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ORIGINAL ARTICLE

X-linked adrenoleukodystrophy: phenotype-genotype correlation in hemizygous males and heterozygous females with *ABCD1* mutations

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Abstract

OBJECTIVES: X-linked adrenoleukodystrophy (X-ALD) causes cerebral adrenoleukodystrophy (cALD), myelopathy and/or adrenal insufficiency in males, and myelopathy/peripheral neuropathy in females. These distinct phenotypes are scarcely linked to a specific mutations. The objective herein was to find a link between the phenotype with the genotype mutation, serum very long-chain fatty acids (VLCFA), and the diet with Lorenzo´s and GTO oils in hemizygous males and heterozygous females.

METHODS: A retrospective study design with follow-up of 45 hemizygous males and 50 heterozygous females carrying mutations in *ABCD1* from 35 unrelated families with X-ALD. Mutation analysis was performed by Sanger sequencing of PCR and/or RT-PCR and the severity of missense mutations was evaluated using GERP++ score and CADD score.

RESULTS: Twenty-five described and eight novel *ABCD1* mutations were identified. Fifteen males and 23 females had severe mutations while 30 males and 27 females had less detrimental ones. cALD developed in 25 males (56%) including nine boys with severe mutations, 10 boys with less detrimental mutations and 6 adults with adrenomyelopathy. Myelopathy and/or adrenal insufficiency developed in 14 males (31%), six were asymptomatic. Adrenal insufficiency developed in two of five boys treated with hematopoietic stem cell transplantation (HSCT). Myelopathy/peripheral neuropathy developed in 26% of females. No correlation was found between the disease severity and the genotype, GERP++ and CADD scores, presence/absence of aberrant ALDP protein or X-inactivation. VLCFA were higher in males than heterozygous females and decreased during Lorenzo´s and GTO oils diet without a clear clinical impact on the disease.

CONCLUSION: The prognosis was unfavourable in most males and significant part of females. Therapy with early HSCT is effective. Thus, the need for early diagnosis with the neonatal screening is crucial.

Abbreviations:

ABCD1	- gene encoding Abcd1, the ATP-binding cassette subfamily D member 1 protein
ABCD2	- gene encoding Abcd2 protein
AMN	- adrenomyeloneuropathy
cALD	- cerebral type of X-ALD
DNA	- deoxyribonucleic acid
C22:0	- docosanoic acid
C24:0	- tetracosanoic acid
C26:0	- hexacosanoic acid
HSCT	- hematopoietic stem cell transplantation
LC-MS/MS	- liquid chromatography tandem mass spectrometry
RNA	- ribonucleic acid
VLCFA	- very long-chain fatty acids
X-ALD	- X-linked adrenoleukodystrophy

INTRODUCTION

X-linked adrenoleukodystrophy (X-ADL) is a peroxisomal disease with an incidence of 1 in 17000 including hemizygote males and heterozygote females (Aubourg, 2007). However, neonatal screening programs revealed a higher prevalence of 1 in 4845–9341 (Wiens *et al.* 2019; Lee *et al.* 2020). X-ALD is caused by mutations in gene *ALDP* encoding the ATP-binding cassette protein Abcd1 in the peroxisomal membrane, which transports CoA-activated very long-chain fatty acids (VLCFA) from the cytosol into the peroxisome (Engelen *et al.* 2014; Kemp *et al.* 2016; Huffnagel *et al.* 2017; Turk *et al.* 2020; Zierfuss *et al.* 2020).

Clinically, X-ALD in males may manifest with several phenotypes. The most severe course of the disease is the progressive cerebral adrenoleukodystrophy (cALD) with devastating inflammatory demyelination. It usually manifests in boys between 5 and 11 years of age but it may also develop in the adolescence or adulthood. The second phenotype is myeloneuropathy. It usually starts in adult males between the ages of 20 and 40 years with gait difficulties and slowly progressive spastic paraparesis, sensory ataxia, impaired vibration sense, sphincter dysfunction, pain in legs, and impotence (Engelen *et al.* 2014; Gong *et al.* 2019; Ozdemir Kutbay *et al.* 2019). The third phenotype is an adrenocortical insufficiency or Addison crisis and the lifetime prevalence in X-ALD males is 80 %. In addition, about 65% of heterozygote females may develop myelopathy, peripheral neuropathy and bladder dysfunction by the age of 60 years (Engelen *et al.* 2014; Kemp *et al.* 2016). These distinct phenotypes of X-ALD are scarcely linked to a specific genetic profile.

To add more to the genotype-phenotype link, we performed a retrospective study on male and female X-ALD patients. Herein, we analysed the course of the disease with respect to the serum VLCFA levels, the impact of therapy with hematopoietic stem cell transplantation (HSCT), and the low-fat diet supplemented with Lorenzo´s and GTO oils, in relation to mutations severity, including CADD and GERP++ scores for missense mutations and X-inactivation in females.

PATIENTS AND METHODS

Ethics

The study was approved by the Ethics committee of the General University Hospital in Prague and was conducted in agreement with the Declaration of Helsinki and institutional guidelines. Written informed consent for molecular analyses was obtained from all patients and the parents of children.

Patients

Forty-five hemizygous males aged between 10-72 years and 50 heterozygous females (10-88 years) carrying mutations in *ABCD1* from 35 unrelated families with X-ALD were included in the study. All the cases were diagnosed in our centre. Therefore, longitudinal data from patient charts collected during a period of 4-27 years were reviewed for the onset and course of the disease and compared to serum VLCFA levels, severity of the mutations and the impact of HSCT or low-fat diet supplemented with Lorenzo´s oil (a 4:1 mixture of glyceryl trioleate and glyceryl trierucate) and GTO oil (glyceryl trierucate). VLCFA were repeatedly analysed since the time of diagnosis till 2017, when the diet was discontinued.

VLCFA Assay

VLCFA, hexacosanoic acid (C26:0) and the ratios between hexacosanoic acid and docosanoic acid (C26:0/C22:0) and tetracosanoic acid and docosanoic acid (C24:0/C22:0) were analysed in serum. Total fatty acids are directly transesterified with acetyl chloride in the presence of methanol. Heptacosanoic acid (C27:0) was used as an internal standard. Gas chromatographic identification of methyl esters was performed by comparison of their retention time with reference retention times (Moser 1991).

Molecular analyses

Genomic DNA and total RNA were isolated from peripheral blood using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) and BiOstic Blood Total RNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA), respectively. Reverse transcription was performed using a high-capacity RNA to cDNA Kit (Applied Biosystems, Carlsbad, CA). Mutation analysis was performed by Sanger sequencing of PCR and/or RT-PCR products using a Big Dye Terminator v3.1 Cycle Sequencing Kit and a 3500xL Genetic Analyzer (Applied Biosystems).

For genotype-phenotype correlation, mutations were divided into two groups. Mutations apparently affecting RNA stability (i.e. frameshift, splicing and nonsense mutations) were classified as severe mutations while missense mutations and in-frame deletions were

Tab. 1. Phenotype, VLCFA levels at the time of diagnosis and mutations severity in 45 hemizygous males and 50 heterozygous females carrying mutations in *ABCD1* for X-linked adrenoleukodystrophy

Gender and phenotype	n (%)	Age (years) at onset (range)	VLCFA (μg/ml) and their ratios at the time of diagnosis*			ABCD1 mutation**		Clinical outcome
			C26:0 controls <0.05	C26:0/C22:0 controls <0.02	C24:0/C22:0 controls < 1.1	severe n (%)	less detrimental n (%)	
Males	45		0.82 ± 0.38*	0.057 ± 0.03*	1.55 ± 0.26*	15	30	20 males died between 7 -75 y
Boys with cALD*** since childhood	19 (42 %)	8.4 (5 - 13)				9 (60 %)	10 (33.3 %)	
Males with cALD*** since adulthood	6 (13 %)	36 (26 - 51)	0.83 ± 0.36	0.062 ± 0.03	1.57 ± 0.27	1 (6.7 %)	5 (16.7 %)	Cerebral symptomatology started in 6 adults many years after the onset of AMN
Males with AMN****	9 (20 %)	21.4 (6 - 40)	0.78 ± 0.39	0.055 ± 0.03	1.55 ± 0.19	3 (20 %)	6 (20 %)	
Males with myelopathy	2 (4.4 %)	48 (40 - 56)	0.5 and 0.58	0.03 and 0.03	1.45 and 1.46	-	2 (6.7 %)	
Males with adrenal insufficiency	3 (6.7 %)	18 (6 - 45)	0.2 - 0.9	0,02 - 0.14	1,46 - 1,8	1+ (6.7 %)	2+ (6.7 %)	+ one boy with severe mutation and one boy with moderate mutation had HSCT
Males asymptomatic	6 (13 %)	13 (7 - 21)	0.62 ± 0.20	0.039 ± 0.02	1.48 ± 0.31	1+ (6.7 %)	5++ (16.7 %)	+ boy had HSCT ++ one boy had PAS and one boy had epilepsy
Females	50		0.60 ± 0.31*	0.034 ± 0.02*	1.26 ± 0.18*	23	27	3 females died at the age of 56, 83 and 88 years
Females with myelopathy and/or neuropathy	13 (26 %)	48 (26 - 70)	0.68 ± 0.30	0.038 ± 0.02	1.32 ± 0.15	7 (30 %)	6 (22 %)	
Females asymptomatic	37 (74 %)	41 (10 - 68)	0.56 ± 0.30	0.032 ± 0.02	1.22 ± 0.18	16 (70 %)	21+++ (78 %)	+++ one girl had cerebral palsy

* Blood VLCFA (very long chain fatty acids) levels in males are higher than in females: $p < 0.05$ for C26:0 and $p < 0.001$ for C26:0/C22:0 and C24:0/C22:0; ** mutations in ABCD1 affecting RNA stability (frameshift, splicing and nonsense mutations) were classified as severe mutations while missense mutations and in-frame deletions were referred as less detrimental mutations. The severity of missense mutations was evaluated using GERP++ score (Davydov et al. 2010) and CADD score (using phred value) (Rentzsch et al. 2019); *** cALD - cerebral type of X-adrenoleukodystrophy; **** AMN - adrenomyelopathy; + HSCT - therapy with hematopoietic stem cell transplantation. Five boys were treated with HSCT, three with early HSCT had normal cognitive functions, while the two other boys had a mild cognitive impairment at the time of HSCT, one stabilised and one died due to severe graft versus host disease. Two boys developed adrenal insufficiency after HSCT; ++ one boy with moderate intellectual impairment had PAS (pervasive autistic spectrum) and one boy had epilepsy; +++ one girl had cerebral palsy due to perinatal asphyxia

regarded as less detrimental. The severity of missense mutations was evaluated using GERP++ score (Davydov *et al.* 2010) and CADD score (using phred value) (Rentzsch *et al.* 2019). X-inactivation patterns were examined in 15 heterozygotes by DNA methylation-based assay at two loci (AR and RP2) using digestion with HpaII (Racchi *et al.* 1998; Machado *et al.* 2014). In 4 cases, mRNA samples were available and the relative expression of the mutated/wt *ABCD1* allele was analysed using amplicon sequencing (NEXTERA XT®) and the Illumina MiSeq platform as described previously (Reboun *et al.* 2016). XCI ratios 75:25 or higher were considered as skewed pattern.

Statistics Analysis

Data were reported in mean ± SD. Comparison of markers between patients groups was carried out using MedCalc, version 19 (MedCalc Software, Belgium). An independent samples t-test with D´Agostino-Pearson test for normal distribution was

Tab. 2. VLCFA levels in five boys with X-linked adrenoleukodystrophy at the time of diagnosis and after hematopoietic stem cell transplantation

Age at HSCT* (years)	Mutation (NM_000033.4)	VLCFA** (µg/ml) and their ratios at the time of diagnosis			VLCFA** (µg/ml) and their ratios after HSCT**		
		C26:0 controls <0.05	C26:0/C22:0 controls <0.02	C24:0/C22:0 controls < 1.1	C26:0 controls <0.05	C26:0/C22:0 controls <0.02	C24:0/C22:0 controls < 1.1
14	c.1850G>A	1.37	0.14	1.46	0.65	0.04	1.49
10	c.649A>G	0.8	0.02	1.22	0.31	0.01	1.02
6	c.1785G>A	0.93	0.08	1.81	0.57	0.05	1.17
6	c.697_900+25del	0.2	0.02	1.82	0.6	0.05	1.46
6	c.1866-10G>A	0.49	0.02	0.8	0.55	0.02	0.92
Mean ± SD		0.76 ± 0.4	0.06 ± 0.05	1.42 ± 0.38	0.54 ± 0.12	0.03 ± 0.02	1.21 ± 0.23

* HSCT - therapy with hematopoietic stem cell transplantation; ** VLCFA (very long chain fatty acids) levels in blood

employed and significance on the false discovery rate was set at < 0,05. The one way analysis of variance (ANOVA) with Tukey's post hoc test was performed by R studio 1.3.for VLCFA ratios in male divided on the basis of diagnosis or mutation severity. *P* value of <0.05 was considered significant. Normality and homogeneity of variance were checked by Levene's test or Shapiro-Wilk normality test respectively.

RESULTS

Patients, clinical symptoms and VLCFA levels

The age of onset, clinical symptoms, and serum VLCFA at the time of diagnosis in 45 hemizygous males and 50 heterozygous females carrying mutations in *ABCD1* are shown in Table 1. Twenty-five (56%) males developed cALD. Of the cALD male patients, 19 cases of cALD developed in children, and 6 cases developed in adult age with adrenomyelopathy and cALD (Table 1). As for the non-cerebral 20 X-ALD patients, nine males had adrenomyeloneuropathy, two myeloneuropathy, three isolated adrenal insufficiency, and last six males were asymptomatic. However, of the asymptomatic, two had other health problems including epilepsy and pervasive autistic spectrum (PAS).

Of the 19 boys with cALD, five were treated with HSCT. Three of HSCT-treated boys had normal cognitive functions, but two of them developed adrenal insufficiency. The fourth boy had a mild cognitive impairment at the time of HSCT but stabilized later, and the last one with moderate cognitive impairment at the time of HSCT died due to severe graft versus host disease. In the heterozygous females, 13 females (26 %) developed myelopathy and/or peripheral neuropathy at the age between 26 and 70 years. On the other hand, 37 females (74%) were asymptomatic (Table 1) except one very premature girl with birth weight 620 g and perinatal asphyxia resulting in cerebral palsy.

At the time of diagnosis, serum VLCFA (C26:0 and the ratios C26:0 to C22:0 and C24:0 to C22:0) were significantly higher in hemizygous males than in heterozygous females (p<0.05, <0.001, and <0.001, respectively) (Table 1). However, there were no significant differences between VLCFA levels and clinical course of the disease in hemizygous males. VLCFA levels decreased but did not normalize in all five males treated with HSCT (Table 2). VLCFA also significantly decreased in 35 hemizygous males during the diet with Lorenzo and GTO oils in comparison to VLCFA at the time of diagnosis (data not shown). However, this long-term decline had no impact on the course of the disease. Interestingly, the ratio C24:0 to C22:0 was higher in 15 males with severe mutations in comparison to 30 males with less detrimental mutations (1.67±0.32 vs. 1.49±0.22, p<0.05), but no other differences were seen in C26:0 or the ratio C26:0 to C22:0.

In heterozygous females, serum VLCFA levels were significantly higher in comparison to healthy controls. However, one female (2.4%) had normal C26:0, six females (14%) had normal C26:0/C22:0, and eight females (19%) had normal C24:0/C22:0. No significant differences in VLCFA were seen between heterozygous females with and without clinical symptoms (Table 1).

Twenty-five known and eight novel ALD mutations were identified

The list of identified mutations in the *ABCD1* gene in 35 families with X-linked adrenoleukodystrophy is shown in Table 3. Twenty-five mutations identified in 28 out of 35 families were described previously. In the other 7 families, eight novel variations were found including family 7 with two novel variants on one allele. Severe mutations in *ABCD1* were found in 15 males, and 23 females, and less detrimental mutations were found in 30 hemizygous males and 27 heterozygous females (Table 1). Out of 15 hemizygous males with severe mutation, 9 boys developed cALD during childhood as the first symptom and one male developed cALD 26 years after the onset of myelopathy. Three patients presented with adrenomyelopathy (AMN), one boy had

Tab. 3. List of mutations in the ABCD1 gene in 35 families with X-linked adrenoleukodystrophy

Family	Males/Females	Mutation (NM_000033.4)	Predicted effect on the protein (NP_000024.2)	Mutation severity* severe (S) and less detrimental (LD)	Reference, notes
1	1 / 3	c.97_100delTACC	p. Tyr33ProfsTer34	S	Dvořáková *et al.* 2001
2	1 / 2	c.112C>T	p.Gln38Ter	S	listed in database++
3	1 / 4	c.293C>T	p.Ser98Leu	LD	Feigenbaum *et al.* 1996
4	2 / 1	c.296C>A	p.Ala99Asp	LD	Dvořáková *et al.* 2001
5	1 / 1	c.303G>C	p.Leu101Phe	LD	present study
6	0 / 1	c.521A>G	p.Tyr174Cys	S	Lachtermacher *et al.* 2000
7**	3 / 1	c.544C>T, c.817G>C	p.Arg182Trp, p.Ala273Pro	LD	present study
8	2 / 0	c.649A>G	p.Lys217Glu	LD	Dvořáková *et al.* 2001
9	1 / 0	c.697_900+25del	p.Ala233_Glu300del	LD	Dvořáková *et al.* 2001
10	2 / 2	c.796G>C	p.Gly266Arg	LD	Ying *et al.* 2011
11	2 / 0	c.799delG	p.Glu267SerfsTer69	S	Krasemann *et al.* 1996
12	1 / 0	c.843C>A	p.Tyr281Ter	S	present study
13	0 / 1	c.887A>G	p.Tyr296Cys	LD	Takano *et al.* 1999
14	1 / 0	c.1018_1019ins50	p.Ser340AsnfsTer13	S	present study
15	1 / 0	c.1092delC	p.Val365Ter	S	Dvořáková *et al.* 2001
16	1 / 1	c.1255G>A	p.Val419Met	LD	present study
17 18+ 19+	1 / 2 1 / 2 1 / 3	c.1415_1416delAG	p.Gln472ArgfsTer83	S	Barcelo *et al.* 1994
20	0 / 1	c.1498G>C	p.Gly500Arg	LD	present study
21	2 / 2	c.1515C>G	p.Ile505Met	LD	listed in database++
22	1 / 1	c.1537A>C	p.Lys513Gln	LD	Yo-Tsen *et al.* 2007
23 24+	1 / 2 0 / 2	c.1553G>A	p.Arg518Gln	LD	Imamur et al. 1997
25	1 / 0	c.1744_1752del	p.Val582_His584del	LD	present study
26	2 / 4	c.1785G>A	p.Trp595Ter	S	Takano *et al.* 1999
27	2 / 0	c.1823G>A	p.Gly608Asp	LD	Dvořáková *et al.* 2001
28	2 / 1	c.1850G>A	p.Arg617His	LD	Fanen *et al.* 1994
29	2 / 3	c.1866-10G>A	p.Lys624ProfsTer15	S	Kemp *et al.* 1995
30	2 / 2	c.1888G>A	p.Glu630Lys	LD	Petrillo *et al.* 2013
31	2 / 1	c.1898G>T	p.Ser633Ile	LD	Dvořáková *et al.* 2001
32	2 / 2	c.1900G>A	p.Ala634Thr	LD	listed in database++
33	1 / 1	c.1903G>A	p.Val635Met	LD	Kemp *et al.* 2001
34	2 / 1	c.1979G>C	p.Arg660Pro	LD	Dvořáková *et al.* 2001
35	0 / 3	c.1991+1G>A	deletion of exon 9 and/or frameshift	S	Kemp *et al.* 2001

* Mutations affecting RNA stability were classified as severe and the missense mutations were evaluated as severe or less detrimental using GERP++ and CADD scores; ** Two novel variants on one allele were identified in family 7; + Unrelated families, as far as we know; ++ Unpublished (in database https://adrenoleukodystrophy.info/mutations-and-variants-in-abcd1)

isolated adrenal insufficiency after HSCT, and the last boy is asymptomatic after HSCT. Among 30 males with less detrimental mutations, 10 boys developed cALD during childhood as the first symptom, 5 males developed cALD 7-20 years after the onset of myelopathy and 10-20 years after the onset of adrenal insufficiency. Six males have AMN, two isolated myelopathy, two isolated adrenal insufficiency, including one boy after HSCT, and 5 males who are younger than 21 years are asymptomatic (Table 1).

For a more detailed classification of missense mutations, the CADD and GERP++ scoring was used. Median scores of CADD and GERP++ in males with cALD (27.9 and 5.34) and males without cerebral impairment (27.8 and 4.93) were similar. Genotype-phenotype correlation in patients with missense mutations was also characterized at protein level using the data of ALD Mutation Database (http://www.x-ald.nl). Variants leading to undetectable protein amounts, mutations p.Ile505Met and p.Arg518Gln were found in patients with myelopathy and mutation p.Val635Met was found in one asymptomatic boy. Contrary, variants p.Lys217Glu and p.Gly266Arg, which do not affect the protein stability, were found in patients with cALD. Thus, the mutations which do not affect the protein stability may be also associated with more severe phenotypes. X-inactivation status was determined in 15 heterozygotes females. Using methylation-based assay, seven heterozygotes showed preferential inactivation of a wild type allele; six had random X-inactivation, while preferential inactivation of a mutated allele was observed in two heterozygotes. Symptomatic as well as asymptomatic females were present in all three categories, including twin females with a random X-inactivation (48/52 and 49/51, respectively), one had progressive spastic paraparesis since the age of 26 years and her monozygotic twin sister is asymptomatic at the age of 33 years. To eliminate the risk of crossing over, the relative expression of the mutated and wild-type *ABCD1* allele was analysed in four heterozygotes in whom the mRNA samples were available. One symptomatic female had balanced expression of the mutated and wild type allele (59/41), while the other three heterozygotes showed preferential expression of the mutated *ABCD1* allele. Two of them, showing the ratio of mutated/wild type alleles 89/11 and 75/25, were asymptomatic. The last one with the ratio of 88/12 was symptomatic from the age of 26 years.

DISCUSSION

cALD is the most severe type of X-ALD, and it manifests in boys and less frequently in adult males and adult females. Our data showed that more than half of male patients developed cALD, 76% of these cases occurred in children and 24% in adults. In females with heterozygous form, 26% developed myelopathy and/or peripheral neuropathy. The impairment of peroxisomal beta-oxidation in patients with X-ALD results in accumulation of VLCFA (C24:0 and C26:0) in plasma and tissues, especially in the brain, spinal cord, adrenal cortex and Leydig cells (Kemp *et al.* 2016; Turk *et al.* 2020). In our study, VLCFA in blood were significantly higher in hemizygous males than heterozygous females. However, VLCFA in females may be false negative in approximately 15-20% of cases (Moser *et al.* 1999; Huffnagel *et al.* 2017). In our study, serum C26:0 was increased in all except one female with heterozygous mutation in *ABCD1* gene, but 14-19% of the females had normal ratios of C26:0/C22:0 and C24:0/C22:0.

Similarly to other studies, blood VLCFA levels in our patients did not correlate with the age or clinical course of the disease. As described previously, the diet with Lorenzo and GTO oils decreased serum VLCFA levels but failed to prevent cALD (Moser *et al.* 1999; Habekost *et al.* 2014). This may be due to the inability to achieve therapeutic exposure levels in the CNS (Moser *et al.* 2005), however it was speculated that the diet might have at least a partial preventive effect in boys before the age of 6 years (Aubourg, 2007).

HSCT is the most effective therapy in males with X-ALD in the prevention of devastating cerebral impairment, especially in young boys with normal cognitive functions or at the early stages of cerebral involvement when the MRI of the brain shows a low Loes score (Mahmood *et al.* 2007). The outcomes of boys after HSCT improved due to early diagnosis, better myeloablative conditioning regimens, and adjunctive treatment (Mallack *et al.* 2019), but even early successful HSCT doesn´t prevent later onset of myelopathy or Addison disease (van Geel *et al.* 2015; Zhu *et al.* 2020). In the present study, two out five boys developed adrenal insufficiency after HSCT. Surprisingly, the progression of cerebral impairment stopped spontaneously in two other patients with cerebral onset of disease, but both developed severe myelopathy and adrenal insufficiency. Furthermore and in agreement with (van Geel *et al.* 2015), the VLCFA levels in our patients did not normalize after HSCT.

Heterozygous females carrying a mutation in *ABCD1* gene may develop mild to severe myeloneuropathy-like phenotype and the onset of disease is strongly associated with age (Engelen *et al.* 2014). In a cross-sectional cohort study with 46 heterozygote females carrying a mutation in *ABCD1* gene, 57-63% females developed symptoms of myelopathy and/or peripheral neuropathy and 28 % faecal incontinence (Engelen *et al.* 2014). The frequency of symptoms increased from 18% in women <40 years of age to 88% in women >60 years (Engelen *et al.* 2014). The average age of symptomatic females in our study was seven years higher than the age of asymptomatic heterozygotes, but this difference was not significant.

So far, more than 850 non-recurrent pathogenic variants in *ABCD1* gene are listed in ALD Mutation Database (http://www.x-ald.nl). In this study, we

identified 8 novel mutant alleles, of which one carries two variants. It may be of importance for genetic counselling in affected families that approximately 5% of probands with X-ALD have de novo *ABCD1* mutations and nearly 1% is associated with gonadal or gonosomal mosaicism (Wang *et al.* 2011). In most studies regarding X-ALD patients, no general genotype-phenotype correlation was recognized, even in siblings carrying the same mutation (Kemp *et al.* 2016, Ozdemir Kutbay *et al.* 2019). In accordance with these data, our study did not reveal a clear correlation between phenotype and genotype and several different phenotypes occurred within one family.

The scoring of missense variants using CADD and GERP++ in our study revealed no correlation with respect to phenotype. In agreement with data from ALD Mutation Database (http://www.x-ald.nl), we showed that mutations with no effect on protein stability do not result in less severe phenotypes. Comparing the early onset of cALD in males with severe and less detrimental mutations in our study, cALD developed in 69% of boys with severe mutations and in 33.8% of boys with less detrimental mutations. However, the difference was not significant due to small number of patients. Interestingly, the ratio C24:0 to C22:0 was higher in males with severe mutations in comparison to males with less detrimental mutations. No differences were seen in C26:0 or the ratio C26:0 to C22:0. It was suggested that other environmental factors and a multitude of modifying genes appear to determine the clinical manifestation in this monogenetic but multifactorial disease (Kemp *et al.* 2016). Several teams focused their studies on X-inactivation pattern in heterozygous females carrying mutations in *ABCD1*, but no association between skewing inactivation and symptoms was observed (Engelen *et al.* 2014; Habekost *et al.* 2014). Similarly, herein we did not find any correlation between phenotype of heterozygous females and X-inactivation.

With increasing knowledge in the field of pathophysiology of X-ALD, new clinical trials were prepared (https://clinicaltrials.gov/). It was shown that methyl esters of the VLCFA induce endoplasmatic reticulum stress, mitochondrial dysfunction, and oxidative stress in cell cultures from spinal cord and brain of abcd1 knockout mice and cultivated fibroblasts from X-ALD patients (van de Beek *et al.* 2017). Therapies with antioxidants documented correction of most biomarkers of oxidative damage and inflammation including some clinical improvement (van de Beek *et al.* 2017; Casasnovas *et al.* 2019; Turk *et al.* 2020). Antioxidants may help to ameliorate the pro-inflammatory state that characterizes myelopathy and slow the disease progression (Casasnovas *et al.* 2019). Other experimental treatment strategies for compensation of *ABCD1* mutations are pharmacological compounds upregulating *ABCD2* gene encoding Abcd2, a homologous peroxisomal transporter - cerebral adrenoleukodystrophy-related protein (Zierfuss *et al.* 2020). In a murine model of X-ALD with defective Abcd1, the therapy with thyroid hormone receptor agonist increased Abcd2 and decreased VLCFA in blood, peripheral organs, and brain (Hartley *et al.* 2017). In a study with primary mixed glial cells, Abcd2 was increased in metformin-treated Abcd1-KO mice and fibroblasts from X-ALD patients (Singh *et al.* 2016).

A recent study with Vorinostat induced *ABCD2* gene expression, and ameliorated VLCFA accumulation in X-ADL macrophages, the most affected immune cells in inflammatory cALD. A clinical trial with Vorinostat in three severely affected children normalised cerebrospinal fluid level of albumin and imunoglobulins, as well as their CSF/serum ratios, although the clinical disease status further progressed (Zierfuss *et al.* 2020). Studies with virus-based gene therapy seem to be very promising. For example, in the Abcd1-/- mouse the intrathecal delivery of recombinant AAV serotype 9 (rAAV9) led to a 20% decrease in VLCFA in the spinal cord compared with control animals (Gong *et al.* 2019). A trial in patients suggested that Lenti-D gene therapy with infusion of autologous CD34+ hematopoietic stem cells transduced ex vivo with the elivaldogene tavalentivec (Lenti-D) lentiviral vector that contains *ABCD1* cDNA may be a safe and effective alternative to HSCT in boys with early-stage cALD (Eichler *et al.* 2020; Zhu *et al.* 2020). In addition, therapy with intrathecal baclofen pumps may reduce muscle tone and decrease the pain in affected patients (Hjartarson *et al.* 2018).

In patients with peroxisomal diseases, VLCFA in the blood is high since birth, thus providing the potential for neonatal screening (Moser *et al.* 1999). The new biomarker, C26:0-lysoPC, is elevated in all males and females with X-ALD, including females with normal plasma C26:0 levels. It was demonstrated that the quantification of C26:0-lysoPC in dried blood spots using liquid chromatography tandem mass spectrometry (LC-MS/MS) is an effective screening assay for the early diagnosis of X-ALD, and the method is already a part of the neonatal screening programs in several countries (Huffnagel *et al.* 2017; Wiens *et al.* 2019; Lee *et al.* 2020)

CONCLUSION

In our study, cerebral adrenoleukodystrophy and/or myeloneuropathy developed in 80% of hemizygous males and myelopathy/peripheral neuropathy developed in 26 % of heterozygous females suggesting that the impact of X-ALD is very severe in most males and a significant part of females. The need for the early diagnosis is crucial. The selective metabolic screening of X-ALD is not beneficial and should be replaced with a neonatal screening program. Therapy with early HSCT is effective and several new therapeutic approaches with promising efficacy were suggested.

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