X-Linked Adrenoleukodystrophy: Molecular and Functional Analysis of the \textit{ABCD1} Gene in Argentinean Patients

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Abstract

X-linked adrenoleukodystrophy (X-ALD) is an inherited metabolic disease associated with mutations in the \textit{ABCD1} gene that encodes an ATP-binding cassette transporter protein, ALDP. The disease is characterized by increased concentrations of very long-chain fatty acids (VLCFAs) in plasma and in adrenal, testicular and nervous tissues, due to a defect in peroxisomal VLCFA \(\beta\)-oxidation. In the present study, we analyzed 10 male patients and 17 female carriers from 10 unrelated pedigrees with X-ALD from Argentina. By sequencing the \textit{ABCD1} we detected 9 different mutations, 8 of which were novel. These new mutations were verified by a combination of methods that included both functional (western blot and peroxisomal VLCFA \(\beta\)-oxidation) and bioinformatics analysis. The spectrum of novel mutations consists of 3 frameshift (p.Ser284fs\textsuperscript{16}, p.Glu380Argfs\textsuperscript{21} and p.Thr254Argfs\textsuperscript{82}); a deletion (p.Ser572_Asp575del); a splicing mutation (c.1081+5G>C) and 3 missense mutations (p.Ala341Asp, p.His420Pro and p.Tyr547Cys). In one patient 2 changes were found: a known missense (p.His669Arg) and an unpublished amino acid substitution (p.Ala195Ser). In vitro studies suggest that p.Ala195Ser is a polymorphism. Moreover, we identified two novel intronic polymorphisms and two amino acid polymorphisms. In conclusion, this study extends the spectrum of mutation in X-ALD and facilitates the identification of heterozygous females.

Introduction

X-linked adrenoleukodystrophy (X-ALD), MIM 300100\textsuperscript{1} is the most common inherited peroxisomal disorder with an estimated birth incidence of 1 in 17,000 newborns (male and female)\footnote{MIM: Mendelian Inheritance in Man, see http://www.ncbi.nlm.nih.gov/omim/}. Clinical manifestations of X-ALD are highly variable even in the same family\footnote{X-linked adrenoleukodystrophy is a genetic disorder that affects the brain and the liver. It is caused by mutations in the \textit{ABCD1} gene. The gene encodes a protein that helps the body break down fatty acids. When this protein is not working properly, fatty acids build up in the brain and liver, leading to deterioration of brain tissue and liver damage. X-linked adrenoleukodystrophy is usually diagnosed in boys, but it can also occur in girls with mutations in the \textit{ABCD1} gene.}. The major forms are childhood cerebral ALD (CCALD) and adrenomyeloneuropathy (AMN). Patients can also present with isolated adrenal insuficiency without neurological involvement (Addison-only). Children affected by the cerebral variant show inflammatory demyelination and usually die within 3–5 years after onset of neurologic symptoms\footnote{X-linked adrenoleukodystrophy is a genetic disorder that affects the brain and the liver. It is caused by mutations in the \textit{ABCD1} gene. The gene encodes a protein that helps the body break down fatty acids. When this protein is not working properly, fatty acids build up in the brain and liver, leading to deterioration of brain tissue and liver damage. X-linked adrenoleukodystrophy is usually diagnosed in boys, but it can also occur in girls with mutations in the \textit{ABCD1} gene.}. Clinical phenotypes are characterized by impaired peroxisomal \(\beta\)-oxidation\footnote{X-linked adrenoleukodystrophy is a genetic disorder that affects the brain and the liver. It is caused by mutations in the \textit{ABCD1} gene. The gene encodes a protein that helps the body break down fatty acids. When this protein is not working properly, fatty acids build up in the brain and liver, leading to deterioration of brain tissue and liver damage. X-linked adrenoleukodystrophy is usually diagnosed in boys, but it can also occur in girls with mutations in the \textit{ABCD1} gene.}. Biochemical diagnosis of X-ALD is based on the measurement of VLCFAs\footnote{X-linked adrenoleukodystrophy is a genetic disorder that affects the brain and the liver. It is caused by mutations in the \textit{ABCD1} gene. The gene encodes a protein that helps the body break down fatty acids. When this protein is not working properly, fatty acids build up in the brain and liver, leading to deterioration of brain tissue and liver damage. X-linked adrenoleukodystrophy is usually diagnosed in boys, but it can also occur in girls with mutations in the \textit{ABCD1} gene.}. Plasma VLCFAs are the most specific and sensitive test for diagnosing X-ALD. VLCFAs are a group of fatty acids that are usually oxidized by peroxisomes to shorter fatty acids. In X-ALD, VLCFAs accumulate because of a defect in peroxisomal \(\beta\)-oxidation. This accumulation leads to abnormal VLCFA metabolism and results in clinical manifestations of the disease.

Patients with mutations in the \textit{ABCD1} gene encoding the peroxisomal ABC transporters ALDP. This protein transports VLCFAs across the peroxisomal membrane\footnote{X-linked adrenoleukodystrophy is a genetic disorder that affects the brain and the liver. It is caused by mutations in the \textit{ABCD1} gene. The gene encodes a protein that helps the body break down fatty acids. When this protein is not working properly, fatty acids build up in the brain and liver, leading to deterioration of brain tissue and liver damage. X-linked adrenoleukodystrophy is usually diagnosed in boys, but it can also occur in girls with mutations in the \textit{ABCD1} gene.}. The \textit{ABCD1} gene was mapped to Xq28, consists of 10 exons spanning 19 kilobases of genomic DNA\footnote{X-linked adrenoleukodystrophy is a genetic disorder that affects the brain and the liver. It is caused by mutations in the \textit{ABCD1} gene. The gene encodes a protein that helps the body break down fatty acids. When this protein is not working properly, fatty acids build up in the brain and liver, leading to deterioration of brain tissue and liver damage. X-linked adrenoleukodystrophy is usually diagnosed in boys, but it can also occur in girls with mutations in the \textit{ABCD1} gene.}. It encodes a 745 amino acid protein\footnote{X-linked adrenoleukodystrophy is a genetic disorder that affects the brain and the liver. It is caused by mutations in the \textit{ABCD1} gene. The gene encodes a protein that helps the body break down fatty acids. When this protein is not working properly, fatty acids build up in the brain and liver, leading to deterioration of brain tissue and liver damage. X-linked adrenoleukodystrophy is usually diagnosed in boys, but it can also occur in girls with mutations in the \textit{ABCD1} gene.}. About 600 unique mutations have been identified in the \textit{ABCD1} gene\footnote{X-linked adrenoleukodystrophy is a genetic disorder that affects the brain and the liver. It is caused by mutations in the \textit{ABCD1} gene. The gene encodes a protein that helps the body break down fatty acids. When this protein is not working properly, fatty acids build up in the brain and liver, leading to deterioration of brain tissue and liver damage. X-linked adrenoleukodystrophy is usually diagnosed in boys, but it can also occur in girls with mutations in the \textit{ABCD1} gene.}. The spectrum of \textit{ABCD1} mutations includes frameshifts, deletions, insertions, missense mutations, and splice site mutations. Approximately 50% of the mutations are de novo and 50% are inherited from the mother. In the present study, we analyzed 10 male patients and 17 female carriers from 10 unrelated pedigrees with X-ALD from Argentina. By sequencing the \textit{ABCD1} we detected 9 different mutations, 8 of which were novel. These new mutations were verified by a combination of methods that included both functional (western blot and peroxisomal VLCFA \(\beta\)-oxidation) and bioinformatics analysis. The spectrum of novel mutations consists of 3 frameshift (p.Ser284fs\textsuperscript{16}, p.Glu380Argfs\textsuperscript{21} and p.Thr254Argfs\textsuperscript{82}); a deletion (p.Ser572_Asp575del); a splicing mutation (c.1081+5G>C) and 3 missense mutations (p.Ala341Asp, p.His420Pro and p.Tyr547Cys). In one patient 2 changes were found: a known missense (p.His669Arg) and an unpublished amino acid substitution (p.Ala195Ser). In vitro studies suggest that p.Ala195Ser is a polymorphism. Moreover, we identified two novel intronic polymorphisms and two amino acid polymorphisms. In conclusion, this study extends the spectrum of mutation in X-ALD and facilitates the identification of heterozygous females.
Materials and Methods

Subjects

The investigation was approved by Institutional Ethics Committee for Health Research, CIEIS (Children’s Hospital, San Roque’s Hospital and Rawson’s Hospital, Córdoba-Argentina). Written informed consents were signed by all participants. The genotype was analyzed in 10 patients from 9 Argentinean and 1 Italian unrelated families. The patients came from different regions of Argentina; the clinical data were evaluated in the Centro de Estudio de las Metabolopatias Congénitas (CEMECO), Universidad Nacional de Córdoba, Hospital de Niños de Córdoba. The diagnoses were performed in our institute through measurements of VLCFA concentration [10]. All patients had elevated concentrations of VLCFA in the plasma. Of the 10 patients 3 had CCALD. One patient died at the age of 11, after bone marrow transplant. Two patients have AMN, one had adolescent cerebral ALD, two have Addison-only disease (AO) and one is asymptomatic (Table 1).

Seventeen family members, including eight obligatory heterozygotes, were evaluated for mutations found in probands. Four females are symptomatic; one of them is 9 years old.

Sample Preparation

Genomic DNA and total RNA were extracted from peripheral blood or from cultured skin fibroblasts. Genomic DNA was isolated using Wizard Genomic DNA Kit (Promega). RNA was prepared from white blood cells or cultured skin fibroblasts using Trizol (Invitrogen) according to manufacturer’s instructions. Messenger RNA was reverse-transcribed using M-MLV Reverse Transcriptase (Promega) and random oligonucleotide hexamers (Amersham-GE Healthcare) according to protocol supplied by the manufacturer.

Fibroblast Cultures

Human skin fibroblasts were obtained from X-ALD patients from Children’s Hospital of Córdoba, Argentina. X-ALD diagnosis was confirmed by VLCFA and ABCD1 gene mutation analysis. Control fibroblast was from male anonymous volunteers (aged 20–50). Cells were grown in HAMF10 supplemented with 10% fetal calf serum, 25 mM HEPES, 100 U/ml penicillin, 100 U/ml streptomycin and 2 mM glutamine. Cell lines were used between passage numbers 6 and 20.

Mutational Analysis of ABCD1 Gene

The whole coding region and part of 5’UTR and 3’UTR of the ABCD1 gene was obtained by four PCR reactions using cDNA as template. Alternatively PCR products were prepared from genomic DNA. The purified PCR products were used as a template for nucleotide sequencing. The complete coding region (both sense and antisense strands) was sequenced to exclude the presence of other mutations [11]. Putative mutations were confirmed by sequencing of a second PCR product amplified from another sample of genomic DNA and by the DNA analysis from mothers and relatives whenever possible.

Computational Analysis

Different online algorithms were used to predict the functional consequences of the tested variants. Sorting Intolerant from Tolerant (SIFT, http://blocks.fhcrc.org/sift/SIFT.html) analysis uses alignments of selected ABCD1 sequences to measure conservation of each amino acid between species and calculates whether the biochemical parameters of the exchanged amino acids are similar or disparate [12]. A SIFT score of less than 0.05 indicates a deleterious amino acid substitution. Polymorphism Phenotyping (PolyPhen, http://genetics.bwh.harvard.edu/pph) uses conservation of sequences and structural predictions to determine functional consequence of each variant [13]. PolyPhen scores of less than 1.5 and 2 are categorized as possibly deleterious and greater than 2 are categorized as probably deleterious. Conservation of the mutated residues was assessed by alignment of orthologous and human ABCD1 sequences with ClustalW2, http://www.ebi.ac.uk/clustalw2/[14]. The possible effect of the intronic changes on the mRNA splicing process was analyzed.

Table 1. Molecular analysis of Argentinean patients with X-linked adrenoleukodystrophy.

| AMN | 1<sup>st</sup> Phenotype | 1<sup>st</sup>cDNA mutation | Protein level | Exon/Intron | Polymorphisms | Exon/Intron | Protein level |
|-----|--------------------------|-----------------------------|--------------|-------------|--------------|-------------|--------------|
| 1   | AMN                      | c.2006A>G                   | p.His669Arg  | 10          | c.55G>T      | 1           | p.Ala195Ser  |
|     |                          |                             |              |             | c.1992-32C>T | 9           |
| 2   | CCALD                    | c.1137dupC                  | p.Glu380Argfs*21 | 3         | c.1992-32C>T | 9           |
|     |                          |                             |              |             | c.2019G>T    | 10          | p.Phe673Phe  |
| 3   | AO                       | c.1022C>A                   | p.Ala341Asp  | 2           | c.1634+14T>A | 6           |
|     |                          |                             |              |             | c.1992-32C>T | 9           |
| 4   | AO                       | c.1081+5G>C                 | Splice mutation | 7         | c.1548G>A    | 6           | p.Leu516Leu  |
|     |                          |                             |              |             | c.1992-32C>T | 9           |
| 5   | Asymptomatic             | c.1640A>G                   | p.Tyr547Cys  | 7           |
| 6   | CCALD                    | c.1714_1725dek12bp          | p.Ser572_Asp575del | 7         |
| 7   | Adolescent cerebral ALD  | c.761delC                   | p Thr254Argfs*82 | 1         | c.1634+14T>A | 6           |
| 8   | --                       | c.1259A>C                   | p.His420Pro  | 1           |
| 9   | AMN                      | c.2006A>G                   | p.His669Arg  | 10          | c.1992-32C>T | 9           |
| 10  | CCALD                    | c.852_853insACTC            | p.Ser284fs*16 | 1         |

1 Nucleotides numbered reflects cDNA numbering with +1 corresponding to the A of the ATG initiation codon in the reference sequence (NM000033), according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

2 CCALD-Childhood adrenoleukodystrophy, AMN-adrenomyeloneuropathy, AO- Addison only.

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using two splicing prediction programs: Maximum Entropy (ME, http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseqacc.html) [15] and Neural Network (NN, http://www.fruitfly.org/seq_tools/splice.html) [16].

**ALD cDNA in vitro Mutagenesis**

A plasmid containing the entire coding region of the human ALD cDNA was kindly provided by Dr. Norimasa Takahashi, University of Toyama, Japan [17]. This plasmid was used to carry out in vitro mutagenesis. Briefly, different degenerate primer pair, introducing point mutation c.55 G>T (p.Ala19Ser), c.1259 A>G (p.His420Pro) or c.1640 A>G (p.Tyr547Cys), were used in a thermal cycling reaction (19 cycles). After amplification of the point mutated strand, the non-mutated parental strand was selectively digested with DpnI for 2 hr at 37°C. Competent XL-blue was transformed using Gene pulser and Capacitance Extender plus (BioRad, Hercules, CA); the resulting colonies were analyzed by sequencing for the presence of the respective point mutation. The three plasmids, carrying human ABCD1 cDNA with point mutations, were used for transfection experiments, together with the non-mutated human ABCD1 plasmid as a control.

**Peroxisomal VLCFA β-oxidation Assay**

The method was carried out as described by Kemp et al [18] except that fibroblasts from healthy controls and patients with XALD were cultured in the absence or presence of 15 umol/L D3-C16:0 for 72 hr. The peroxisomal β-oxidation activity was calculated by measurement of the amount of intracellular deuterium-labeled hexadecanoic acid (D3-C16:0) present in nmol/mg of protein. Fatty acids were analyzed by electrospray ionization mass spectrometry [19].

**Western Blot Analysis**

Pelleted cultured fibroblast was resuspended in PBS with complete Mini (Roche) and disrupted by sonication. Protein concentration was measured by the method of Lowry [20]. Equal amounts of protein extract (50 ug per lane) were loaded on SDS polyacrylamide gels, electrophoresed and transferred to a PVDF membrane. Membranes were blocked with 2% BSA in TBPS before incubation with a primary antibody anti-ALDP 1D6 (1:2000; Euromedex) that binds to a 279–482 fragment. The secondary antibody was Goat anti mouse-IRDye800 (1:10000). A prestained molecular weight standard (BioRad) was used. Proteins were detected with Odyssey Infrared Imaging System (LI-COR).

**Results**

**ABCD1 Mutation Spectrum**

Full coding sequence and exon-intron boundaries of the ABCD1 gene were analyzed in 27 unrelated subjects: 10 male patients and 17 female relatives whose heterozygous status was confirmed. We identified nine different mutations: one missense mutation was previously described (p.His669Arg) and eight were new ones (Table 1). These new changes included 3 frameshifts (p.Ser284fs*16; p.Glu380Argfs*21; p.Thr254Argfs*82); a deletion (p.Ser572_Asp573del), a splicing mutation (c.1081+5G>G) and three single base pair substitutions (p.Ala341Asp, p.His420Pro and p.Tyr547Cys). These new mutant alleles that are supposed disease causing mutations were confirmed on two independent PCR products and on mothers’ DNA when available. We have uploaded the mutations and polymorphisms in the X-ALD database and registered in Human genetic database [21].

**Figure 1. Western blot analysis of transient transfected X-ALD fibroblasts.** Each line was loaded with the same amount of total protein extracts, as verified with anti-β-actin protein. Line 1: healthy fibroblasts, line 2: X-linked adrenoleukodystrophy fibroblasts, line 3: fibroblasts expressing wild type ALDP-GFP, line 4: fibroblasts expressing ALDP-GFP (c.55G>T, p.Ala19Ser), line 5: fibroblasts expressing ALDP-GFP (c.1640A>G, p.Tyr547Cys).

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residues, p.Leu303_Glu498. Due to this mutation at the RNA level is r.907_1494del (Table 1). Although sample traces of normal transcripts are also detectable in the patient (data not shown), ALDP was not observed by western blot analysis (Figure 3) and peroxisomal β-oxidation was deficient (Figure 4).

For ME, theoretically, the higher score is more likely that the sequence is a true splice site. For the normal allele, the program calculated a score of 9.22 indicating that the splicing is done (Figure 7A). The ME program estimated a score of 5.49 for this splicing change, thus the probability that this splice site is recognized could be lower. Furthermore, according to the NN program, a gt sequence downstream from the mutation could be used as a cryptic 5' donor splice site (Figure 7B).

In patient 1 (Table 1), we identified two different substitutions, one in exon 1 and other in exon 10 (p.Ala19Ser and p.His669Arg). The last one has been identified previously as a disease causing mutation in the X-ALD database (http://www-x-ald.nl), but p.Ala19Ser has not been reported before. When a construct carrying substitution p.Ala19Ser was transfected into X-ALD fibroblasts, ALDP was detected by western blot analysis (Figure 1) and peroxisomal β-oxidation was restored to control levels (Figure 2). This demonstrates that the p.Ala19Ser may represent a SNP. As expected, SIFT predicted that p.Ala19Ser is tolerated with a score of 0.05 (Table 2). PolyPhen predicted that p.Ala19Ser can be a benign variant although the alanine residue at position 19 was highly conserved between different species (Figure 5).

Finally, five polymorphisms were detected: 3 novel intronic changes (c.1634+14T>A, c.1992-32C>T and c.55G>T or p.Ala19Ser) and 2 previously reported (c.1548G>A, p.Leu516Leu and c.2019G>T, p.Phe673Phe).

Discussion

More than 1200 mutations in the ABCD1 gene, of which 47% are non-recurrent mutations, have been identified and listed in the X-ALD database. The mutation spectrum includes missense (61%), frameshifts (22%), nonsense (10%), and insertions and

Figure 2. Peroxisomal β-oxidation was measured using stable-isotope labeled VLCFA (modified from Kemp et al., 2004). D3-C16:0 levels in normal (positive control), X-ALD fibroblast (mock) and X-ALD transfected fibroblasts with a construct with either wild type (WT) and each mutant ALDP. doi:10.1371/journal.pone.0052635.g002

Figure 3. Western blot analysis of X-ALD patients. Each line was loaded with the same amount of total protein extracts, as verified with anti-β-actin protein. Line 1: healthy fibroblasts, line 2: X-linked adrenoleukodystrophy fibroblasts, line 3: c.1081+5G>C, line 4: p.Ala341Asp. doi:10.1371/journal.pone.0052635.g003
deletions (6%). Because in 0.1% of affected males the plasma C26:0 level is at borderline of the healthy subjects and 15% of female heterozygotes have normal levels of VLCFA [5], genetic analysis is the only reliable method to determine X-ALD [22].

The present study describes the genetic analysis of 10 X-ALD patients and 17 female relatives from 9 unrelated Argentinean families and 1 Italian family. We identified 14 genomic changes, 9 were mutations, (8 of which are novel and private, and one known mutation) and 5 were polymorphisms (3 new and 2 known).

As shown in Table 1, the mutations were widely distributed along the ABCD1 gene and included missense, frameshift and splicing mutations. The majority of X-ALD patients in our study group had non-recurrent (89%) mutations, except two patient (one of them Argentinean and the other of Italian origin) that had the same mutation p.His669Arg. This missense mutation was previously reported in the X-ALD database that refers to two unpublished cases in which the only data available is the lack of protein on western blot.

Validation of new mutations was performed according to the Human Genome Variation Society (HUGO). Nonsense and frameshift mutations, and those that alter highly conserved nucleotides in the splice site (position +1, +2, –1 and –2) can be predicted to affect the primary transcript or protein synthesis. Therefore the frameshift changes p.Ser284fs*16, p.Glu380Argfs*21 and p.Thr254Argfs*82 are causing disease mutations.

To define the effect of missense or intronic changes in non-conserved positions is more difficult. For these cases, we determined that changes were pathogenic using the following criteria: 1) mutations were found as the only change in the coding region and 5' UTR and 3' UTR, with the exception of polymorphisms found in several patients (Table 1), 2) the expression level and activity in β-oxidation was affected and 3) the mutation changes a conserved amino acid residue (Figure 5).

For the p.Ala341Asp, p.Tyr547Cys and p.His420Pro alleles, we did not observe protein in western blots and the level of β-oxidation was deficient (Figures 1, 2, 3, 4 and data not shown). These results confirm that p.Ala341Asp, p.Tyr547Cys and p.His420Pro are the causing disease mutations in each patient. Besides, SIFT predicted that both mutations are non-tolerated and PolyPhen predicted that these variants are possibly and probably damaging, respectively (Table 2). The mutated residues Ala341 and Tyr547 were evolutionarily conserved while the residue His420 is not conserved in all species (Figure 5).

In patient 4 we describe a novel intronic mutation c.1081+5G>C. This substitution affects the 5' donor site of intron 2 and results in the loss of four exons in the mature mRNA (Figure 6). Study of the endogenous protein by western blotting

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**Table 2. Results of computational analysis for missense changes in ABCD1 gene from Argentinean patients.**

| Missense Change | p.Ala19Ser | p.Tyr547Cys | p.His420Pro | p.His669Arg | p.Ala341Asp |
|-----------------|------------|-------------|-------------|-------------|--------------|
| Multiple sequence alignment MSA | Highly conserved | Highly conserved | Relatively conserved | Conserved | Highly conserved |
| http://www.ebi.ac.uk/clustalw2/ | | | | | |
| SIFT | Tolerated | Non-tolerated | Tolerated | Tolerated | Non-tolerated |
| http://blocks.fhcrc.org/sift/SIFT.html | | | | | |
| PolyPhen | Benign | Probably damaging | Probably damaging | Benign | Possibly damaging |
| http://genetics.bwh.harvard.edu/pph | | | | | |

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*Figure 4. Peroxisomal β-oxidation was measured using stable-isotope labeled VLCFA (modified from Kemp et al., 2004). D3-C16:0 levels in normal, X-ALD (mock) and X-ALD fibroblast from patients; c.1081+5G>C and c.1022C>A (p.Ala341Asp).*

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showed that the mutant transcript is not translated (Figure 3); therefore, the levels of β-oxidation are deficient (Figure 4). According to information obtained from the combination of ME and NN programs, this genomic change would generate a loss of normal splice site and the creation of a new cryptic donor site, which could cause the loss of 81 nucleotides and the deletion of 27 amino acids (Figure 7). Both programs clearly predicted that the mutated sequence at position c.1081+5G>C is no longer recognized as a 5' donor splice site. It was confirmed experimentally that splicing of intron 2 was affected, although there were deleted the 3 following exons.

The bioinformatics studies are predictive; however, the results were consistent in most cases with functional studies, except for p.Ala19Ser.

In patient 1 (Table 1) we identified two different one-base substitutions, one new in exon 1 (c.55G>T) and one known in exon 10 (c.2006A>G, http://www-x-ald.nl), both of them causing an amino acid change (p.Ala19Ser and p.His669Arg, respectively). The p.Ala19Ser allele had similar expression and β-oxidation levels to wild type ALDP in the experiments of transient expression in X-ALD fibroblast (Figures 1 and 2). On the other hand, according to the literature when the change p.His669Arg is present, ALDP was not observed in western blot. Besides the fact that p.His669Arg has been found as the only mutation in other patients where no protein was detected in western blot also suggests that p.Ala19Ser is a SNP. However, p.Ala19Ser is highly conserved in all species unlike p.His669 (Figure 6) and both were predicted to be “bening” by the Polyphen program and “tolerated” by the SIFT program (Table 2). Moreover, there are
not studies about its frequency in normal populations. One of the questions to be answered is how these amino acid changes affect the structure and function of ALDP. Different groups investigated the dimerization of the COOH-terminal half of ALDP [17,23] and found that it could dimerize with ALDP and the regions of C-terminal amino acids 361–506 and 631–745 were likely involved in the dimerization [23]. They also suggested that if the subdomain involved in dimerization was incorrectly folded, it failed to interact with its partner correctly. Therefore, an alternative would be that p.His420Pro mutant might be misfolded on the peroxisomal membranes and unable to interact correctly with each ALDP or other peroxisomal ABC proteins such PMP70 or ALDRP [23–27] and are recruited to proteasomes for degradation.

We detected 3 novel intronic SNPs, 2 of them highly represented in our population c.1992-32C>T and c.1634+14T>A present in 6 and 2 patients respectively.

We could confirm the carrier status of 17 women under study, of which 4 are symptomatic. Our result, in accordance with the published data, confirms that there is no correlation between genotype-phenotype. Segregation analysis suggests that in addition to the disease causing ABCD1 mutations and environmental factors, other genetic autosomal inherited factors are involved in the clinical manifestation of X-ALD [28].

Some studies have been published describing clinical and biochemical aspects of X-ALD patients from Latin America [29–31], while only a few studies focused at the molecular level [32,33]. Overall, this study represents the molecular and functional characterization of the largest cohort of Argentinean X-ALD patients studied to date. In addition, it also increases the...
mutational spectrum of ABCD1 gene allelic variants, and facilitates genetic counseling and prenatal diagnosis in affected families as well as in further prevention and therapy.

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Author Contributions

Conceived and designed the experiments: CA AMOR. Performed the experiments: CA HM SK. Analyzed the data: CA. Contributed reagents/materials/analysis tools: CA MRM CA SK LD RDK MM AMOR. Wrote the paper: CA AMOR.

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