Norway since 2001

Systematic screening
- pedigree screening – not systematically performed before this study
- screening of boys and adult males with addisonism
- screening of other groups of neurological disorders, like HSP and PPMS
- newborn screening – not yet implemented in Norway

Extended family screening. After identifying X-ALD probands, the next step is extended family screening. Bezman [76] describes well the challenges of approaching families struck by X-ALD, asking them to engage in pedigree exploration and testing of family members at risk of having the mutation. This may be difficult, particularly at a time when a boy with CCER is severely ill or have recently died. Such screening will often reveal presymptomatic X-ALD in a younger brother or cousin, adding to the family's trauma. Mothers of affected boys may find themselves in a clinch – being caregivers of or grieving for their son, while at the same time having to cope with the realization that they themselves are affected by a neurological disorder. However, equally problematic is approaching families who have lost a son to X-ALD, long after the event, ripping open old

wounds. Some family members, *at risk* according to the pedigree, may be reluctant to face the possibility that they themselves may harbor the gene that had such a devastating effect in their relative. Bezman recommends sensitive and careful pedigree assembly at the time of diagnosis unless the opportunity to identify other affected family members should be missed.

In our survey, we screened the affected kindreds as extensively as possible. Half of the kindreds were explored until no "loose ends" remained. However, among the 73 *at risk* subjects identified through pedigree screening, only half of them could be excluded by way of VLCFA or mutational analysis. Thirty-six subjects remained unclarified, either because we were unable to reach them, or because they declined diagnostic clarification. Twelve of these were girls < 16 years of age; it is highly likely that some of them are actually presymptomatic heterozygotes. Other *at risk* subjects were, based on age, gender, apparent health status and production of healthy offspring, unlikely carriers.
However, our experience with the patient described in Paper 5 was thought-provoking: During our extended family screening, we might well have reasoned that a male > 60 years, with no sign of addisonism, walking strenuous hikes in the mountains, could hardly have X-ALD – and we would have been wrong.

Screening for X-ALD. Systematic screening of particular patient groups with differential diagnoses to X-ALD has been discussed in this thesis. Screening subjects with HSP from South East Norway (55 % of the Norwegian population) [136] led to identification of two of the 22 kindreds described in Paper 1; extending the screening to the remainder of the HSP population in Norway might have yielded more X-ALD subjects. Also, female HSP subjects were screened by way of VLCFA analysis only; as in Paper 4, there is a risk that AMN females with false negative VLCFA results have been missed.
In Paper 3 we screened a large part of the adult male population with non-autoimmune PAI in Norway. This revealed no X-ALD subjects not already known to us, but illustrated the point that even when PAI and myelopathy occur together,

the AMN diagnosis may be delayed.

Screening PPMS patients in Oslo (Paper 4) did not capture any X-ALD subjects. Although the methodological weaknesses of that study mandate cautious interpretation, at least we showed that misdiagnosis of X-ALD as PPMS was not frequent.

All these screening efforts included only parts of the relevant patient populations in Norway. Registry studies, or studies performed long after initial diagnosis, easily suffer from incomplete participation, "consent fatigue" or unwillingness to embark on new diagnostic voyages once a diagnosis has been made. Given the very serious consequences both of making the diagnosis of X-ALD, and of missing it (with ramifications for the extended family), we recommend screening for X-ALD at onset or early in the diagnostic process for these patient groups. Suggestions for screening are presented in Table 12.

Table 12. Suggestions for screening patient groups for X-ALD.

- Boys with Attention deficit hyperactivity disorder (ADHD) – see text
- Boys < 18 years with Addison's disease
- Adult males ≥ 18 years with non-autoimmune Addison's disease (negative 21OHAb)
- HSP patients without other apparent or genetically verified cause of HSP
- PPMS patients, particularly if dominated by myelopathy, or if associated with peripheral neuropathy
- Patients with neurological disorders associated with Addison's disease

Note: Male subjects may be screened using VLCFA levels in blood. For female subjects, VLCFA is unreliable; consider mutational analysis of the *ABCD1* gene.

Of particular concern is whether boys with ADHD should be tested for X-ALD. This has not been touched upon in this thesis; however, several of the Norwegian boys who died from CCER were initially evaluated for ADHD or similar symptoms of cognition and behavior – as is well-known from the international experience [2, 95]. The clinical problem is that if the diagnosis is delayed, then the

cerebral demyelination may advance too far for HCST to be effective. From an epidemiological point of view, though, boys in whom CER is initially mistaken for ADHD will hardly be "missed", since the true diagnosis will rapidly be revealed. However, the few cases of "arrested cerebral ALD" (estimated at 10 % [2]) are thought-provoking – in theory, such cases could go undetected for years, until MRI was performed revealing the underlying cause. How to approach investigation of the large group of boys with ADHD and other problems of cognition and behavior is beyond the scope of this thesis – however, child psychiatrists and other involved personnel ought to be aware of the small possibility that X-ALD may be the underlying cause.

Newborn screening. The controversy regarding the incidence at birth of X-ALD will find its solution through newborn screening [81] (see section 1.4). This is now under implementation in some American states [84], and frequency estimates are eagerly anticipated (preliminary results reportedly suggest 1:30,000 [164]). However, some caution should perhaps be exercised, when these figures arrive. The frequency estimates for X-ALD have so far been based largely on symptomatic subjects. Our own research (Papers 1 and 2) and that of others [8, 9, 36, 37] indicate a more severe course for both male and female subjects than previously envisioned, with an age-dependent penetrance that may approach 100 %. Yet, as discussed in section 5.2.1 below, X-ALD may also be mild. With earlier and more comprehensive diagnosis, we may find that the disease spectrum is changed, with less severe forms becoming relatively more frequent.

To conclude the epidemiological discussion, this project has greatly improved our knowledge about the occurrence of X-ALD in Norway. Although inclusion of affected subjects may not have been complete, unidentified subjects may exist and there are still some unexplored parts of the pedigrees, our study seems to have captured at least as many subjects with X-ALD as have previous reports, relative to population size. In contrast to other studies, *at risk* subjects have been tracked and accounted for, and may tentatively be included in the epidemiological

estimates (see Table 9, page 89). The challenges encountered during patient inclusion in this study must have afflicted previous studies as well. Incomplete capture of Norwegian X-ALD subjects is not a likely explanation for the discrepancy between Norwegian and American incidence estimates. Newborn screening will hopefully resolve this controversy.

5.2 Phenotype studies

5.2.1 Spectrum of male phenotypes

Cerebral involvement in adults. The distribution of phenotypes among X-ALD males in Norway (see Table 13) was comparable to previous studies [7, 30, 73-78]. However, one important finding was that no Norwegian male diagnosed with X-ALD (except for one man, previously described by Sanaker [165], who died from cancer at the age of 41) had died without developing cerebral ALD. Other research [36, 37] has demonstrated a high rate of conversion to CER among adult males with AMN: after about 10 years of follow-up, between 19 and 63 % had developed CER with fatal outcome.

Our cohort is small, and the poor outcome for X-ALD males might be due to chance. Furthermore, there is a clear risk that X-ALD males without cerebral symptoms have been missed by our search, as this part of the study was performed retrospectively. Finally, several elderly Norwegian AMN males who are alive today have so far disproved this pessimistic outcome. Despite these factors, our findings are indeed consistent with a bleaker view on the prognosis of AMN, with a higher proportion of male subjects developing CER during their lifetimes than previously thought.

Whereas the cerebral demyelination in X-ALD is marked by an autoimmune inflammatory response, the triggering factor for this process might be non-inflammatory or external. For instance, head trauma has been linked to precipitation of CER [38]. Conceivably, other forms of acute cerebral damage (like ischemic stroke, frequent in elderly patients) might trigger a similar inflammatory

reaction. In theory, this might even affect elderly females. So far, only a few cases of cerebral involvement in females have been reported [166-170]. However, keeping in mind the surprisingly high frequency of manifesting heterozygotes revealed when studied systematically and in a population-based setting [8, 171], female cohorts ought to be followed prospectively with regards to function and cerebral involvement even late in life.

Table 4. Phenotype assignment of Norwegian subjects with X-linked adrenoleukodystrophy

	Alive		Deceased		Total
	n (%)	Age (range)	n (%)	Age (range)	n (%)
Males					
CCER	0	–	14 (67)	9 (6-17)	14 (41)
Adol-CER	2 (15)	14 (14)	2 (10)	26 (21-31)	4 (12)
Adult-CER	0	–	0	–	0
AMN	6 (46)	49 (23-72)	1 (5)	41 (41)	7 (21)
AMN-CER	0	–	4 (19)	49 (37-57)	4 (12)
ADD	5 (38)	12 (9-17)	0	–	5 (15)
Total	13	–	21	–	34
Females					
AMN	6 (23)	65 (55-78)	3 (100)	81 (67-87)	9 (31)
SIGNS	5 (19)	52 (42-81)	0*	–	5 (17)
ASYMP	11 (42)	37 (17-50)	0*	–	11 (38)
Not examined	4 (15)	52 (31-61)	0*	–	4 (14)
Total	26	–	3	–	29

Abbreviations (for definition of phenotype categories, see Table 2)
ADD = Addison only phenotype
Adol-CER = Adolescent cerebral ALD
Adult-CER = Adult cerebral ALD
AMN = adrenomyeloneuropathy
AMN-CER = AMN with cerebral leukodystrophy
ASYMP = Asymptomatic
CCER = Childhood cerebral adrenoleukodystrophy
SIGNS = Neurologic signs only
n = Number of identified subjects, percentage in parentheses
age = Age at examination or (for deceased subjects) at death, median value with range in parentheses

* No deceased females without history of AMN were identified as X-ALD heterozygotes.

Table 13. Results of Norwegian X-ALD survey. From Horn et al, Pediatr Neurol 2013;48:212-219

The severe outcomes observed in the Norwegian cohort, and the results from longitudinal follow-up of adult AMN males from the USA and the Netherlands [36, 37], serve to obscure the clear demarcation between the fatal CCER of childhood X-ALD and the adult male phenotype, thought to be more benign. Perhaps development of CER in males is more a question of when, rather than if. If so, there would be a need for finding safe and tolerable treatment options for CER – for adult males as well. Regular monitoring with MRI might be necessary throughout adult life, not only during childhood. If any efficacious primary prophylaxis for CER is found, then this more bleak prospect might justify treating all males, regardless of symptoms.

Consider this thought experiment: About one third of X-ALD males develop cerebral ALD in childhood. Most of those who survive adulthood develop AMN in adulthood (this may occur even in boys successfully treated with HCST for cerebral ALD [118]. In the Dutch study [37] 63 % of consecutive AMN males developed cerebral ALD during the follow-up period of about 10 years. Taken together, these figures may indicate that as many as three out of four X-ALD males will, eventually, develop cerebral ALD during their lifetimes. If the risk of cerebral ALD continues to increase even beyond the limited observation period of the Dutch study, the proportion ultimately developing cerebral ALD might in principle approach the observation in the Norwegian cohort (95 %).

X-ALD may also be mild. Then, on the other hand, the case of mild X-ALD presented in Paper 5 and brief descriptions in other reports [7, 30, 67, 68] illustrate that the spectrum of X-ALD phenotypes may be wider and more unpredictable still. Cases with “arrested cerebral ALD” are believed to constitute 10 % of CER cases [2]. We cannot really know the frequency of the very mild phenotypes – mild enough to go undetected – until for instance newborn screening allows us to identify the entire X-ALD population and follow it prospectively. However, when such a comprehensive diagnostic strategy is introduced and follow-up is improved – will those “extra” new cases be mild phenotypes (previously undetected), or will

they be severe phenotypes (previously misdiagnosed)?
The existence of mild phenotypes even in males is important and could be a source of hope. But the widening of the disease spectrum may actually make counselling, prognostic estimates and therapeutic decisions more difficult. As long as we cannot predict severity and long-term outcome, hard choices may become harder, for instance regarding dramatic therapeutic interventions in childhood, or selective abortion of male fetuses.
In theory, there might exist hitherto unknown protective factors (genetic or other) diverting some subjects from the common course of X-ALD. This highlights the need for further research into the natural history of X-ALD, of population-based studies avoiding bias towards the most severely affected cases, and of a continued search for predictors or biomarkers that may help identifying subjects at risk of progression and thus in need of therapy.

5.2.2 Women with X-ALD

Age-dependent penetration in heterozygotes. It was well-known that some female relatives of X-ALD males might have themselves have symptoms [2, 7]. Textbook knowledge, even referred to by genetic counsellors at our hospital, states that about 20 % of females develop myelopathy similar to the AMN phenotype found in males, although at a later age and without adrenal failure. However, when examining mothers of boys with X-ALD, Dr. Sakkubai Naidu found that half of them had varying degrees of neurological signs or symptoms [7]. In contrast, in the Australasian X-ALD survey [74], only 7 % of heterozygotes were reported to have neurological symptoms. However, that study did not describe the age composition of the female cohort, and classification was based on referral letters, not systematic clinical evaluation. In all, the phenotypic spectrum among females was poorly mapped; females had to a large part been overlooked, or regarded simply as carriers and relatives of male subjects [5].

Previous review articles [2] had suggested that at least 65 % of heterozygotes had developed AMN at the age of 60 years. However, our Paper 1 was the first in

which an age-dependent penetrance was shown in detail, in a population-based setting without selection bias [35, 171]. All ASYMP females in Norway were < 50 years of age, with median age of 37. All those with symptomatic AMN were > 50 years of age, with median age 65.

The most interesting category was the subjects in the SIGNS group, who were surprised to find that they, despite normal functioning, had subtle neurological signs indicating long tract involvement. Although the age range of this group was large (42 to 81 years), the median age was 52, and placed this group in-between the ASYMP and the AMN group. Our interpretation was that females heterozygous for the *ABCD1* mutation are neurologically unaffected at young age, and with rising age they start developing signs of long tract involvement that later on turns into symptomatic and disabling myelopathy.

This age-dependence of severity of neurological involvement is reflected in the findings from Paper 2. As both studies are small (based on 22 and 8 females, respectively), caution must exercised in the interpretation.

Comparison with other studies on heterozygotes. Our findings are supported by evidence from two other studies, published shortly after our own. The Dutch cohort [8] consisted of 46 females from 26 kindreds. The authors estimated that they captured 25-30 % of the Dutch female X-ALD population, although application of the prevalence figures found in Norway would indicate that there are roughly 90 heterozygotes in the Netherlands[5]. Neurological signs and symptoms attributable to myelopathy or peripheral neuropathy were recorded. The women were grouped according to age categories 18-39 (24 % of the women), 40-59 (59 %) and > 60 years (17 %). Similar to our findings, the women in the youngest group had few signs or symptoms of neurological involvement; only 2/11 were found to be symptomatic. In the middle age group (which might be compared age-wise to our SIGNS subjects), a variety of neurological signs or symptoms led to

[5] The authors have, so far, not published updated frequency data from the Netherlands. However, if the Norwegian estimates are reliable, the cohort presented by Engelen and coworkers would actually constitute about 50 % of the female Dutch X-ALD population.

82 % being classified as “symptomatic”. In the oldest age category, 7/8 (88 %) were classified as “symptomatic”, and all seven women were diagnosed with myelopathy.

In the descriptive cohort study from the South Region of Brazil, Habekost [9] described the phenotypes of 33 X-ALD heterozygotes belonging to 16 kindreds, with mean age 41.2 $\pm$ 11.9 years (range 25-61). Fourteen of the kindreds were from the state of Rio Grande do Sul (population 11 million), 1 family was from Santa Catarina state (population 6.5 million) and 1 family was from the state of Mato Grosso (population 3 million) (Laura Jardim, email correspondence August 27th 2014). The number of kindreds relative to population size was 1.3, 0.15 and 0.33 kindreds per million inhabitants, respectively for the three states, markedly lower than the 4.5 per million found in Norway. The number of females relative to the total population was 0.16:100,000, compared to 0.52:100,000 in Norway. Therefore, the Brazilian survey apparently captured only a fraction of the heterozygotes in that region (if the prevalence of X-ALD heterozygotes in South Brazil is similar to that in Norway, there should be roughly 200 heterozygotes in that region).

As in the Dutch study and ours, asymptomatic women were markedly younger than the symptomatic ones. A surprisingly high proportion, 29/33 (87 %), was classified as symptomatic based on presence of symptoms of neuropathic pain, sphincter dysfunction, paresthesia or paresis. Only 13 % were asymptomatic. However, the patient material, representing perhaps one sixth of the actual population, may have been selected towards inclusion of those more severely affected: the authors report loss of follow-up and may have missed inclusion of asymptomatic females. Furthermore, classification as “symptomatic” in this study was done by the patients themselves. This may be a more sensitive, and less specific, way of classification than in our study, where classification as “symptomatic” was made by the neurologist, requiring that the reported symptoms were compatible with a neurological understanding of myelopathy and neuropathy.

To conclude the phenotype discussion, our studies and those of others indicate a more severe course of X-ALD in both males and females than previously envisioned. On the other hand, mild cases obviously exist as well. Newborn screening may, again, be the only method sensitive enough to allow clarifying the true spectrum of X-ALD phenotypes.

5.3 Genetic studies

***De novo* mutations.** The most important genetic finding in Paper 1 was the high frequency of definite and possible *de novo* mutations. As discussed in section 1.6, previous studies [76, 79, 98] have found *de novo* mutations to be surprisingly uncommon, considering the expected rate of *de novo* mutations and the large spectrum of unique, "private" mutations observed in the different populations.

The high frequency of *de novo* mutations we observed may be a result of chance – due to the small size of the source population. Still, our patient material contains a larger proportion of single-member kindreds, and relatively more kindreds, than found in other populations. We interpret this as yet another indication that our search strategy was good, and that we were able to capture not only large, well-established X-ALD kindreds, but single cases as well.

Three of the four kindreds in which a *de novo* mutation was possible, but not confirmed, would not have been included in the definition used by Wang [79], as the new mutation was suspected in the mother of the index case. Furthermore, as discussed in section 3.6 and section 5.1.3 (Extended family screening), the observation of the mild phenotype in a 61 years old male made us realize the danger in ruling out X-ALD in an elderly male, based on clinical symptoms alone.

Still, these families illustrate an important point: in our survey – which we believe has less center-bias and a more comprehensive inclusion strategy than previous studies – the sudden appearance of X-ALD in a previously healthy family was not at all uncommon. We have afterwards discovered another X-ALD kindred in which the mother has a possible *de novo* mutation, and the patient presented in

Paper 5 in whom no evidence of X-ALD was found in the pedigree. These findings provide a strong argument for screening strategies like newborn screening, if the aim is to provide young boys and older males with appropriate diagnosis and follow-up for adrenal failure and cerebral demyelination (see section 1.4).

Genotype-phenotype correlations. It is a common truism that "there are no genotype-phenotype correlations in X-ALD" [2, 63, 69, 95, 164]. This is based on the observation that even in a single pedigree with a single *ABCD1* mutation, all forms of X-ALD may be observed: Both cerebral and non-cerebral forms, both males with and without adrenal failure, both symptomatic and asymptomatic women. Age at onset and at death appears to vary, without any correlation with the gene defect. Differing courses have even been seen among monozygotic twins [172] (although in our cohort, two monozygotic twins had a remarkably similar presentation at age 14 with MRI-determined onset of Adol-CER and subclinical PAI [35]). Importantly, it is the unpredictability of whether CER will occur or not, that has been the chief argument against a genotype-phenotype correlation in X-ALD.

However, this argument rests on a concept that all the diverse manifestations of X-ALD are phenotypic aspects of the same genotype. This may not be true. As discussed in section 1.2.2 (Myeloneuropathy), a modern view on X-ALD is that the myeloneuropathy due to slow axonal degeneration is the basic phenotype. It is seen to varying degrees in most or all subjects, regardless of gender, but with age-dependent penetrance. Evidence from the *Abcd1* knockout mouse [45] supports this view. The difference seen between males and females in terms of the age at onset and rate of progression of myeloneuropathy is perhaps reflected in differences in the genetic condition (females have a normal copy of the gene, males have not) and the biochemical effect (males have higher VLCFA levels than females do); it seems likely that at least the very existence of myeloneuropathy in a X-ALD subject may be some sort of a genotype effect. If so, the question would be whether the severity of the myeloneuropathy, the age at onset and rate of

progression, may differ between families with different mutations, and whether they might actually be consequences of the genotype.

The cerebral demyelination in X-ALD is occurring far more erratically than the myeloneuropathy. Although recent research [35-37] indicates that a large proportion of X-ALD males, perhaps the majority, are at risk of developing CER during their lifetimes, this may happen at very different times in life. The vulnerability for developing CER may be due to the genetic defect, and the biochemical consequences in cells and tissues. But some evidence [38] suggests that the triggering factor may be external and thus not related to the genotype at all.

We believe genotype-phenotype-correlations in X-ALD should be studied by separating the cerebral and the myeloneuropathy phenotypes, which appears to be un- or not intimately related processes. This is difficult to achieve in males, since at least one third of males are either torn away due to CER in childhood or adolescence, or they are transplanted and no longer follow a natural course. Even among adult males, CER may suddenly occur and disrupt the otherwise slow progression of myeloneuropathy [37].

In females, on the other hand, CER appears to be very rare. We now have established that the majority of heterozygotes develop myeloneuropathy during adult life, with varying degrees of severity. As larger cohorts of women with X-ALD are being diagnosed and classified, we may start looking for genetic or other factors associated with age at onset and rate of progression of their myeloneuropathy in longitudinal follow-up. For that purpose, paraclinical methods such as MRI studies [129], neurophysiology [90] and small nerve fiber testing [104] may prove useful.

To conclude the genetic discussion, *de novo* mutations were more frequent in our study than previously reported. This finding may actually fit better with the expected frequency and with the high number of unique mutations described in the *ABCD1* gene. This strengthens the argument for newborn screening for X-ALD. Our study did not look for genotype-phenotype correlations. However, our

observations add support to the hypothesis of AMN as the basic phenotype in X-ALD; hence it might be worthwhile to study the severity of the myeloneuropathy (disregarding the cerebral demyelination) as a possible phenotypic effect of the genotype.

5.4 Small nerve fiber involvement in X-ALD

The most important finding in Paper 2 was the observation that small nerve fiber involvement was indeed frequent in a cohort of X-ALD subjects of both genders, with a wide range of ages, and with varying degrees of neurological manifestations. This had not previously been shown, although other reports have found a variety of neurophysiologic pathology in the long tracts of the spinal cord and in peripheral large nerve fibers [65, 90, 91].

X-ALD is considered to be a disorder of demyelination – as reflected in the term "leukodystrophy" – due to the demyelinative changes seen in the lesions of cerebral ALD. In contrast, the pathological lesions seen in the long tracts of AMN patients has been categorized as a dying-back axonopathy [86, 87]. Furthermore, the majority of peripheral nerve lesions have been described as axonal rather than demyelinating [65], although some have found a predominance of demyelinating pathology [66, 91].

Pathological studies [86] of the spinal cord of AMN subjects have found atrophy of the corticospinal and dorsal column tracts – both of which contain myelinated fibers. So far, atrophy of the spinothalamic tracts has not been found – but neither have these tracts been explicitly described as normal. In one reported case [58], MRI showed hyperintense lesions corresponding to the spinothalamic tract at the level of the thalamus.

Our findings are interesting, because they demonstrate involvement of nerve fibers with scanty or none at all myelin sheaths. In theory, the axonopathy seen in AMN might be secondary to some form of myelin dysfunction, either due to an autoimmune response (not observed in AMN) or to some other harmful effect of

the pathological composition of the myelin sheaths. However, such a theory would not readily explain why even small, unmyelinated nerve fibers are affected – even in young subjects with few signs or symptoms of dysfunction of the long, myelinated tracts. Our findings might therefore contribute to the understanding of the pathophysiology of X-ALD.

Therapeutic trials in AMN are inherently difficult to perform, due to the rarity of the disorder and the slowly progressive nature of the myeloneuropathy. The mutational heterogeneity, and the so-far unclarified issue of genotype-phenotype correlations, contributes to these difficulties: which patients should be entered into trials aimed at evaluating agents that might slow or arrest – or even improve – the progressive myeloneuropathy? How should they be scored, what measures could be used to predict that the intervention really was able to provide long-term benefits for the patients? And how can such observations be made in a realistic time-frame, with acceptable retention of trial subjects? An accessible biomarker is greatly desired to enable such studies. Based on our findings in Paper 2 small nerve fiber pathology, nigh-universally present in adult X-ALD subjects, may be a useful biomarker for therapeutic trials. In particular, IENFD measurements, although slightly invasive, may be repeated to monitor progression and/or therapeutic effects [173]. However, further studies needs to be done to develop this theory.

In males with X-ALD, the disability due to the myelopathy often dominates the clinical situation. Among females, on the other hand, clinicians have noted that complaints of unexplained pain are frequent (Sakkubai Naidu, Patrick Aubourg, personal communications). Also, the Dutch study of X-ALD heterozygotes showed that problems with fecal incontinence and other sphincteric disturbances are common [8]. It may be that symptoms such as these may partly be explained by small nerve fiber dysfunction. If so, this might direct symptomatic therapies for these problems.

6 Conclusions

- Prevalence and incidence of X-ALD in Norway was lower than expected, based on estimates used in the literature. Paradoxically, we identified a higher number of subjects and kindreds, relative to the population size, than in previous population surveys. Newborn screening, when implemented, may resolve the question of the true incidence of X-ALD.

- Phenotypes of males with X-ALD were compatible with the emerging view that AMN, a slowly progressive degeneration of the long tracts, is the basic phenotype in X-ALD. Cerebral demyelination, while most frequent among young boys, developed also in older males: 95 % of deceased males had developed cerebral demyelination prior to death.

- We found evidence of age-dependent penetrance of X-ALD among females, with young subjects being asymptomatic, elderly subjects having symptomatic myeloneuropathy, and middle-aged subjects showing subtle neurologic signs of emerging myeloneuropathy.

- Small nerve fiber involvement was surprisingly frequent among both males and females, increasing with age and severity of the disease. These findings support the theory that the basic pathology in X-ALD is that of primary axonal damage. Most or all X-ALD heterozygotes will, eventually, develop some degree of neurological involvement. This may contribute to understanding of pain and sphincter disturbances among female subjects.

- *De novo* mutations were frequent in the Norwegian X-ALD population; 19 % of probands had definite de novo mutations, compared to 4-5 % in the literature. Another 19 % of kindreds showed a possible *de novo* mutation. A high frequency of *de novo* mutations strengthens the case for newborn screening for X-ALD, in order to provide timely treatment for adrenal failure, cerebral demyelination and to allow genetic counselling for females.

7 Future perspectives

Several groups around the world, particularly in the USA (Baltimore and Minneapolis), France (Paris), the Netherlands (Amsterdam), Spain (Barcelona), Germany (Wermsdorf) and Austria (Wien), are engaged in clinical, epidemiological, biochemical and genetic research on X-ALD. The clinical classification of the disease is still undergoing change. Therapeutic agents are eagerly sought, although clinical trials are challenging, not least due to the rarity of the disease. We have recently joined a European collaborative effort applying for COST funding. Hopefully, the great insight we have gained in the Norwegian X-ALD population may be put to use in furthering our understanding of this disorder.

Several areas of research need to be looked into:

When newborn screening is implemented…

- What *is* the true incidence of X-ALD?
- What is the true natural history, the spectrum of phenotypes, when all affected subjects are identified at birth and followed longitudinally?

If AMN really is the basic phenotype…

- Can the severity of AMN be evaluated? Age at onset and rate of progression?
- Is the severity related to genotype? Are there genotype-phenotype correlations after all, when the cerebral demyelination is not taken into consideration?
- Are there predictors or biomarkers for the severity of AMN? A role for small nerve fiber measurements, perhaps?
- Can we develop therapies modifying the progression of AMN?

If CER really is an epiphenomenon, distinct from the underlying axonopathy…

- Will it really affect most or all males? What about females, when they get older?
- What triggers or predisposes for CER? Genetic factors or environmental?
- Is it once-in-a-lifetime, or can it recur after successful HCST?
- Can risk of CER be prevented once-and-for-all (in childhood, maybe), or must it be treated whenever it develops?
- Is there a role for any of the myriad of new immunomodulatory drugs?

Specific symptoms of AMN…

- What is the basis for pain syndromes and sphincteric disturbances? Small fiber neuropathy? Can we treat them better?
- How important is really the testicular involvement in X-ALD males? How shall we manage it?
- Endocrinologic function in females – are they really unaffected?
- A majority of our AMN patients displayed a curious dystrophy of the toe nails. Could this be a part of their phenotype?

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Appendix (in Norwegian)

Utkast til program for utredning og oppfølging av pasienter med X-bundet adrenoleukodystrofi

Utarbeidet av Morten Horn, overlege/klinisk stipendiat, Nevrologisk avd. OUS-Ullevål, juni-2015.
Basert på retningslinjer for nyfødtscreening-programmet i New York State publisert 2015 (Vogel et al, Mol Genet Metab 2015;114:599-603).

1. Utredning for X-ALD

X-ALD kan gi symptomer hos menn i alle aldre og hos voksne kvinner, og gir særlig dramatiske symptomer hos gutter i tidlig skolealder. Noen kliniske situasjoner bør gi særlig mistanke om X-ALD:

- Gutter < 18 år som utvikler binyrebarksvikt (Addisons sykdom).
- Voksne menn med ikke-autoimmun binyrebarksvikt (dvs negative 21-hydroxylase-antistoffer)
- Gutter i alderen 4-8 år som utvikler fallende kognitive-/skoleprestasjoner, ADHD-lignende bilde, tap av visuospatiale og motoriske ferdigheter.
- Unge menn (20-40 års alder) og noe eldre kvinner (30-50 år eller eldre) som utvikler langsomt progressiv spastisk paraparese, evt. med kliniske eller nevrofysiologiske tegn til polynevropati. Kan ses hos pasienter (feil-) diagnostisert med hereditær spastisk paraparese eller primær progressiv multippel sklerose.
- Voksne menn, med eller uten forutgående myelonevropati eller binyrebarksvikt, som utvikler progressivt demensbilde der MRI viser hvit substans-forandringer suspekt på X-ALD.

Diagnosen X-ALD stilles vha. klinisk bilde, MRI og klinisk nevrofysiologi, ultralange fettsyrer i blod (VLCFA) og mutasjonsanalyse (*ABCD1*-genet).

- Klinisk bilde: Se over.
- MRI: Ved cerebral ALD ses symmetriske, konfluerende høysignallesjoner med utgangspunkt i splenium (occipitalt mønster, 80 %) eller genu (frontalt mønster, 20 %) av corpus callosum og økende utbredelse utover i hvit substans, evt. med kontrastoppladning i lesjonens ytterkanter.

- Klinisk nevrofysiologi: Typisk funn er sensorimotorisk polynevropati med dominerende axonale forandringer, mest uttalt i underekstremitetene.

- VLCFA: Typisk funn er forhøyet absolutt nivå av C26:0 (NB! Bør tas fastende), samt forhøyet ratio av C26:0/C22:0 og evt. også C24:0/C22:0. Samtlige menn med X-ALD har patologiske VLCFA fra fødsel og gjennom livet. Blant kvinner har 15-31 % normale VLCFA-nivåer i blod, selv om VLCFA-studier i fibroblastkultur vil øke sensitiviteten til opptil 95 %. Hos kvinner må derfor mutasjonsanalyse anvendes for å utelukke X-ALD. Prøve sendes til Seksjon for biokjemisk genetikk, Avdeling for medisinsk biokjemi, OUS-Rikshospitalet.

- Mutasjonsanalyse: Alle pasienter med X-ALD har mutasjon i *ABCD1*-genet. Minst 700 unike mutasjoner finnes, typisk har hver familie sin «private» mutasjon. *ABCD1*-genet på kromosom Xq28 undersøkes etter klinisk rutine ved Avdeling for medisinsk genetikk, OUS-Ullevål sykehus. Det gjøres sekvensering av alle ti kodende regioner og ekson-intron-overganger, samt MLPA for å utelukke store delesjoner og duplikasjoner.

2. Familiescreening

Når en pasient med X-ALD diagnostiseres bør det tegnes slektstre (pedigree). Alle slektninger som ut fra pedigree kan være bærere av genfeilen bør tilbys utredning for å avklare bærerstatus, etter følgende generelle retningslinjer:

- Den som har fått diagnosen stilt (evt. foresatte til mindreårige pasienter) avgjør selv om han/hun vil informere sine slektninger.

- Den som skal informere sine slektninger oppfordres til å tenke grundig over hvorvidt den enkelte slektning vil ønske informasjon om at han/hun kan være bærer.

- Slektninger som får informasjon om mulig X-ALD bør få enkel tilgang til lege som kan svare på spørsmål om sykdommen, og effektivt henvise videre til diagnostisk avklaring.

- Slektninger som selv har symptomer på X-ALD kan utredes av relevant spesialist. Slektninger som er asymptomatiske henvises genetisk veiledning etter vanlig rutine.

- Menn som er sønner av en mannlig X-ALD-pasient har i teorien 0 % risiko for å arve genfeilen. Kvinner som er døtre av en mannlig pasient har 100 % risiko, forutsatt ekte farskap. Menn og kvinner som er barn av en kvinnelig

X-ALD-pasient har 50 % risiko for å arve genfeilen. Grunnet relativt høy forekomst av *de novo*-mutasjoner er det ikke gitt at moren til en X-ALD-pasient selv har genfeilen. Det er uklart hvor vanlig mutasjoner på kjønnscelle nivå er; søsken av pasienter med *de novo*-mutasjoner kan også arve genfeilen, men vi vet ikke hvor hyppig dette skjer.

3. Oppfølging

Pasienter som får stilt diagnosen X-ALD trenger bred og kompetent oppfølging og informasjon om sykdommen. Oppfølgingen er ofte tverrfaglig, og involverer bl.a. fastlege, barne- og/eller voksen-endokrinolog og nevrolog, genetiker, nevroradiolog, hematolog (ved benmargstransplantasjon), klinisk ernæringsfysiolog, nevropsykolog, barne- og ungdomspsykiatri og sykepleietjenester. Grunnet sykdommens sjeldenhet og kompleksitet bør pasientene følges ved (eller i samarbeid med) universitetssykehus.

1. Gutter inntil 10 år (typisk diagnostisert gjennom familie-screening eller grunnet binyrebarksvikt):

- Klinisk vurdering hos barnelege/-endokrinolog årlig
- s-cortisol og p-ACTH hver 6. måned
- Klinisk vurdering hos barnelege/-nevrolog årlig
- Cerebral MRI uten kontrast hver 6. måned
    - Ved patologi på MRI; supplere med kontrastundersøkelse
- Rutinemessig nevropsykologisk testing er ikke påkrevet

2. Gutter 10-18 år

- Klinisk vurdering hos barnelege/-endokrinolog årlig
- s-cortisol og p-ACTH hver 6. måned
- Klinisk vurdering hos barnelege/-nevrolog årlig
- Cerebral MRI uten kontrast årlig
    - Ved patologi på MRI; supplere med kontrastundersøkelse

3. Menn > 18 år

- Behandlende lege bør være obs. på muligheten for latent binyrebarksvikt

- Spesiell aktsomhet ved infeksjoner, traumer, operasjoner -> sjekke binyrebarkfunksjon
- s-cortisol og p-ACTH årlig
- Klinisk vurdering hos nevrolog årlig
- Cerebral MRI uten kontrast årlig
    - Ved patologi på MRI; supplere med kontrastundersøkelse

4. Jenter opp til 18 år

- Ikke evidensgrunnlag for å anbefale særskilt oppfølging
- Fra 16 års alder har kvinner rett til å be om presymptomatisk testing
- Vær særlig obs. på problematikk med uforutsett svangerskap hos jenter som kan være bærere av X-ALD -> kan ha behov for «akutt» genetisk veiledning
- Utvis aktsomhet ved bruk av NSAIDs til jenter med (mulig) X-ALD

5. Kvinner > 18 år

- s-cortisol og p-ACTH som baseline ved diagnosetidspunkt, siden ved behov
- Utvis aktsomhet ved bruk av NSAIDs til kvinner med X-ALD
- Klinisk vurdering hos nevrolog hvert 2. år
- MRI av cerebrum og medulla spinalis vurderes avhengig av klinikk

4. Behandling

- VLCFA-senkende tiltak: Diettomlegging og Lorenzos olje. Retningslinjer for diettråd og eventuell bruk av Lorenzos olje vil bli utarbeidet av fagmiljøet ved OUS. Per i dag er det ingen konsensus i internasjonal litteratur vedrørende VLCFA-senkende tiltak.
- Behandling av binyrebarksvikt: Cortisolsubstitusjonstereapi. Gutter og voksne menn med latent eller klinisk manifest binyrebarksvikt behandles etter vanlige retningslinjer av barne- eller voksenendokrinolog. Viktig at pasienten/foreldrene/fastlege/annet helsepersonell er aktsom ved interkurrent sykdom der økning av cortisolsubstitusjon er nødvendig.

Endokrinologer som følger gutter og menn med X-ALD bør være obs. på muligheten for utvikling av testikulær svikt.

- Behandling av cerebral ALD: Benmargstransplantasjon. Allogen hematopoietisk stamcelletransplantasjon (HCST) er etablert som behandling av gutter og ungdommer med cerebral ALD i Norge, men forekommer så sjelden at hvert tilfelle krever særskilt vurdering. For voksne menn med cerebral ALD foreligger ingen erfaring eller retningslinjer i litteraturen. Forslag til kriterier for HCST er:
    - Gutt/ung mann < 21 år med verifisert X-ALD-diagnose
    - Cerebral demyelinisering på MRI med Loes score $\geq$ 1 og $\leq$ 9
    - Kontrastladende eller raskt progredierende lesjoner på MRI
    - Normal nevrologisk status (Neurological Function Score < 1)
    - Performance IQ $\geq$ 80

  Ved indikasjon for HCST, fravær av beslektet donor og med kontrastladende lesjoner på MRI kan det være aktuelt med inklusjon i internasjonale studier av autolog HCST med genetisk modifisert benmarg (genterapi).
- Symptomatisk behandling for adrenomyelonevropati hos voksne.
    - Spastisitet (trening, fysioterapi, medikamenter)
    - Gangvansker (fampiridin)
    - Smerter (vurdere medikamenter mot nevropatisk smerte)
    - Vannlatnings- og avføringsproblemer (antimuskarin-medikamenter, andre tiltak)
- Genetisk veiledning og tilbud knyttet til reproduksjon og prenatal diagnostikk.