UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

ASPECTOS MOLECULARES DA ADRENOLEUCODISTROFIA LIGADA AO X: EPIDEMIOLOGIA, PARADIGMAS DIAGNÓSTICOS E POTENCIAIS GENES MODIFICADORES

FERNANDA DOS SANTOS PEREIRA

Orientadora: Dra. Laura Bannach Jardim

Tese de Doutorado

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LISTA DE ABREVIATURAS

ABCD1: do inglês *ATP-Binding Cassette transporter subfamily D member 1*

ACTH: do inglês a*drenocorticotropic hormone*

ALDP: do inglês *adrenoleukodystrophy protein*

ALDRP: do inglês *adrenoleukodystrophy-related protein*

AMN: adrenomieloneuropatia

APP: proteína precursora amilóide

CALD: do ingles *childhood cerebral X-ALD*

CGH: do inglês *comparative genomic hibridization*

CNV: do inglês *copy number variant*

LCFA: do inglês *long chain fatty acids*

MAPH: do ingles *multiplex amplification and probe hybridization*

MLPA: do ingles *multiplex ligation-dependent probe amplification*

P70R: do inglês *PMP70-related protein*

PAS: do inglês *periodic acid Schiff*

PLP: proteína proteolipídica

PMP70: do inglês *peroxisomal membrane protein*

POMC: do inglês *proopiomelanocortin*

QMPSF: do ingles *quantitative multiplex PCR of short fluorescent fragment*

RNM: ressonância magnética

SNC: sistema nervoso central

SNPs: do inglês *single nucleotide polymorphisms*

TNF-α: do inglês *tumor necrosis factor-α*

VLCFA: do inglês *very long chain fatty acids*

X-ALD: do inglês *X-linked adrenoleukodystrophy*

RESUMO

A adrenoleucodistrofia ligada ao X (X-ALD) é a doença peroxissomal mais freqüente, com uma incidência de 1:20.000 homens na população geral, sendo identificada em todos os grupos étnicos. O gene envolvido na X-ALD é o gene *ABCD1* (*ATP-Binding Cassette transporter subfamily D member 1*), que codifica uma proteína de membrana peroxissomal, ALDP. Mutações nesse gene são relacionadas ao acúmulo de ácidos graxos de cadeia muito longa (*very long chain fatty acids* – VLCFA) em todos os tecidos e fluidos corporais, especialmente no sistema nervoso e glândulas adrenais. Até o momento, mais de 1200 mutações já foram descritas neste gene. Três fenótipos principais são claramente identificados entre homens afetados: a forma cerebral (CALD), caracterizada por resposta inflamatória no SNC, a forma não inflamatória, AMN e a insuficiência adrenal isolada, também chamada de Addison-only. Não há correlação entre o tipo de mutação e o fenótipo clínico e uma possível explicação para essa alta variabilidade fenotípica poderia ser a ação de fatores ambientais e genes modificadores. Nesta tese está descrita, provavelmente, a primeira série de casos de pacientes sul-americanos com X-ALD.

Foram avaliados aspectos moleculares da X-ALD em uma série de 38 famílias procedentes desde a Argentina até o Amazonas, relacionando-os com dados epidemiológicos e com a avaliação da taxa de sensibilidade e especificidade da análise de VLCFA em mulheres; e foi realizada a busca por potenciais genes modificadores que atuem, por CNV, na variabilidade fenotípica da X-ALD. Encontraram-se 36 mutações diferentes: apenas uma delas recorreu em 3 famílias. Doze destas mutações foram novas: a sua morbidade foi sustentada principalmente pelos graves rearranjos ou códons de parada prematuros por elas produzidos (p.Pro623Leu, p.Glu577X, p.Tyr33_Pro34fsX34, p.Arg538fs, p.Ala232fsX64, p.Trp137fsX57, p.Leu628Glu, p.Ile481Phe, p.Ala95fsX11, p.Gln55X, p.Arg401Gly e p.Ser358fsX42). Quatro situações (ou 10% da série) foram documentadas como mutações *de novo*. Como era de se esperar, não foi possível determinar nenhuma correlação entre genótipos e fenótipos. Uma das famílias foi identificada a partir de uma menina com CALD e desvio completo da inativação do X. Através da curva de Kaplan-Meyer, descrevemos a idade média de início das três formas principais nos homens: o início do Addison–only foi em média (IC de 95%) aos 7,4 (5,4-9,4) anos, da CALD, aos 10,9 (9,1-12,7) anos, e da AMN, aos

26,4 (20,3-32,5) anos. Descrevemos também a sobrevida média da CALD como de 24,7 (19,8-29,6) anos. Na presente série, 54 dos 87 afetados (ou 62%) apresentavam o fenótipo CALD, uma taxa maior do que a descrita na literatura (de 45 a 57%). Procuramos alguma evidência que vinculasse esse aparente excesso de formas desmielinizantes com a latitude de origem dos casos, à semelhança da esclerose múltipla: nossos resultados foram negativos.

Em um subgrupo de famílias foi possível comparar o *status* genético final de 50 mulheres (heterozigotas versus normais) decorrente da investigação molecular, com os diagnósticos prévios levantados pela investigação bioquímica dos VLCFA. Encontramos uma elevada taxa de casos VLCFA falso-negativos, de 72,5%, muito maior do que a descrita na literatura (20%) e encontramos também dois casos de mulheres com VLCFA falso-positivos. Concluímos que o uso dos VLCFA deve ser evitado no aconselhamento genético.

Finalmente, voltamos aos hemizigotos, em busca de genes candidatos a modificadores de fenótipos, através da associação dos mesmos com específicas variações no número de cópias (*copy number* variation - CNV) em 29 genes selecionados por seu papel na inflamação, metabolismo dos lipídios ou regeneração do sistema nervoso. A estratégia foi investigar CNVs destes genes através da técnica da Multiple Ligation-dependent Probe Amplification (MLPA) em um grupo de 85 pacientes espanhóis e brasileiros: 39 com AMN e 46 com CALD. Conseguimos identificar CNVs em nove destes genes. Interações potenciais entre estes genes foram inferidas a partir da ferramenta da web Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) versão 9. A análise das redes criadas identificou três nódulos principais, com a POMC (Proopiomelanocortina) sendo a proteína central. Nós então sugerimos estes genes e a própria POMC como genes candidatos a modificadores do fenótipo X-ALD.

ABSTRACT

The X-ALD is the most frequent peroxisomal disease with a pan-ethnic incidence of 1:20,000 males in the general population. The gene involved in X-ALD is the *ABCD1*, which encodes a protein of the peroxisomal membrane, ALDP. Mutations in this gene are related to the accumulation of VLCFA in all tissues and body fluids, especially in the nervous system and adrenal glands. To date, more than 1200 mutations have been described in the gene. Three phenotypes are clearly identified among affected men: the cerebral form (CALD), characterized by inflammation in the CNS, the non-inflammatory form, adrenomyeloneuropathy (AMN), and Addison-only. There is no genotype-phenotype correlation, in spite of the high phenotypic variability observed in affected relatives. This thesis probably describes the first case series of South American patients with X-ALD.

We have evaluated the molecular features of X-ALD, correlating them with epidemiological data in a series of 38 South American families. We have also evaluated the sensitivity and specificity of VLCFA in women, and have searched potential modifier genes that may act by CNV in the male phenotypic variability of X-ALD. We found 36 different mutations, only one recurring in three families. Twelve were new mutations: its morbidity was sustained mainly by major rearrangements or stop codons produced by them (p.Pro623Leu, p.Glu577X, p.Tyr33_Pro34fsX34, p.Arg538fs, p.Ala232fsX64, p.Trp137fsX57, p.Leu628Glu, p.Ile481Phe, p.Ala95fsX11, p.Gln55X, p.Arg401Gly and p.Ser358fsX42). Four (or 10% of the series) were documented as *de novo* mutations. As was expected, no genotype-phenotype correlation was found. One family was identified from a girl with CALD and complete deviation of X inactivation. By Kaplan-Meyer analysis, we have described the onset of the three main forms in men: the mean (95% CI) age at onset of Addison-only was 7.4 (5.4 - 9.4) years, of CALD, 10.9 (9.1 - 12.7) years, and of AMN, 26.4 (20.3 - 32.5) years. We have also estimated the survival of CALD as 24.7 (19.8 to 29.6) years. In this series, 54 of the 87 affected male (or 62%) had the phenotype CALD, a rate higher than that described in the literature (45-57%). We look for any evidence that relates the apparent excess of demyelinating forms with latitude of origin of cases, like multiple sclerosis: the results were negative.

In a subgroup of families, the molecular analyses allowed us to compare the genetic status of 50 women (heterozygous versus normal) with the previous biochemical diagnoses raised up by VLCFA. A high rate of false negative VLCFA was disclosed, of 72.5%, significantly greater than the 20% described in literature. We have also found two false positives VLCFA results in females. We conclude that the use of VLCFA should be avoided in genetic counseling.

Finally, we turned to hemizygotes, in search of candidate genes that could modify the X-ALD phenotype, chosen by for their role in inflammation, lipid metabolism or in the regeneration of the nervous system. The strategy was to investigate CNVs of these genes through the technique of Multiple Ligation-dependent Probe Amplification (MLPA) in a group of 85 Spanish and Brazilian patients: 39 with AMN and 46 with CALD. We have identified nine CNVs in these genes. Potential interactions between these genes were inferred from the tool Web Search Tool for the Retrieval of Interacting Genes / Proteins (STRING) version 9. The analysis of network nodes identified three key created, with POMC (proopiomelanocortin) being the core protein. We then suggested they own and POMC genes as candidate genes modifying the phenotype of X-ALD.

1. INTRODUÇÃO

A adrenoleucodistrofia ligada ao X (X-ALD – OMIM 300100) é a doença peroxissomal mais freqüente, caracterizada pelo catabolismo inadequado dos ácidos graxos de cadeia muito longa completamente saturados (ou VLCFA, do inglês *very long chain fatty acids*). Nessa condição, a degradação dos VLCFA encontra-se impedida ou muito limitada (Moser *et al*., 2001). O acúmulo de VLCFA ocorre em todos os tecidos e fluidos corporais, mas seu impacto clinicamente relevante ocorre na substância branca cerebral, nervos periféricos, glândulas adrenais e testículos.

Como diz o seu nome, a X-ALD é uma doença ligada ao X: afeta mais intensamente os homens ligados entre si através de mulheres (mães, avós maternas, tias, etc). As mulheres heterozigotas também podem apresentar manifestações clínicas um pouco mais tênues e tardias. A X-ALD é causada por mutações no gene *ABCD1* e apresenta uma grande variabilidade fenotípica entre indivíduos afetados da mesma família, independente do tipo de mutação e do local do gene em que ela ocorre. O crescente conhecimento e identificação das diversas formas fenotípicas da X-ALD leva a uma estimativa de incidência de homens portadores de 1:20.000 na população geral (Berger *et al*., 2006), tendo sido identificada em todos os grupos étnicos.

2. REVISÃO DA LITERATURA

2.1 CARACTERÍSTICAS FENOTÍPICAS DA X-ALD

Utilizando como critérios a idade de início, envolvimento clínico e a taxa de progressão dos sintomas neurológicos, Moser e colaboradores (Moser *et al*., 2001) subdividiram as variações da X-ALD em 7 fenótipos em homens e 5 em mulheres heterozigotas. Estes critérios estão listados na tabela 1. Embora essa subdivisão seja um tanto arbitrária, ela tem-se mostrado útil para estudos de mecanismos patogênicos e aconselhamento genético (Moser *et al*., 2001).

Tabela 1: Fenótipos X-ALD em hemizigotos.

Fenótipos em hemizigotos	Descrição	Freqüência relativa estimada
Cerebral infantil (CALD)	Início entre 3 e 10 anos de idade. Déficit progressivo de comportamento, cognitivo e neurológico, muitas vezes levando à total incapacidade dentro de 3 anos. Ocorrência de lesões inflamatórias demielinizantes no cérebro.	31-35%
Cerebral adolescente	Semelhante à CALD, porém de início entre 11 e 21 anos, com progressão um pouco mais lenta.	4-7%
Adrenomieloneuropatia (AMN)	Início entre 29±9 anos, com progressão ao longo de décadas. Envolve principalmente cordão espinhal, com axonopatia distal inflamatória moderada ou ausente. Aproximadamente 40% apresentam ou desenvolvem envolvimento cerebral com variados graus de resposta inflamatória e de progressão mais rápida.	40-46%
Cerebral adulto	Distúrbios de comportamento e demência. Algumas vezes ocorre a presença de déficit focal sem precedência de AMN. Presença de resposta inflamatória na substância branca. Progressão similar à forma CALD	2-5%
Olivo-ponto-cerebelar	Envolvimento principalmente d tronco cerebral e cerebelo na adolescência ou fase adulta.	1-2 %
Addison isolada	Basicamente insuficiência adrenal sem envolvimento neurológico aparente. Início antes dos 7,5 anos. Eventualmente desenvolvem AMN.	Varia com a idade. Mais de 50% na infância
Assintomáticos	Presença da alteração gênica e bioquímica sem demonstração de déficit neurológico ou adrenal. Estudos detalhados muitas vezes apresentam hipofunção adrenal ou sinais discretos de AMN.	Diminui com a idade. Comum em < de 4 anos. Muito rara em > de 40 anos

(Adaptada de Moser *et al*., (2001).

2.1.1 Fenótipos em pacientes X-ALD do sexo masculino

2.1.1.1 Forma cerebral infantil (CALD):

A média de idade de início varia de 3 a 10 anos (figura 1) e sua freqüência relativa apresenta estimativas que variam dos 31%-35% a 45-57% (Moser *et al*., 2001; Dubey *et al*., 2005; Raymond *et al*., 2010; http://www.x-ald.nl). As alterações comportamentais iniciais incluem instabilidade emocional, comportamento hiperativo ou retraído, baixo

rendimento escolar ou uma combinação desses sintomas. Dificuldade na compreensão da fala em um ambiente barulhento ou ao telefone são sintomas iniciais comuns e refletem dificuldades na discriminação auditiva. O déficit de atenção pode evoluir rapidamente e inclui evidentes sinais de disfunção do lobo parietal (Moser *et al*., 2001). A deficiência visual é um sintoma inicial em aproximadamente 33% dos pacientes. Em estágios mais avançados da doença, a visão é totalmente perdida e identifica-se a atrofia óptica. Uma vez que os sintomas neurológicos iniciam, progridem rapidamente (Moser *et al*., 2001).
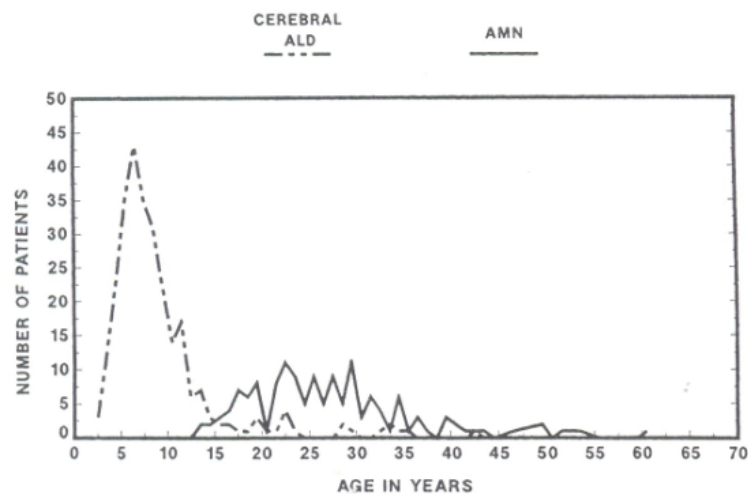
Figura 1: Idade de início dos sintomas neurológicos nas formas cerebral e AMN da X-ALD. (Fonte: Moser *et al*.,2001).

Estudos de acompanhamento de pacientes indicam que informações significativas do prognóstico podem ser obtidas pela subdivisão dos pacientes de acordo com a idade e gravidade das anormalidades identificadas por imagens de ressonância magnética (Moser *et al*., 2001). A gravidade do envolvimento cerebral foi associada a essas imagens através de um sistema de quantificação desenvolvido para X-ALD por Loes e colaboradores (Loes *et al*., 1994). Essa escala tem 34 pontos, graduados em intervalos de 0,5 unidades e está baseada na localização das alterações da substância branca em regiões específicas do cérebro e no grau de atrofia focal ou global. Um grau de 0 a 0,5 é considerado normal.

2.1.1.2 Forma cerebral adolescente:

Os sintomas se iniciam entre 11 e 21 anos e sua progressão nesses pacientes assemelham-se muito à CALD, porém ocorrem um pouco mais lentamente (Moser *et al*., 1987). Este fenótipo tem uma freqüência relativa estimada entre 4% e 7% dos casos.

2.1.1.3 Adrenomieloneuropatia (AMN):

A adrenomieloneuropatia (AMN) caracteriza-se por mielopatia e neuropatia. A deficiência neurológica é de progressão lenta, porém dentro de 5 a 10 anos os distúrbios de marcha tornam-se graves e faz-se necessário o uso de uma bengala ou cadeira de rodas. Distúrbios urinários são notados nas segunda ou terceira décadas de vida. A freqüência relativa estimada desse fenótipo da X-ALD varia de 40% a 46% dos pacientes.

Existem algumas evidências de que haja algum envolvimento cerebral em certos pacientes com AMN. Kumar e colaboradores (Kumar *et al*., 1995) demonstraram anormalidades na ressonância magnética cerebral (RNM) restritas às fibras do trato corticoespinhal na ponte, no pedúnculo cerebral e na cápsula interna. Sugere-se que os casos AMN sem envolvimento cerebral tenham uma progressão clínica mais lenta. Anormalidades cerebrais equivalentes a CALD ainda podem aparecer na história natural de um indivíduo com AMN, equivalendo a uma conversão ao fenótipo desmielinizante e tendo uma progressão neurológica tão grave quanto na forma CALD pura (Kumar *et al*., 1995).

Depressão e distúrbios emocionais são comuns e tornam-se mais graves com o avanço da doença. Impotência iniciando entre os 20 e 30 anos é uma ocorrência comum (Powers *et al*., 1980).

2.1.1.4 Forma cerebral adulta:

O termo "forma cerebral adulta" é aplicado ao paciente com alteração bioquímica para X-ALD que desenvolve sintomas cerebrais depois dos 21 anos de idade, mas que não tem sinais de envolvimento medular. Essa forma é relativamente rara, tendo em torno de 23 casos sido reportados na literatura (Laureti *et al*., 1996), com uma

freqüência relativa estimada entre 2% à 5% dos casos. A idade de início varia entre os 20 e 50 anos. Os sintomas se parecem com esquizofrenia acompanhada de demência ou déficit cerebral específico. Enquanto a presença da doença de Adisson é um indício crucial no diagnóstico, a função adrenal pode ser normal em 30% a 50% dos pacientes (Moser *et al*., 2001).

2.1.1.5 Doença da Addison isolada (Addison-only):

A X-ALD é uma das causas importantes de insuficiência adrenal nos pacientes do sexo masculino. Laureti e colaboradores identificaram a X-ALD em 35% dos pacientes do sexo masculino que foram anteriormente diagnosticados com insuficiência adrenocortical idiopática (Laureti *et al*., 1996).

O fenótipo Addison-only é possivelmente a primeira manifestação clínica de quase todos os hemizigotos, iniciando-se a partir dos 2-3 anos de idade. Portanto, é um fenótipo transitório na maioria das vezes, convertendo-se em CALD ou em AMN na medida em que o tempo passa. No entanto, uma parcela dos hemizigotos poderá manter seu quadro limitado ao Addison.

2.1.1.6 Hemizigotos assintomáticos

A ausência de qualquer manifestação clínica – neurológica ou adrenal – é muito incomum, mas ainda assim pode ocorrer em hemizigotos.

2.1.2 Heterozigotas X-ALD sintomáticas:

O segmento mais negligenciado entre indivíduos afetados pela X-ALD são as mulheres portadoras. Vinte a 50% das mulheres heterozigotas podem manifestar algum sintoma (Jangouk *et al*., 2012). Estima-se que a freqüência de heterozigotas seja a mesma que a dos hemizigotos (1:20.000): no entanto, como muitos desses homens podem morrer sem diagnóstico, sugere-se que as heterozigotas correspondam a 1:14.000 mulheres da população geral (Bezman *et al*., 2001).

Heterozigotas para X-ALD podem desenvolver alteração de marcha, hipertonia e sintomas urinários, manifestações equivalentes à AMN dos homens (Tabela 2). Alguns

estudos sugerem que aproximadamente metade das heterozigotas apresenta algum grau de envolvimento neurológico (Moser *et al*., 1991a). A média de idade de início dos sintomas é muitas vezes maior que a dos homens e a doença é de natureza moderada (van Geel *et al*., 1997),

Tabela 2: Fenótipos X-ALD em heterozigotas.

Fenótipo	Descrição
Assintomática	Sem evidência de envolvimento adrenal ou neurológico.
Mielopatia moderada	Alterações sensoriais distais e aumento dos reflexos do tendão inferior nas extremidades inferiores com incapacidade ausente ou moderada.
Mielopatia moderada a grave	Sintomas e patologia semelhante à AMN, mas de início mais tardio.
Envolvimento cerebral	Raramente visto na adolescência e ligeiramente comum em heterozigotas de mais idade.
Insuficiência adrenal clinicamente evidente	Insuficiência adrenal clinicamente evidente.

Adaptada de Moser *et al*., 2001.

A doença cerebral como uma manifestação em heterozigotas tem sido raramente documentada. Somente 3% das mulheres heterozigotas para X-ALD apresentam doença inflamatória cerebral. Isso vai de encontro à alta freqüência de adrenomieloneuropatia, a qual está presente em aproximadamente 50% delas (Moser *et al*., 1991b). Mulheres heterozigotas, assim, parecem ser suscetíveis à axonopatia não inflamatória, sendo resistentes à doença inflamatória cerebral.

Finalmente, as heterozigotas raramente apresentarão insuficiência adrenal, mesmo a subclínica.

2.2 NEUROPATOLOGIA DA X-ALD

2.2.1 Neuropatologia da CALD

Estudos *postmortem* em cérebros de pacientes CALD mostraram que o córtex está intacto, mas que a mielina é substituída por um tecido marrom acinzentado (Moser *et*

al., 2001). A neuropatologia da CALD é caracterizada pela progressiva degeneração da substância branca cerebral com lesões demielinizantes que são confluentes, geralmente simétricas e apresentam progressão ora caudorostral (65%), ora rostrocaudal (Moser *et al*., 2001). A substância branca do cerebelo também é afetada, embora em menor extensão do que a substância branca do cérebro (Ferrer *et al*., 2010).

A atrofia que afeta os tratos piramidais no tronco cerebral e medula espinhal é observada no estágio mais avançado da doença. Em contraste ao grave envolvimento da substância branca, o córtex cerebral, córtex e núcleos cerebelares e substância cinzenta do tronco cerebral são amplamente poupadas (Ferrer *et al*., 2010) (figura 2).

Três zonas histopatológicas com uma seqüência espaço-temporal podem ser definidas nas lesões demielinizantes da CALD. A primeira zona ou borda periférica, na qual a destruição da mielina ocorre inicialmente, contêm macrófagos sudanofílicos e PAS-positivos espalhados, mas os axônios são preservados. Ela é intimamente seguida por uma segunda zona, na qual existem muitos macrófagos carregados de lipídios com inclusões lamelares e alguns axônios sobreviventes mielinizados. A terceira zona, no centro da lesão, consiste de uma densa rede de fibrilas gliais com ausência de oligodendróglia, bainha de mielina e, muitas vezes, axônios. Cavidades e depósito de cálcio também podem estar presentes na terceira zona, geralmente refletindo um estágio avançado da lesão (Ferrer *et al*., 2010).

A alteração mais notável é a presença de infiltrados inflamatórios na borda das lesões da substância branca, justamente atrás da demielinização frontal ativa (figura 2). Um pouco de infiltrado inflamatório também pode ser encontrado na depressão das áreas demielinizadas. As células inflamatórias são linfócitos T CD4^{+} e CD8^{+}, alguns deles contendo granzima B e são raros os linfócitos B (Ito *et al*., 2001; Hudspeth *et al*., 2007). De maneira importante, lifócitos CD8^{+} são observados em áreas da substância branca afastadas das lesões (Ito *et al*., 2001).
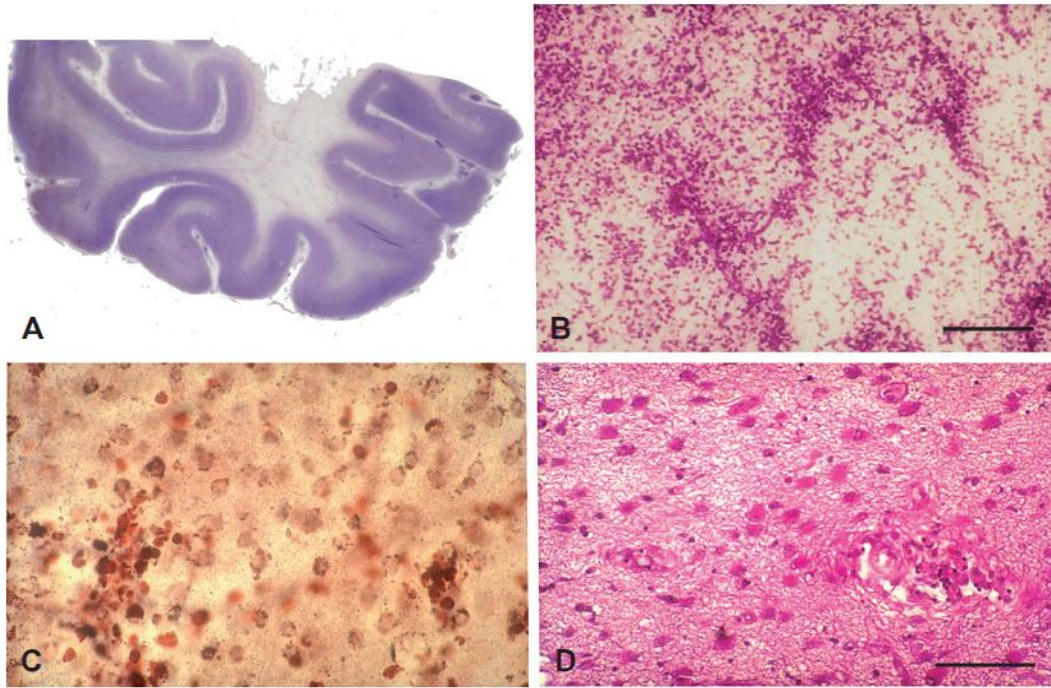
Figura 2: A) Forma cerebral infantil (CALD) da X-ALD. A perda de mielina e axônios na substância branca do lobo temporal. B) Infiltrado inflamatório na substância branca na borda da lesão desmielinizante. C) Células CD68^{+} infiltrando na zona intermediária entre a borda da desmielinização e a área de desmielinização. D) Astrócitos hipertróficos na área de desmielinização. Seções de parafina: A, B, D. hematoxilina e eosina; C. Imuno-histoquímica para CD68. B, bar = 200 mm; C, D, bar = 50 mm. (Ferrer *et al*., 2010).

2.2.2 Neuropatologia da AMN

A neuropatologia da AMN é caracterizada pela perda dos axônios ao longo dos tratos da medula espinhal, principalmente fascículo dorsal e tratos piramidais, com perda apenas secundária da mielina. Não há infiltração de células mononucleares hematógenas, com alterações inflamatórias restritas à microglia ativada. Desta maneira, lesões típicas na medula espinhal na AMN são manifestadas como uma mielopatia tipo *dying-back* (Ferrer *et al*., 2010). Neurônios do sistema motor não parecem estar muito afetados, enquanto os neurônios do gânglio dorsal, aparentemente preservados em número, apresentam atrofia e inclusões lipídicas anormais nas mitocôndrias (Powers *et al*., 2001), aumentando a possibilidade de que, além do defeito peroxissomal, a diminuição de função mitocondrial possa contribuir para a mielopatia através da falha de transporte axonal dependente de ATP nos tratos espinhais envolvidos (Powers *et al*., 2005).

Pela mesma fisiopatologia, a neuropatia periférica é uma característica na AMN, embora normalmente superada por sintomas na medula espinhal. Como na medula espinhal, a principal patologia nos nervos periféricos surge como uma axonopatia (Powers *et al*., 2000; Dubey *et al*., 2005). Inclusões trilamelares também são observadas nas células de Schwann.

2.3 ASPECTOS BIOQUÍMICOS DA X-ALD

A principal anormalidade bioquímica é o aumento de VLCFA saturados e não-ramificados, especialmente ácido tetracosanóico ($C_{24:0}$) e ácido hexacosanóico ($C_{26:0}$). Esse acúmulo pode ser encontrado em todos os tecidos, fluidos corporais e cultura de células (Moser *et al*., 2001). O acúmulo da VLCFA é a única alteração bioquímica conhecida por estar presente em todas as formas clínicas da X-ALD, incluindo indivíduos pré-sintomáticos. Em mulheres heterozigotas, o acúmulo dos VLCFA é reduzido quando comparado com hemizigotos e pode levar a um diagnóstico falso-negativo do *status* de portadora na investigação bioquímica (Moser *et al*., 2001).

Os VLCFA são parcialmente absorvidos da dieta, mas resultam principalmente da síntese endógena através do alongamento de ácidos graxos de cadeia longa (LCFA) (Moser *et al*., 2001). A síntese de VLCFA saturados, monoinsaturados e poliinsaturados compartilha de uma mesma rota enzimática, que ocorre no lado citoplasmático da membrana endoplasmática (Osei *et al*., 1989; Denic *et al*., 2007). Rizzo *et al*. (1984) não encontraram diferença nos níveis de $C_{26:0}$ em fibroblastos X-ALD cultivados em meio livre de lipídios ou sob condições padrão de cultura (Rizzo *et al*., 1984). Além disso, a restrição alimentar de VLCFA não diminui os níveis de $C_{26:0}$ no plasma de pacientes X-ALD (Moser *et al*., 2001).

O mecanismo exato que relaciona o excesso de VLCFA à degeneração axonal na AMN ou à inflamação e demielinização na CALD permanece desconhecido. Os VLCFA normalmente são encontrados como constituintes de lipídios complexos, tais como gangliosídios, fosfatidilcolina e frações ésteres de colesterol da mielina cerebral e são também encontrados nas proteínas proteolipídicas (Singh *et al*., 2010).

O mecanismo de toxicidade do excesso de VLCFA foi investigado utilizando diversas culturas primárias de células neurais de ratos e demonstraram-se alterações nos

níveis celulares de cálcio, alterações nas funções mitocondriais e morte celular (Hein *et al*., 2008), embora estudos da citotoxicidade dos VLCFA utilizando ácidos graxos livres possam conduzir a uma limitação de interpretação dos dados, já que a maioria dos ácidos graxos encontrados nas células não está livre, mas complexada em fosfolipídios ou outras espécies de lipídios. Mesmo assim, esses estudos mantêm seu crédito por produzirem efeitos semelhantes aos vistos na patologia. Por exemplo, além de produzir uma resposta imunológica, a incorporação excessiva de VLCFA a lipídios complexos pode desestabilizar as membranas celulares (Singh *et al*., 2010). Sabe-se que o ácido hexocosanóico ($C_{26:0}$) altera as propriedades fisiológicas e estruturais das membranas. (Ho *et al*., 1995).

Enfatizando os resultados descritos *in vitro*, o dano oxidativo também foi documentado em estudos clínicos da X-ALD. Tanto pacientes AMN quanto CALD têm aumento da peroxidação lipídica e diminuição de antioxidantes não enzimáticos (Vargas *et al*., 2004; Deon *et al*., 2006). Danos às proteínas foram relatados nas células mononucleares periféricas de pacientes X-ALD (Pujol *et al*., 2002). Além do acúmulo excessivo de VLCFA, a perda funcional do gene *ABCD1* resulta na perda funcional dos peroxissomos e na síntese de plasmalógenos (lipídios com vinil-ésteres com atividade antioxidante) (Singh *et al*., 2010). A biosíntese de plasmalógenos utiliza acil-CoA gerada pela via da β-oxidação peroxissomal (Hayashi *et al*., 1997) e, supostamente, a inativação do gene *ABCD1* resulta em menor quantidade de plasmalógenos devido à falta de substrato (acil-CoA). Dessa maneira, alterações metabólicas nos níveis de VLCFA e plasmalógenos podem atuar sinergicamente para aumentar o estresse oxidativo na X-ALD (Singh *et al*., 2010).

Powers *et al*, (1992) sugeriram que o excesso de VLCFA estimula astrócitos, células perivasculares e macrófagos a iniciar uma cascata dependente de TNF-α (fator α de necrose tumoral) que leva à demielinização mediada principalmente por citocinas e por células T e, em menor grau, pelo sistema do complemento e células B. A demielinização inflamatória na X-ALD se assemelha a da esclerose múltipla, onde células inflamatórias são principalmente macrófagos e linfócitos T, com infreqüentes células B, embora a ocorrência de resposta autoimune mediada por auto-anticorpos não esteja clara na X-ALD (Schmidt *et al*., 2001).

Além do sistema nervoso central (SNC), tecido nervoso periférico, testículos e glândulas adrenais estão patologicamente envolvidas na AMN e na forma cerebral

inflamatória (CALD) (Moser *et al*., 2001). Contudo, a inflamação não tem sido observada fora do SNC. Assim, antígenos lipídicos contendo VLCFA devem ser específicos do SNC e, possivelmente, incluem gangliosídios e lipoproteínas específicos do SNC, como a proteína proteolipídica (PLP) (Berger *et al*., 2006).

2.4 ASPECTOS GENÉTICOS DA X-ALD

O gene da X-ALD foi mapeado na região Xq28 (figura 3A) através de estudos de ligação com o gene G-6-PD (Migeon *et al*., 1981) e com marcadores polimórficos (Aubourg *et al*., 1987; Willems *et al*., 1990). Ele foi localizado em 1993 e logo identificado, em 1994, pelo grupo parisiense (Mosser *et al*., 1993; Fanen *et al*., 1994). Este gene codifica uma proteína de membrana peroxissomal e tem estrutura geral de um cassete transportador de ligação de ATP, sendo denominado cassete transportador de ligação de ATP, membro 1 da subfamília D (*ATP-Binding Cassette transporter subfamily D member 1 – ABCD1*). O gene *ABCD1* abrange aproximadamente 19kb, contém 10 éxons (figura 3B), transcreve um mRNA de 4,3kb e este codifica uma proteína composta por 745 aminoácidos (Kemp *et al*., 2010).

Até março de 2012, o banco de dados de mutações na X-ALD (http://www.x-ald.nl) continha 1251 mutações descritas. Destas, 771 (62%) eram mutações de sentido trocado, 274 (22%) mutações de mudança de quadro de leitura, 123 (10%) mutações sem sentido, 43 (3%) inserções ou deleções de aminoácidos e 40 (3%) grandes deleções de um ou mais éxons. A maioria das famílias X-ALD (47%) tem mutações únicas, não recorrentes, ou seja, exclusivas de uma única família.
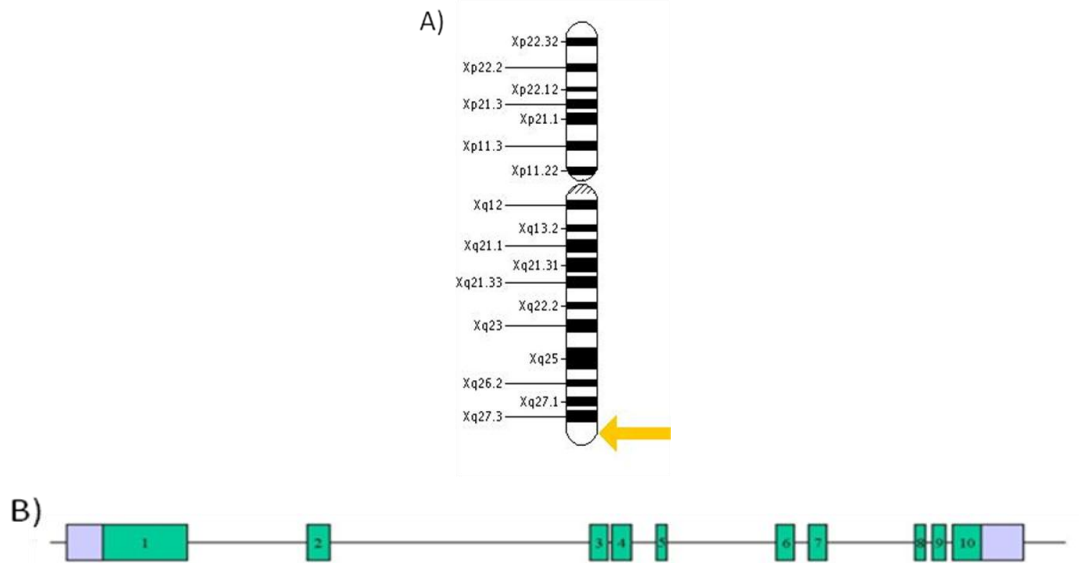
Figura 3: A) Diagrama mostrando a localização do gene *ABCD1* no cromossomo X. B) Distribuição dos 10 éxons e 9 íntrons que compõem o gene *ABCD1*.(Fonte: www.blobs.org/science/metabolism).

Não há correlação entre o tipo de mutação e o fenótipo clínico. Essa afirmação está baseada em quatro observações principais: (a) todos os fenótipos clínicos da X-ALD podem recorrer dentro de um mesmo núcleo familiar (Pollard *et al*., 1995); (b) mutações que são conhecidas por causar uma completa perda de atividade da proteína, tais como grandes deleções, podem ser associadas com todos os diferentes fenótipos clínicos (Fouquet *et al*., 1997); (c) a deleção idêntica de 2 nucleotídeos no éxon 5 pode levar a todo espectro clínico da X-ALD (Troffer-Charlier *et al*., 1998); e (d) gêmeos monozigóticos têm sido descritos com fenótipos clínicos claramente diferentes (Sobue *et al*., 1994; Korenke *et al*., 1996; Wilichowski *et al*., 1998; Berger *et al*., 1999). Contudo, todos esses argumentos não excluem a possibilidade de que a atividade residual da ALDP possa prevenir o desenvolvimento da forma cerebral inflamatória em pacientes X-ALD, levando assim ao fenótipo moderado (Berger *et al*., 2006).

A proteína codificada pelo gene *ABCD1*, chamada proteína da adrenoleucodistrofia (ALDP), estruturalmente representa um meio transportador ABC, com somente um domínio transmembrana hidrofóbico e um domínio de ligação de nucleotídeo hidrofílico e, supostamente, dimeriza-se para tornar-se uma unidade funcional (Higgins *et al*., 1992) (figura 4). Três outros meio-transportadores ABC em mamíferos, estruturalmente similares à ALDP, foram identificados: a proteína relacionada à ALDP (ALDRP) (Lombard-Platet *et al*., 1996; Holzinger *et al*., 1997), uma proteína de membrana peroxissomal de 70kDa (PMP70) (Kamijo *et al*., 1990; Gärtner *et al*., 1992) e uma

proteína relacionada à PMP70 (P70R) (Shani *et al*., 1997), com respectivamente 63%, 33% e 25% de homologia com a ALDP. ALDRP, PMP70 e P70R são codificadas pelos genes *ABCD2, ABCD3* e *ABCD4*, respectivamente (Berger *et al*., 2006).

Uma importante questão era se a ALDP dimeriza-se como um homodímero ou forma heterodímeros com um padrão de heterodimerização específico ou forma diferentes heterodímeros em diferentes tipos celulares tecido-específicos de acordo com a disponibilidade individual dos meio-transportadores ABC (Berger *et al*., 2006).
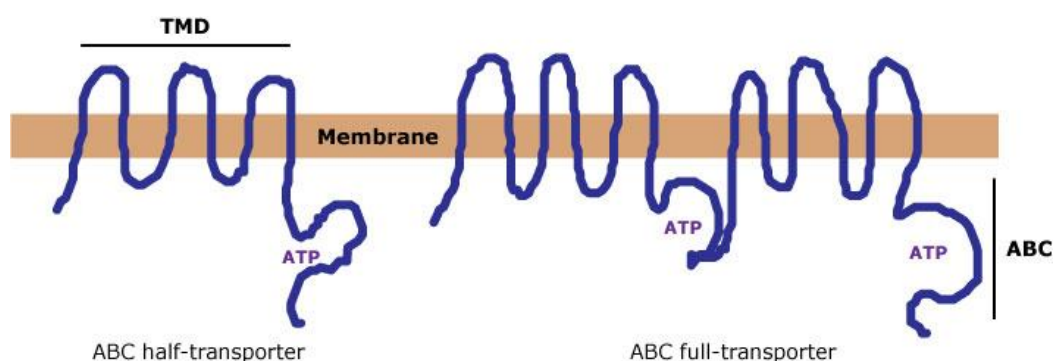
Figura 4: A estrutura típica de um transportador ABC é composta por dois domínios transmembrana (TMD) e dois cassetes de ligação de ATP (ATP-binding cassete - ABC). Existem vários arranjos diferentes dos domínios TMD e ABC encontrado entre as proteínas humanas ABC. Porém, em geral, um transportador funcional completo é construído na seqüência da composição TMD-ABC-TMD-ABC. Os quatro domínios podem estar presentes em um polipeptídeo (como o MDR P-glicoproteínas), ou podem ser formados por dimerização de dois meio-transportadores ABC, TAP1 (ABCB2) e TAP2 (ABCB3). Fonte: www.x-ald.nl.

Liu e colaboradores mostraram que tanto a homo quanto a heterodimerização ocorrem entre as metades C-terminais de ALDP, ALDRP e PMP70. Duas mutações na X-ALD localizadas na metade C-terminal da ALDP (p.Pro484Arg e p.Arg591Gln) afetam tanto a sua homo quanto a heterodimerização (Liu *et al*., 1999). Está estabelecido que a ADLP, ALDRP e PMP70 são pelo menos parcialmente redundantes em suas funções e têm sobreposição por substratos específicos (Kemp *et al*., 2007). Além disso, a superexpressão de PMP70 em fibroblastos X-ALD corrigiu parcialmente a β-oxidação dos VLCFA e a superexpressão da ALDRP resultou na completa correção da degradação dos VLCFA (Moser *et al*., 1999).

2.5 DIAGNÓSTICO DA X-ALD

O diagnóstico inicial da X-ALD é suspeitado a partir de uma apresentação clínica e de imagens cerebrais por ressonância magnética compatíveis (a Figura 5 exemplifica algumas) e é realizado através da análise bioquímica dos VLCFA e da análise molecular do gene *ABCD1* (Moser *et al*., 2001).
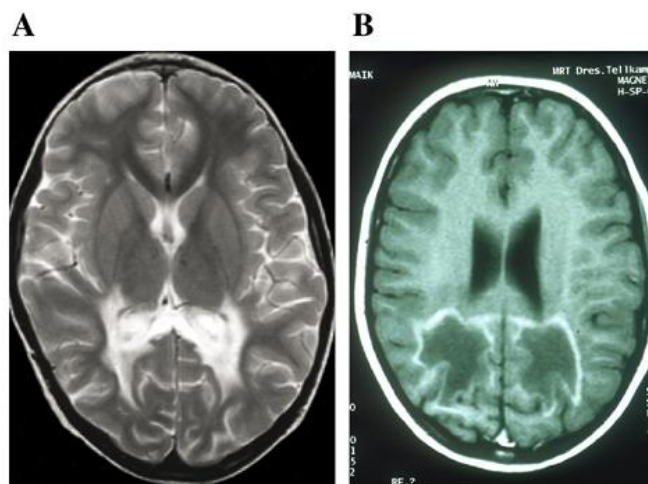
Figura 5: (A) Ressonância magnética em T2 do cérebro em um menino de 6 anos de idade, com a forma cerebral da X-ALD. As lesões características são simétricas e desmielinizante localizadas no lobo parieto-occipital. (B) Ressonância magnética em T2 do cérebro de um menino de 8 anos de idade, com a forma cerebral da X-ALD . Aumento das áreas inflamatórias adjacentes à desmielinização, marcadas por gadolina. (Berger *et al*., 2006).

Até o momento, a análise de VLCFA no plasma é o teste diagnóstico mais freqüentemente utilizado para diagnosticar a X-ALD. Os dois parâmetros atualmente analisados são a concentração de $C_{26:0}$ e a razão $C_{26:0}/C_{22:0}$. Altas concentrações de $C_{26:0}$ e proporções anormais de $C_{26:0}/C_{22:0}$ (e também de $C_{24:0}/C_{22:0}$) no plasma são muito sugestivas de X-ALD, sendo encontradas desde o nascimento em meninos afetados e em muitas mulheres heterozigotas (Liu *et al*., 1999; Valianpour *et al*., 2003). Os níveis de VLCFA monoinsaturados também estão aumentados na X-ALD, embora em menor extensão do que VLCFA saturados.

O diagnóstico bioquímico de homens afetados tem aproximadamente 100% de exatidão, desde que o plasma tenha sido coletado em jejum. Esse não é o caso para as mulheres, onde as análises de VLCFA podem detectar entre 85% e 95% das heterozigotas somente. Assim, um nível normal de VLCFA no plasma, não exclui a possibilidade da mulher ser heterozigota para X-ALD (Berger *et al*., 2006).

Desde a descoberta do gene *ABCD1* em 1993 (Mosser *et al*., 1993), o diagnóstico pode ser confirmado por análise molecular. A análise de mutações é o método mais confiável para a identificação de heterozigotas, uma vez que a mutação tenha sido identificada em um homem afetado ou familiar heterozigota obrigatória (Boehm *et al*., 1999; Ferrer *et al*., 2010). Embora não adicione nenhuma informação sobre o curso clínico do paciente, estudos genéticos são obrigatórios em todos os casos para confirmar o *status* da mulher em risco de ser heterozigota (Moser *et al*., 2007).

2.6 TERAPIAS NA X-ALD

A crescente atividade na área da genética molecular e o melhor entendimento da patogênese da doença promovem tentativas na elaboração de terapias eficazes. A reposição de esteróides para a insufuciência adrenal é uma terapia efetiva e prontamente disponível, mas sem efeitos sobre as manifestações neurológicas. Para estas, a terapia eficaz se limita ao transplante de medula óssea, indicado para a forma cerebral, desde que descoberta a tempo (com um índice de Loes de 9 ou menos). Abordagens farmacológicas tradicionais, incluindo o óleo de Lorenzo e imunosupressão (Korenke *et al*., 1997) trazem pouco ou nenhum benefício. Outras terapias específicas estão sob avaliação, incluindo terapia gênica (Berger *et al*., 2006).

2.7 VARIAÇÃO NO NÚMERO DE CÓPIAS DE GENES E A X-ALD

A falta de correlação genótipo-fenótipo na X-ALD é de especial interesse pelo fato de que em uma mesma família, portanto com a mesma mutação, é possível identificar todos os fenótipos clínicos da X-ALD. Mesmo mutações conhecidas por causar a perda completa da atividade da ALDP, como grandes deleções, podem estar associadas a todos os fenótipos, incluindo a forma AMN de início tardio (Moser *et al*., 2001). Estes dados sugerem que fatores adicionais, tais como genes modificadores, fatores epigenéticos, eventos ambientais e estocásticos podem atuar sozinhos ou em conjunto para modular a expressão dos diferentes fenótipos observados.

Entre os mais importantes conhecimentos derivados da conclusão do Projeto Genoma Humano está o reconhecimento da grande quantidade de polimorfismos de nucleotídeos únicos (*single nucleotide polymorphisms* – SNPs) como a maior fonte de variação genética. Isso levou à especulação de que a grande variabilidade fenotípica nas populações humanas era devido a alterações de base única. Estudos populacionais para identificar determinantes genéticos de doenças freqüentes implicaram diversos SNPs na susceptibilidade para algumas doenças humanas como diabetes (Saxena *et al*., 2007), degeneração macular (Edwards *et al*., 2005; Klein *et al*., 2005), câncer (Easton *et al*., 2007; Hunter *et al*., 2007) e doença de Crohn (Duerr *et al*., 2006). Contudo, estudos mais recentes têm reforçado o papel da variação genética estrutural na modulação da expressão gênica e fenotípica. Por variações genéticas estruturais, referimo-nos a variações que contenham deleções, duplicações, inversões ou grandes fragmentos repetitivos (Redon *et al*., 2006; Sharp *et al*., 2006).

Variações no número de cópias (*copy number* variants – CNV) são definidas como ganho ou perda de segmentos de DNA de tamanho maior que 1kb dentro do genoma humano (Feuk *et al*., 2006). Alguns estudos indicam que CNVs afetam entre 12-15% do genoma humano (Carter *et al*., 2007), enquanto outros sugerem que cerca de 0,2% do genoma varia em número de cópias entre indivíduos (McCarroll *et al*., 2008). As CNVs podem se originar por alguns mecanismos mutacionais, tais como junções terminais não homólogas, retrotransposons e recombinação homóloga não alélica, a qual parece ser a mais freqüente em humanos (Schrider *et al*., 2010).

CNVs podem ocorrer *de novo* ou serem herdadas, sendo a primeira a maneira mais provável de contribuição para o desenvolvimento de doenças genéticas esporádicas (Willcocks *et al*., 2008). O processo de duplicação ou deleção pode interromper um número variável de genes, resultando em produtos gênicos alternativos ou alterações na expressão alélica. Contudo, a interrupção de regiões regulatórias no genoma também pode levar à função gênica alterada. Provavelmente essa diversidade seja responsável por uma significativa proporção da variação fenotípica (Redon *et al*., 2006).

Diversas características das CNVs suportam seu papel na patogênese de doenças. Primeiro, apesar de menos abundante que os SNPs, tem sido sugerindo que as CNVs explicariam melhor a variação genética do que SNPs, em relação ao seu número absoluto (Tuzun *et al*., 2005). Por seu tamanho de milhares de bases, as CNVs muitas vezes incluem (e podem interromper) seqüencias funcionais de DNA. Segundo, parece

existir um enriquecimento de CNVs em direção aos atualmente conhecidos "genes do ambiente sensorial", isto é, genes que não são necessariamente críticos para o desenvolvimento inicial do embrião, mas nos ajudam a entender e interagir com as alterações ambientais (Sebat *et* al., 2004; Tuzun *et al.*, 2005). Isso inclui receptores olfatórios, genes de resposta imune e resposta inflamatória, sinalização celular e moléculas de adesão, proteínas estruturais e canais de íons. Terceiro, como em outras formas de variação genética, seleção purificadora e seleção adaptativa parecem ter influenciado a freqüência da distribuição das CNVs, sugerindo a significância de seu papel funcional (Nguyen *et al.*, 2006; Redon *et al.*, 2006). Atualmente, dados disponíveis sugerem que muitas CNVs conferem maior risco à doenças do que SNPs - em alguns casos, essas variantes de susceptibilidade a doenças baseadas em CNVs parecem aumentar o risco em 30%.

Uma recente comparação do impacto relativo dos SNPs e das CNVs na expressão gênica indicou que uma substancial proporção (18%) da variabilidade de expressão gênica era atribuída a CNVs maiores que 40kb de tamanho (Stranger *et al.*, 2007). Especialmente, 53% dos genes cuja expressão era influenciada por CNVs apresentavam CNVs correspondentes fora de genes verdadeiros, sugerindo que muitos podem afetar seqüencias regulatórias.

As CNVs podem influenciar nos fenótipos por diferentes mecanismos moleculares, como dosagem gênica, desmascarando alelos recessivos ou polimorfismos funcionais, efeitos de posição, interrupção ou fusão gênica (Zhang *et al.*, 2009). Seu papel tem sido relacionado a inúmeras doenças complexas, como esquizofrenia (Xu *et al.*, 2008), doença celíaca (Fernandez-Jimenez *et al.*, 2010), diabetes (Grayson *et al.*, 2010) e câncer (de Leeuw *et al.*, 2011; Jin *et al.*, 2011).

Como CNVs têm sido observadas entre indivíduos idênticos (Bruder *et al.*, 2008), elas permanecem como uma possível explicação para a variação fenotípica entre gêmeos monozigóticos em geral e, em particular, para a heterogeneidade fenotípica na X-ALD. Ademais, estudos do genoma têm demonstrado que CNVs raras alterando genes na via do neurodesenvolvimento estão implicados no autismo (Sebat *et al.*, 2007) e esquizofrenia (Walsh *et al.*, 2008), o que as vincula também a efeitos sobre o sistema nervoso.

Para completa exploração do impacto das CNVs nos fenótipos, são necessárias técnicas de alta resolução, como por exemplo array CGH (*comparative genomic*

hibridization) e métodos alternativos, tais como MLPA (*multiplex ligation-dependent probe amplification*) (Schouten *et al*., 2002), MAPH (*multiplex amplification and probe hybridization*) (Armour *et al*., 2000; Sellner *et al*., 2004) ou QMPSF (*quantitative multiplex PCR of short fluorescent fragment*) (Saugier-Veber *et al*., 2006), permitindo a detecção de pequenas CNVs e fornecendo uma estimativa quantitativa do número de cópias.

3. OBJETIVOS

3.1 Objetivo Geral

Estudar os aspectos moleculares da X-ALD, relacionando-os com dados epidemiológicos, métodos diagnósticos e potenciais genes modificadores.

3.2 Objetivos Específicos

1. Descrever o espectro mutacional de famílias sul-americanas com X-ALD e compará-lo com o descrito para outras populações.

2. Determinar a proporção de casos X-ALD herdados e de casos devidos à mutação *de novo* na população estudada.

3. Descrever a proporção de fenótipos masculinos, calcular a sobrevida média dos casos CALD identificados dentro das famílias estudadas e buscar potenciais associações com suas origens geográficas.

4. Diagnosticar mulheres heterozigotas em famílias X-ALD e descrever sua proporção em relação às não afetadas.

5. Correlacionar os níveis plasmáticos de VLCFA com a análise molecular em mulheres pertencentes a famílias com X-ALD, descrevendo a sensibilidade e especificidade da análise de VLCFA.

6. Explorar potenciais genes modificadores entre alguns envolvidos na resposta inflamatória, metabolismo de lipídios e regeneração do sistema nervoso, através de potenciais associações de seus CNVs com os fenótipos CALD e AMN através da técnica do MLPA.

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5. RESULTADOS

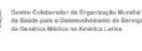Os resultados desta tese serão apresentados sob a forma de três artigos.

Artigo 1 – submetido à PLoS ONE

PLoS ONE

Mutations, clinical findings and survival estimates in South American patients with X-linked adrenoleukodystrophy
--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Article
Full Title:	Mutations, clinical findings and survival estimates in South American patients with X-linked adrenoleukodystrophy
Short Title:	ALD Mutations in South America
Corresponding Author:	Laura Bannach Jardim Hospital de Clínicas de Porto Alegre Porto Alegre, Porto Alegre BRAZIL
Keywords:	X-linked Adrenoleukodystrophy; ABCD1 gene mutations; de novo mutations; survival; affected females
Abstract:	In this study, we analyzed the ABCD1 gene in X-linked adrenoleukodystrophy (X-ALD) patients and relatives from 38 unrelated families from South America, as well as phenotypic proportions, survival estimates, and the potential effect of geographical origin in clinical characteristics. Methods: X- ALD patients from Brazil, Argentina and Uruguay were invited to participate in molecular studies to determine their genetic status, characterize the mutations and improve the genetic counseling of their families. All samples were screened by SSCP analysis of PCR fragments, followed by automated DNA sequencing to establish the specific mutation in each family. Age at onset and at death, male phenotypes, genetic status of women, and the effect of family and of latitude of origin were also studied. Results: We identified thirty-six different mutations (twelve novel). This population had an important allelic heterogeneity, as only p.Arg518Gln was repeatedly found (three families). Four cases carried de novo mutations. Intra-familiar phenotype variability was observed in all families. Out of 87 affected males identified, 65% had the cerebral phenotype (CALD). The mean (95% CI) ages at onset and at death of the CALD were 10.9 (9.1-12.7) and 24.7 (19.8-29.6) years. No association was found between phenotypic manifestations and latitude of origin. One index-case was a girl with CALD who carried an ABCD1 mutation, and had completely skewed X inactivation. Conclusions: This study extends the spectrum of mutations in X-ALD, confirms the high rates of de novo mutations and the absence of common mutations, and suggests a possible high frequency of cerebral forms in our population.
Order of Authors:	Fernanda dos Santos Pereira
	Ursula Matte
	Clarissa Troller Habekost
	Raphael Machado de Castilhos
	Antonette Souto El Husny
	Charles Marques Lourenço
	Angela M Vianna-Morgante
	Liane Giuliani
	Marcial Francis Galera
	Rachel Honjo
	Chong Ae Kim
	Juan Politei
	Carmen Regla Vargas
	Laura Bannach Jardim

Suggested Reviewers:	Gerald Raymond Johns Hopkins School of Medicine Raymond@kennedykrieger.org
	Ann Moser The Johns Hopkins University mosera@kennedykrieger.org
Opposed Reviewers:	

December 13, 2011.

To the editors of PLoS ONE

Dear sirs.

I am sending enclosed the manuscript entitled "Mutations, clinical findings and survival estimates in South American patients with X-linked adrenoleukodystrophy", to be considered for publication in *PLoS ONE*.

All co-authors have approved the publication of the manuscript, which has never been published before, in whole or in part. I also warrant that the present work has been approved by the Ethics Committees from the institutions at which the work was performed, which follow the Code of ethics of the World Medical Association (Declaration of Helsinki) and the standards established by the author's Institutional Review Board and granting agency.

We believe our manuscript is suitable for publication in PLoS ONE. This paper presents one of the first description of the mutations found in South American patients with X-linked adrenoleukodystrophy. It also describes the survival estimates for disease onset and for death in the obtained cohort, tests the possible association of phenotypes with the latitude of origin (in a way similar to multiple sclerosis), and finally describes a girl with the cerebral form of this disease.

We have looked at the list of PLoS ONE academic editors. Since our paper covers an X-linked disorder, we suggest Emanuele Buratti to handle our manuscript.

Yours sincerely,

Laura B. Jardim, MD, PhD
Chief, Medical Genetics Service
Hospital de Clínicas de Porto Alegre
Rua Ramiro Barcelos 2350
90.035-903, Porto Alegre, Brazil
Phone (55)(51) 3359 8011
Fax (55)(51) 3359 8010
e-mail: ljardim@hcpa.ufrgs.br

www.hcpa.ufrgs.br

Ramiro Barcelos 2350 - 90035-903 - Porto Alegre/RS - Brasil
Tel.: [51] 3359.8011 - Fax: [51] 3359.8010

1   **Mutations, clinical findings and survival estimates in South American**

2   **patients with X-linked adrenoleukodystrophy**

3

4   Fernanda dos Santos Pereira[1, 3], Ursula Matte[1,4], Clarissa Troller Habekost[2,4],

5   Raphael Machado de Castilhos[2,3], Antonette Souto El Husny [2], Charles

6   Marques Lourenço[7], Angela M. Vianna-Morgante [8] , Liane Giuliani [10], Marcial

7   Francis Galera[11], Rachel Honjo [9], Chong Ae Kim[9], Juan Politei[12], Carmen Regla

8   Vargas [2,5] and Laura Bannach Jardim[2,3,4,6]

9

10   [1]Gene Therapy Center and [2]Medical Genetics Service, Hospital de Clínicas de

11   Porto Alegre; Post-Graduation Programs in [3]Medical Sciences and in [4]Genetics

12   and Molecular Biology, and Departments of [5]Analysis and of [6]Internal Medicine,

13   Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [7]Hospital de

14   Clinicas de Ribeirão Preto, [8]Department of Genetics and Evolutionary Biology,

15   Institute of Biosciences, and [9]Children Institute, Department of Genetics,

16   Universidade de São Paulo, Brazil; [10]Department of Pediatrics, Universidade

17   Federal do Mato Grosso do Sul, Campo Grande, Brazil; [11] School of Medicine,

18   Universidade de Cuiabá, Cuiabá, Brazil; [12] Neuromuscular Disorders and

19   Neuropathic Pain Section, Neurology Department, Hospital Juan A. Fernández,

20   Buenos Aires, Argentina.

21

22   Corresponding author:

23   Laura Bannach Jardim, MD, PhD

24   Medical Genetics Service,

25   Hospital de Clinicas de Porto Alegre

26   Rua Ramiro Barcelos 2350

27   90.035-903, Porto Alegre, Brazil

28   Phone: 55 51 3359 8011

29   Fax: 55 51 3359 8010

30   Email: ljardim@hcpa.ufrgs.br

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32   **Short title**: X-ALD Mutations in South America

33

Abstract

34   In   this   study,   we   analyzed   the   *ABCD1*   gene   in   X-linked

35   adrenoleukodystrophy (X-ALD) patients and relatives from 38 unrelated families

36   from South America, as well as phenotypic proportions, survival estimates, and

37   the potential effect of geographical origin in clinical characteristics. **Methods**: X-

38   ALD patients from Brazil, Argentina and Uruguay were invited to participate in

39   molecular studies to determine their genetic status, characterize the mutations

40   and improve the genetic counseling of their families. All samples were screened

41   by SSCP analysis of PCR fragments, followed by automated DNA sequencing

42   to establish the specific mutation in each family. Age at onset and at death,

43   male phenotypes, genetic status of women, and the effect of family and of

44   latitude of origin were also studied. **Results**: We identified thirty-six different

45   mutations (twelve novel). This population had an important allelic heterogeneity,

46   as only p.Arg518Gln was repeatedly found (three families). Four cases carried

47   *de novo* mutations. Intra-familiar phenotype variability was observed in all

48   families. Out of 87 affected males identified, 65% had the cerebral phenotype

49   (CALD). The mean (95% CI) ages at onset and at death of the CALD were 10.9

50   (9.1-12.7) and 24.7 (19.8-29.6) years.   No association was found between

51   phenotypic manifestations and latitude of origin. One index-case was a girl with

52   CALD who carried an *ABCD1* mutation, and had completely skewed X

53   inactivation. **Conclusions**: This study extends the spectrum of mutations in X-

54   ALD, confirms the high rates of *de novo* mutations and the absence of common

55   mutations, and suggests a possible high frequency of cerebral forms in our

56   population.

1. Introduction

X-linked adrenoleukodystrophy (X-ALD, OMIM #300100) is a neurodegenerative disease characterized by great clinical expression variability even within the same family. Several phenotypes are recognized in males according to the age of onset, affected organs and rate of the progression of neurologic symptoms. The X-ALD clinical spectrum ranges from the rapidly progressive childhood cerebral form (CALD), which typically leads to severe disability and death during the first decade, to the milder adrenomyeloneuropathy (AMN) that usually manifests between ages 20 and 30 years and may be compatible with survival into the eighth decade, to pure Addison's disease [1]. Whereas AMN is characterized mainly by a noninflammatory "dying-back" axonopathy, involving the long spinal tracts, the inflammatory nature of the demyelinating lesion in CALD resembles those found in multiple sclerosis (MS), the most common central demyelinative disease. It is postulated that modifier genes or environmental factors are involved in the pathogenesis of these highly variable phenotypes [1, 2, 3].

X-ALD is caused by a defect in the gene for the adenosine triphosphate (ATP)-binding cassette protein, subfamily D, member 1 located on Xq28 (*ABCD1*) [1]. X-ALD protein (ALDP) [4], is a structural protein related to the transport of very long chain fatty acids (VLCFA) across peroxisome membranes. The *ABCD1* gene contains 10 exons, spanning 20kb of genomic DNA and ALDP contains 745 amino acids.

Since its identification, 1236 mutations have been reported in the *ABCD1* gene of which 582 (47%) appear to be private (http://www.x-ald.nl). Mutations have been found throughout the entire gene, but they are not distributed evenly. There is a clustering of mutations in the transmembrane domain (47%), in the ATP-binding domain (34%), and in exon 5 (8%), which is not part of any of this domains. The remaining 11% of mutations are spread throughout the gene. No promoter mutations or complete gene deletions have been reported [5].

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The main biochemical abnormality associated with X-ALD is the accumulation of unbranched saturated VLCFA in plasma and tissues, due to impaired β-oxidation in peroxisomes. The increase in VLCFA levels provides a reliable diagnostic tool for prenatal and postnatal identification of affected males. In females, however, VLCFA levels present false-negative results in around 20% of the obligate carriers [1]. Mutation analysis is therefore the best approach in order to improve genetic counseling to the families.

X-ALD is the most common peroxisomal disorder with a hemizygote frequency of 1:21,000 in USA [6] and of at least 1:35,000 in South Brazil [7]. Although no differences in distribution of mutations were found among different populations [5, 8, 9, 10], little is known about differences in X-ALD epidemiology in particular countries and continents. Specially, there is a relative lack of knowledge about the X-ALD epidemiology on subtropical regions of the world. This knowledge may not only help these populations, but may also help the identification of environmental factors that may modify X-ALD phenotype.

The present study aimed to describe the *ABCD1* mutations in a case series of X-ALD families from South American patients, most of them Brazilian individuals, the rate of *de novo* mutations, phenotypes and survival estimates in the affected males found in this population.

113 **2. Material and methods**

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115 **Ethics Statement**

116   The present work has been approved by the Ethics Committees from the
117 institutions at which the work was performed, which follow the Code of ethics of
118 the World Medical Association (Declaration of Helsinki) and the standards
119 established by the author's Institutional Review Board and granting agency.

120   **Population**

121   Patients originated from Brazil, Argentina and Uruguay, were previously
122 diagnosed by VLCFA analysis. Most of them were followed-up in the main
123 institution where the present study was carried out, while others were
124 ascertained by their physicians in other sites. Families received genetic
125 counseling and appropriate management, as described elsewhere [7]. These
126 families were invited to participate in the present study, which was approved by
127 the local Ethics Committee. Variables such as age, age at onset of symptoms,
128 age at death, male phenotypes, genetic status (in women, such as obligate
129 carriers), the family and the latitude of origin were also studied.

130   **Methods**

131   After consent, blood was collected from the index-case and DNA was
132 extracted by the salting out procedure [11]. Using 10 PCR reactions it was
133 possible to screen the entire coding sequence of the *ABCD1* gene and intron-
134 exon boundaries using the protocol described by Boehm et al [12]. All samples
135 were screened by Single Strand Conformational Polymorphism (SSCP) analysis
136 followed by automated DNA sequencing to establish the specific mutation in
137 each family. The different conditions used for SSCP were: 6% and 8%
138 polyacrilamide-agarose gel eletrophoresis(PAGE) at room temperature, 8% and
139 10% PAGE at 4°C. These conditions were chosen at random. Amplicons with
140 mobility shift were purified with Exo-SAP (GE Healthcare) and submitted to
141 automated sequencing on ABI 3100 Genetic Analyzer using BigDye v3.0 (Life
142 Technologies). Mutations were confirmed by reverse strand PCR sequencing.
143 Family relatives were screened for the specific mutation by PCR and automated
144 sequencing.

145 The percent of mutations assigned at each exon was compared to those
146 described in the literature at http://www.x-ald.nl. (17.10.2011). The mutations
147 were also mapped according to the protein domains described by Kumar et al
148 [13].

149 When an affected male was no longer available, an obligate carrier or a
150 female relative with elevated VLCFA was chosen as the family's index case.

151 Patient characteristics are given as mean ± SD and range. Categorical
152 variables such as normal, homozygote women versus heterozygote women
153 were compared through chi-square test. Kaplan-Meyer curves were used to
154 describe survival until disease onset, according to phenotypes, and to describe
155 survival, in CALD. All tests were 2-sided. Test results were considered
156 significant at the 0.05 level.

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158 **3. Results**

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160   From 2007 to 2011, thirty-eight families entered the study. Of those,
161 thirty-six were Brazilian families, 20 of them originating from Rio Grande do Sul,
162 the Southern most state of Brazil. The clinical characteristics of some of these
163 latter families have been previously reported [7]. We have also recruited one
164 family from Uruguay and one family from Argentina to the present case series.
165 Twenty-four families lived around parallel 30th South (Capricorn tropic),
166 whereas 14 families lived between Equator and parallel 20th South.

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168   **3.1 Molecular results**

169   Sequencing of the exons with mobility shift detected by SSCP, revealed
170 thirty-six different sequence variations in the 38 families (Table 1). Twelve
171 index-cases (or 31,5% of the total sample of families) carried new mutations.
172 The other 26 carried 24 mutations already described in the literature: 14 were
173 missense mutations and 10 were nonsense or frameshift mutations.
174 Insertion/deletion of one aminoacid, or major deletions were not detected
175 among our patients.

176   The already described p.Arg518Gln mutation [14] was found in three
177 families from Rio Grande do Sul: one of these affected families was due to a *de*
178 *novo* mutation in the maternal germ line. We can not rule out the possibility that
179 the other two p.Arg518Gln pedigrees have a common ancestral origin, given
180 their geographical proximity. All the other detected mutations were found in
181 unique pedigrees.

182   Eight of the twelve new sequence variations were considered pathogenic
183 mutations due to the creation of premature stop codon – either by nonsense
184 mutation or as consequence of deletions, insertions or duplication (Table 1).
185 The last four novel sequence variations (p.Pro623Leu, p.Leu628Glu,
186 p.Ile481Phe and p.Arg401Gly), if not causative of the disease, were linked to X-
187 ALD genotype by DNA study of hemizygous affected males.

188   The incidence of mutations in exons 1A and in exon 3 to 6 were very

189   similar to the reported in other populations (http://www.x-ald.nl). There seemed

190   to be less mutations than expected in exons 1B and 1C, where we found 8,4%

191   and 5,5% of our cases versus the expected 18% and 19%; and more mutations

192   than expected in exon 2, with 14% of observations versus the expected 4%,

193   (http://www.x-ald.nl) (Figure 1).

194   In 35 families, information on the genetic status of the oldest transmitting

195   mother was available. In this subset of families, we were able to define four *de*

196   *novo* mutations (two in the affected male) (Figure 2).

197   From the remaining 34 families where the mutation was inherited, 21

198   men (2-53 years of age) and 77 women older than 18 years old were tested by

199   molecular methods. Of those, thirty-eight females (or 49%) and thirteen males

200   (or 62%) of the studied relatives also carried mutations. These proportions were

201   in accordance with a priori mendelian risks (chi-square ns).

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3.2 Clinical characteristics of the present sample

204   Index-cases comprised 30 males and 8 females - six obligate carriers or

205   biochemically proven heterozygotes, and two symptomatic women.

206   Clinical characteristics of the affected men, index-cases as well as the

207   other affected relatives were described in Table 2. The family history of up to

208   three generations, plus biochemical and molecular studies of the men alive

209   identified 87 affected men in these 38 genealogies. At the end of this

210   investigation, 6 asymptomatic, 6 Addison-only, 54 CALD (27 already deceased),

211   and 14 AMN (3 already deceased) had been recognized. These phenotypes

212   were distributed among the families, with no peculiar clustering in any pedigree

213   (data no shown). Kaplan-Meyer curves on age at onset of each phenotype, and

214   on survival of the cerebral forms were presented in Figures 3. No differences on

215   proportion of phenotypes, on ages at onset or on survival (data not shown) of

216   CALD were seen, according to the latitude of origin of the affected patient

217   (Figure 3B and Table 1).

218   Two symptomatic women were the index-cases of their respective

219   families. Case 55 was a 63 years old woman with a progressive, pure spastic

220 paraplegia and urinary incontinence with 10 years of disease duration. There
221 was no previous occurrence in the family. Her VLCFA profile was highly
222 suggestive of a heterozygous state for X-ALD; the molecular analysis revealed
223 the presence of the mutation p.GLy510Ser in one of her alleles (Table 2).

224   Case 54 was a 15 years old girl with clinical, biochemical and MRI
225 abnormalities similar to those found in boys with the childhood cerebral
226 phenotype (Figure 4). Motor and cognitive deterioration started at 6 years of
227 age; VLCFA analysis was compatible with the heterozygous state (C26:0 of
228 2.02, normal range: 0.78 to 1.54; C26:0-C22:0 of 0.051, normal range: 0.01 to
229 0.03). She had completed skewed X-inactivation in blood cells, as determined
230 by the human androgen receptor locus (HUMARA) methylation assay. Her G-
231 banded karyotype was normal and the array-comparative genome hybridization
232 (a-CGH) analysis revealed no X chromosome copy-number alterations (44K X-
233 chromosome platform, design 2008, Agilent Technologies, Santa Clara, USA).
234 Gene sequencing of this girl revealed the presence of the mutation
235 p.Ser358fsX42 in one of her alleles (Table 1), probably on the active X
236 chromosome.

237

3.3 Genotype/Phenotype relationships

239   Some families were considered informative regarding the detected
240 phenotypes: families with only CALD patients (more than one CALD patient),
241 and heterogeneous families (with at least one CALD and one AMN). After that,
242 the affected domain of the ALDP was defined: only NBF (20 families) and TMD
243 (10 families) domains were considered. Distribution of these domains among
244 the families characterized as CALD or heterogeneous was equal (ns, chi-
245 square).

246

247 **4. Discussion**

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249   The present results probably describe the first case series of South

250 American mutations related to X-ALD. Although it is usually said that all ethnic

251 groups are affected by this disease, there is scarce knowledge about

252 differences in frequency of X-ALD phenotypes or genotypes in our region, as

253 well as in other subtropical regions of the world. Our results suggest some

254 peculiarities such as a higher incidence of mutations in exons 2 and 8-9, and of

255 cerebral forms, than the expected.

256   More than a thousand different mutations at *ABCD1* gene have been

257 related to X-ALD. There are no common mutations for this disease: the most

258 recurrent in other reports was p.Gln472fsX555, a frameshift mutation detected

259 in 6.4% of cases recorded. At least 47% of all reported mutations have been

260 found in single pedigrees in the world (http://www.x-ald.nl). Our rate of 34% of

261 new mutations is in agreement with this overall picture, moreover if we

262 remember that even the only recurrent mutation in our case series

263 (p.Arg518Gln) has emerged at least once from a *de novo* phenomenon (Table 1

264 and Figure 2).

265   We have found 4/35 *de novo* mutations, or 10%: they have occurred in

266 two men and in two women (Figure 2). In a previous large series, the rate of the

267 detected *de novo* mutations had been of 5% in affected male probands [7]. The

268 actual numbers might be much higher, if at least three generations (up to the

269 grand-mothers) would be checked. The absence of any frequent mutation, even

270 in restricted populations, states against the existence of distant common

271 ancestors for contemporary patients. The same phenomenon appeared in our

272 sample.

273   In our sample, the proportions of missense, frameshift and nonsense

274 mutations were similar to those found worldwide (http://www.x-ald.nl). In

275 contrast, a possible cluster of mutations at two amplicon regions, exons 2 and

276 8-9, was observed (Figure 1).

277   The occurrence of CALD in a girl with a skewed X-inactivation in our

278 case series deserves consideration. The present female patient resembled the

279 one reported by Hershkovitz et al [15]. Both girls have clinical, biochemical and
280 MRI abnormalities similar to those seen in boys with CALD. Two important
281 differences must be stressed, however. Our patient did not have a positive
282 family history of X-ALD, nor an X chromosome deletion.

283 Skewing of inactivation is frequently observed in heterozygotes for
284 *ABCD1* mutations, and it was found to correlate with neurological
285 manifestations [16]. As far as we know, skewing *per se* was never related to
286 CALD in a woman. The occurrence of CALD in our patient points to the X
287 chromosome bearing the c.1074-1075insA mutation in *ABCD1* to be the active
288 one, rendering "the patient totally deficient in ALDP and equivalent to an
289 affected male" [15].

290 Our phenotype proportions in males seemed to differ from the expected
291 ones, reported in literature. In the present study, all efforts to obtain a complete
292 family history of up to three generations were done, plus biochemical and
293 molecular studies of the men alive. We identified 87 affected men in 38
294 genealogies (including the *de novo* pedigrees), or 2.2 cases per family, a rate
295 similar to those found by others in larger series [6]. However, our rates of CALD
296 (62%) were higher than the expected 45-57% [1, 17, 18, http://www.x-ald.nl].
297 We are aware that underdiagnosis would be the best explanation to the present
298 numbers, moreover because only 6% of our series comprised Addison-only
299 disease (expected to be between 8 and 20%). However, if underdiagnosis of
300 AMN and Addison-only was operating here, we would be unable to explain why
301 the molecular results obtained in male relatives studied after genetic counseling
302 of the nuclear family were near as those expected *a priori*. If the clinical
303 characterization of our sample was insufficient, a higher proportion of carriers
304 found by chance would be expected.

305 If the present observation of a high proportion of CALD in our sample
306 was correct, the hypothesis of an environmental factor should be considered.
307 Head trauma has been postulated as a risk factor for the cerebral form in X-ALD
308 [17], but we did not observe such phenomena. Inflammation and altered
309 immune responses are present in CALD, whose demyelinating lesion is very
310 similar to those seen in multiple sclerosis (MS) [19, 20]. Among other factors,
311 latitude is clearly related to MS prevalence, and environmental modifiers which

312 vary with latitude, such as ultraviolet radiation, have been postulated to MS [21].
313 By analogy, we speculated if differences in latitude could also be related to a
314 higher prevalence and/or natural history of CALD. Our data did not support this
315 association, though the small latitude range of the present observation might
316 have precluded a final conclusion.

317 We believe that further studies on X-ALD prevalence, mutation patterns,
318 natural history and clinical course after bone marrow transplantation in countries
319 outside Europe and USA are necessary. This knowledge will further help the
320 management of these families, as well as may help the understanding of
321 disease mechanisms.

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404 **7. List of Tables**

405

406 Table 1 - Mutations found in the present study

407

408 Table 2 - Clinical characteristics of the affected men in the present families

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410

411 **8. List of Figures**

412

413 Figure 1 – Distribution of the mutations observed in this series according to the
414 amplicon regions (exons), and compared with the expected proportions
415 (http://www.x-ald.nl).

416

417 Figure 2 - The four pedigrees where *de novo* mutations were detected.

418

419 Figure 3 – Kaplan–Meier survival curves. (A) Overall disease onset of the main
420 phenotypes Addison-only, cerebral (CALD) and adrenomyeloneuropathy
421 (AMN). Disease onset were significantly different (log rank test, p< 0.001). (B)
422 Disease onset of CALD, according to latitude of origin of the patient (ns). (C)
423 CALD survival until death.

424

425 Figure 4 – MRI of the female patient 54, showing the typical pattern of white
426 matter abnormality found in X-ALD.

Figure 1- Distribution of the mutations observed in this series according to the amplicon regions (exons), and compared with the expected proportions (http://www.x-ald.nl). Inside of the columns, the absolute numbers.

Distribution of mutations in ALDP
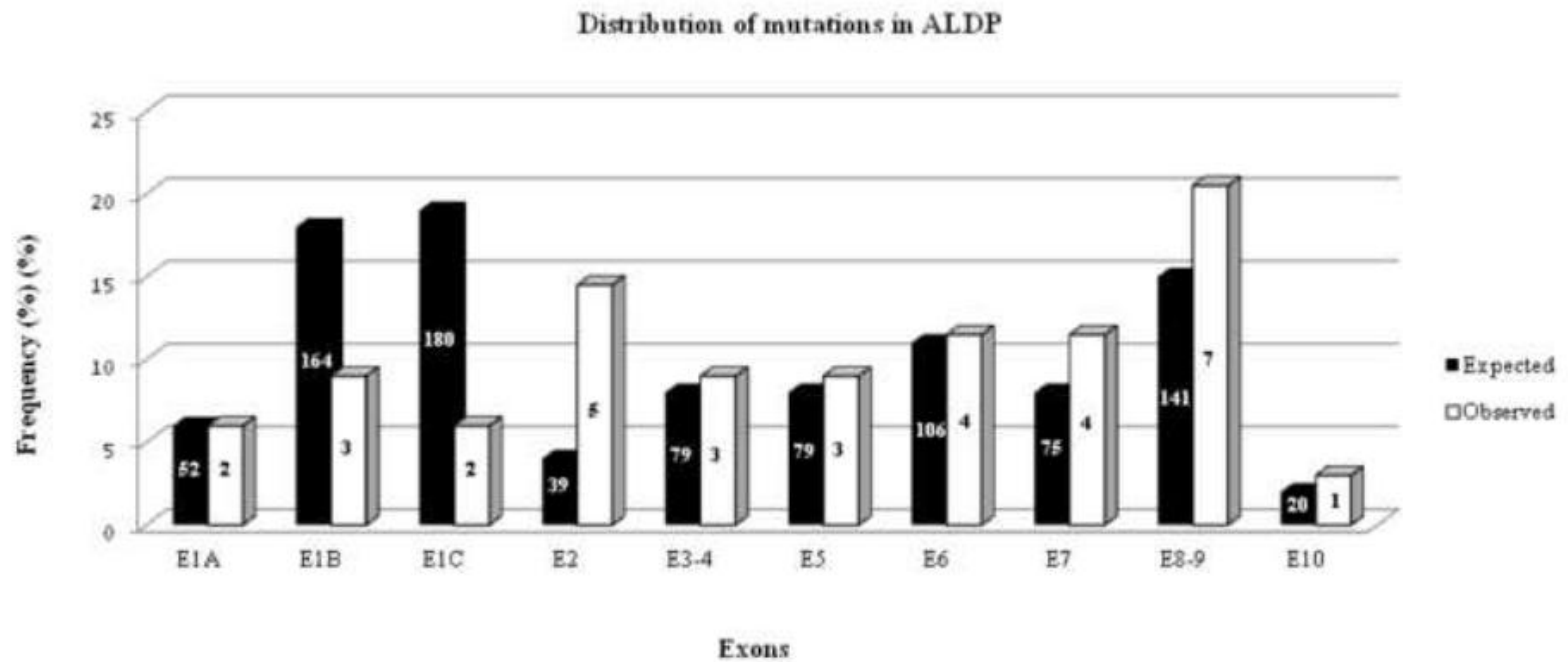
Figure 2 – The four pedigrees where *de novo* mutations were detected.

a) Pedigree's family 1 (p.Glu512Ser)
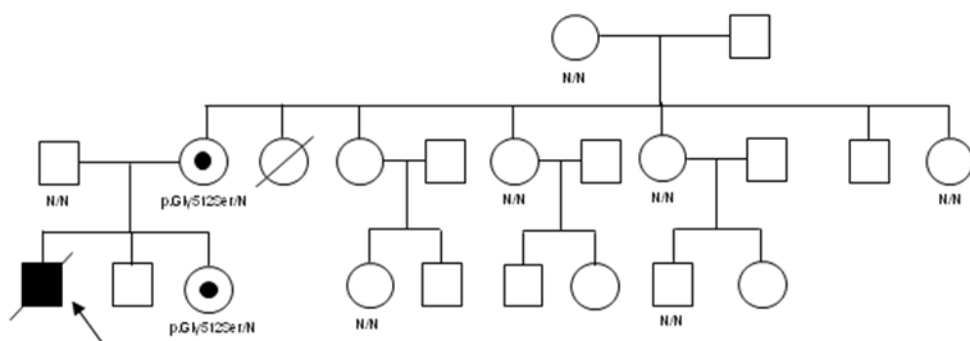
b) Pedigree's family 20 (p.Arg538fs)
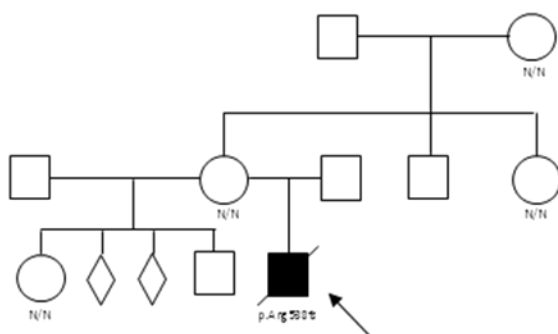
c) Pedigree's family 31 (p.Arg518Gln)
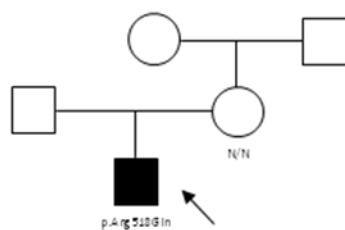
d) Pedigree's family 33 (p.Thr632Pro)
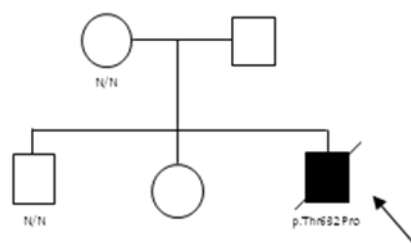
Figure 3 – Kaplan-Meier survival curves. (A) Overall disease onset of the main phenotypes Addison-only, cerebral (CALD) and adrenomyeloneuropathy (AMN). Disease onset were significantly different (log rank test, p>0.001). (B) Disease onset of CALD, according to latitude of origin of the patients (ns). (C) CALD survival until death.

Figure 3

Figure 4 – Female patient 54 MRI, showing the typical pattern of white matter abnormality found in ALD-X

Figure 4 – Female patient 54 MRI, showing the typical pattern of white matter abnormality found in ALD-X

Table 1 - Mutations found in the present study.

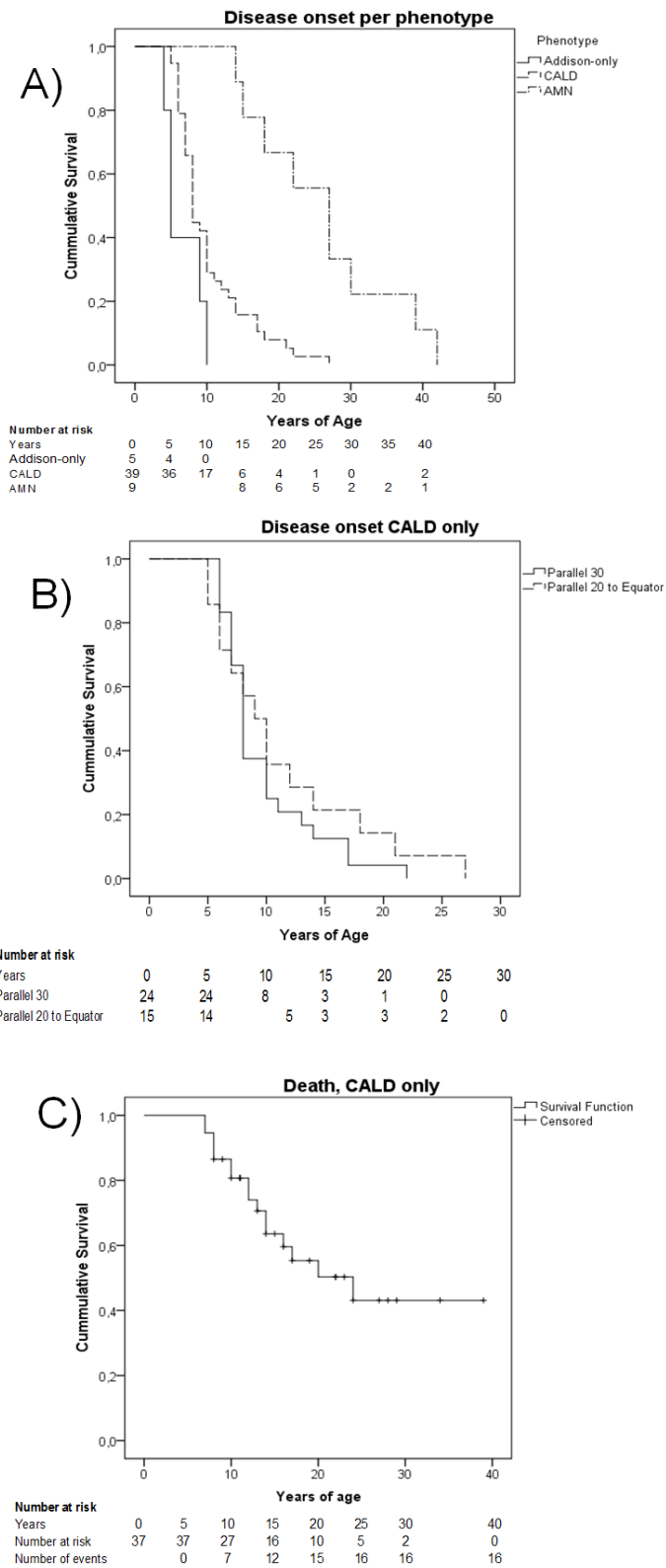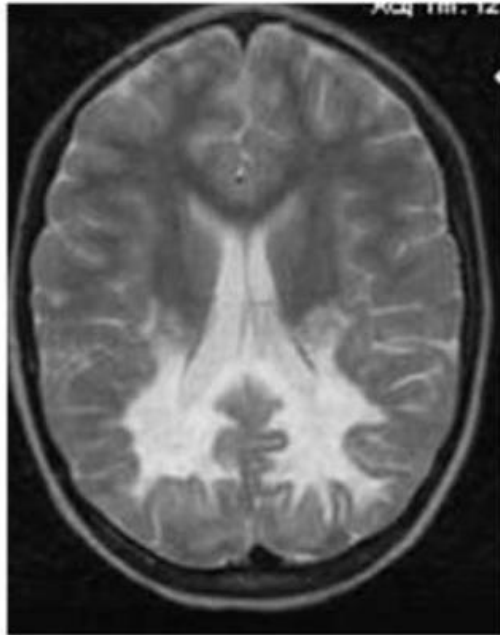| Family/Index case	Phenotype at diagnosis	Mutation	Exon/IVS	Mutation type	Effect on protein (cDNA)	Effect on protein (mRNA)	Protein localization	Origin of mutations	Origin of family
1 / Female	asymptomatic	p.Gly512Ser (Feigenbaum V et al. 1996)	E6	Missense	c.1534G>A	GGC>AGC	NBF	*de novo*	Southern Brazil
2 / Female	asymptomatic	p.Ser606Leu (Fanen P *et al.*, 1994)	E8	Missense	c.1817C>T	UCG>UUG	NBF	Inherited	Southern Brazil
3 / Male	AMN	p.Trp601X (Gartner J et al.,1998)	E8	Stop codon	c.1802C>A	Truncated	NBF	Inherited	Southern Brazil
4 / Female	asymptomatic	p.Arg617His (Fanen P *et al.*, 1994)	E8	Missense	c.1850G>A	CGC>CAC	NBF	ND	Southern Brazil
5 / Male	AMN	**p.Pro623Leu**#	E9	Missense	c.1868C>T	CCC>CUC	NBF	Inherited	Southern Brazil
6 / Male	AO	p.Trp326X (Barcelo A et al, 1996)	E2	Stop codon	c.978G>A	Truncated	TMD	Inherited	Southern Brazil
8 / Female	asymptomatic	**p.Glu577X**#	E7	Stop codon	c.1729C>T	Truncated	NBF	Inherited	Southern Brazil
9 / Male	asymptomatic	p.Arg554His (Smith KD et al., 1999)	E7	Missense	c.1661G>A	CGU>CAU	NBF	Inherited	Southern Brazil
10 / Male	CALD	p.Arg518Gln (Imamura A et al., 1997)	E6	Missense	c.1553G>A	CGG>CAG	NBF	Inherited	Southern Brazil
11 / Male	AO	**p.Tyr33_Pro34fsX34**#	E1A	Frameshift + stop codon	c.99_102delC	Truncated	-	Inherited	Southern Brazil
12 / Female	asymptomatic	p.Gly266Arg (Fuchs S et al., 1994)	E7	Missense	c.1653insG	Truncated	TMD	ND	Southern Brazil
20 / Male	CALD	**p.Arg538fs**#	E6	Frameshift	c.1614_1615dup27	Elonged	NBF	*de novo*	Southern Brazil
21 / Male	CALD	**p.Ala232fsX64**#	E2	Frameshift + stop codon	c.696_697del11	Truncated	TMD	Inherited	Southern Brazil
22 / Male	CALD	**p.Trp137fsX57**#	E1B	Frameshift + stop codon	c.411_412insC	Truncated	TMD	Inherited	Northern Brazil
23 / Male	asymptomatic	p.Trp679X (Waterham HR et al, 1998)	E10	Stop codon	c.2037G>A	Truncated	NBF	ND	Southern Brazil
24 / Male	AO	p.Tyr296Cys (Takano H et al., 1999)	E2	Missense	c.887A>G	UAU>UGU	TMD	Inherited	Southern Brazil
27 / Male	CALD	**p.Leu628Glu**#	E9	Missense	c.1883T>A	CUG>GAG	NBF	Inherited	Southern Brazil
29 / Male	CALD	p.Pro546fsX? (Fanen P et al. 1994)	IVS6	Frameshift + stop codon	IVS+1g>a	Splicing error ?	NBF	Inherited	Northern Brazil
31 / Male	CALD	p.Arg518Gln (Imamura A et al., 1997)	E6	Missense	c.1553G>A	CGG>CAG	NBF	*de novo*	Southern Brazil
32 / Male	CALD	p.Arg401Trp (Takano H et al., 1999)	E3	Missense	c.1201C>T	CGG>UGG	-	ND	Southern Brazil
33 / Male	CALD	p.Thr632Pro (http://www.x-ald.nl)	E9	Missense	c.1894A>C	ACC>CCC	NBF	*de novo*	Southern Brazil
36 / Male	CALD	p.Arg518Gln (Imamura A et al., 1997)	E6	Missense	c.1553G>A	CGG>CAG	NBF	Inherited	Southern Brazil
37 / Male	CALD	p.Ser358X (Coll MJ et al., 2005)	E2	Stop codon	c.1073C>G	UCA>UGA	TMD	Inherited	Southern Brazil
38 / Male	CALD	**p.Ile481Phe**#	E5	Missense	c.1441A>T	AUC>UUC	NBF	Inherited	Northern Brazil
39 / Male	AMN	p.Arg389Gly (Krasemann EW et al., 1996)	E3	Missense	c.1165C>G	CGC>GGC	-	ND	Argentina
40 / Male	AMN	p.Gln472fsX83 (Barceló A et al., 1994)	E5	Frameshift + stop codon	c.1415_1416delAG	Truncated	-	Inherited	Uruguay
41 / Male	CALD	**p.Ala95fsX11**#	E1B	Frameshift + stop codon	c.283_284ins9	Elonged	TMD	Inherited	Southern Brazil
44 / Male	CALD	p.Ser606Pro (Feigenbaum V et al. 1996)	E8	Missense	c.1816T>C	UCG>CCG	NBF	Inherited	Northern Brazil
45 / Male	CALD	**p.Gln55X**#	E1A	Stop codon	c.163C>T	Truncated	-	Inherited	Northern Brazil
46 / Male	CALD	p.Glu199Lys (http://www.x-ald.nl)	E1C	Missense	c.595G>A	GAG>AAG	TMD	ND	Northern Brazil
49/ Male	CALD	p.Trp132X (Pan H et al., 2004)	E1B	Stop codon	c.396G>A	TGG>TGA	TMD	Inherited	Northern Brazil
50/ Male	CALD	p.Glu477fsX80 (Valadares ER et al., 2011)	E5	Frameshift + stop codon	c.1430delA	Truncated	NBF	ND	Northern Brazil
51/ Female	asymptomatic	p.Pro623fsX? (Kemp S et al., 1995)	IVS8	Frameshift + stop codon	c.1866-10G>A	Splicing error ?	NBF	ND	Northern Brazil
52/ Male	CALD	**p.Arg401Gly**#	E3	Missense	c.1201C>G	CGG>GGG	-	Inherited	Southern Brazil
54/Female	CALD	**p.Ser358fsX42**#	E2	Frameshift + stop codon	c.1074_1075insA	Truncated	TMD	ND	Northern Brazil
55/ Female	AMN	p.Gly510Ser (http://www.x-ald.nl)	E6	Missense	c.1528G>A	GGC>AGC	NBF	ND	Northern Brazil
56/Male	CALD	p.Asp200Asn (Takano H et al., 1999)	E1C	Missense	c.528G>A	GAC>AAC	TMD	Inherited	Northern Brazil
57/Male	CALD	p. Pro560Leu (Braun A et al., 1995)	E7	Missense	c.1679C>T	CCG>CTG	NBF	Inherited	Northern Brazil

The number of family: the registration number in records of our lab. AMN: adrenomyeloneuropaty; AO: Addison only; #: new mutations identified in this study; NBF: nucleotide-binding fold; TMD: Transmembrane Domin; ND: not determined.

Southern Brazil: those families who lived near parallel 30th South; Northern Brazil: those families who lived between Equator and parallel 20th South. Argentina and Uruguay lies on parallel 30th or southern to it.

Table 2 – Clinical characteristics of the affected men in the present families

Clinical phenotypes	Number of cases (%)	Latitude of origin Parallel 30	Alive	Age at investigation (years)	Age at onset (years)	Age at death (years)
				Mean, *CI 95%** (range)		
Asymptomatic	6 (7 %)		6/6	6.4 *1.9-10.8* (2-14)	-	-
Addison-only	6 (7 %)		5/6	15.2 *5.3-25* (5-34)	7.4 *5.4-9.4* (4-10)	10[a]
CALD	54 (62 %)	39/54	22/49[#]	16.5 *13.9-19* (7-39)	10.9 *9.1-12.7* (5-27)	24.7[b] *19.8-29.6* (7-24)
AMN	14 (16 %)	11/14	11/14	40.2 *32.5-47.8* (20-53)	26.4 *20.3-32.5* (15-42)	*Unknown*
Unknown	7 (8 %)		--			
Total	87 (100 %)		44/75	19.7	12.5	-

				16.2-32.2 (2-53)	10.2-14.7 (4-42)	

CALD: Cerebral form, AMN: Adrenomyeloneuropathy
a. one case, b. 16 cases

* Means estimated as survival functions (see Figure 3).
# number of valid cases, 5 losses in follow up

Artigo 2 – submetido ao Clinical Biochymistry

Corresponding Author: Dr. Laura Bannach Jardim, MD, PhD

Corresponding Author's Institution: Universidade Federal do Rio Grande do Sul

First Author: Fernanda S Pereira, MSc

Order of Authors: Fernanda S Pereira, MSc; Clarissa T Habekost, MD; Ursula Matte, PhD; Daniella Coelho; Marion Deon, PhD; Angela Sitta, PhD; Carmen R Vargas, PhD; Laura Bannach Jardim, MD, PhD

Abstract: X-linked adrenoleukodystrophy (X-ALD) is caused by a defect in the gene encoding the adenosine triphosphate (ATP)-binding cassette ABCD1. The gene defect is related to an increase of completely saturated, very long chain fatty acids (VLCFA) in body fluids. Former studies in large samples have shown that VLCFA is abnormal in 99% of males with X-ALD, and in 80-85% of affected females. (a) Aims: to compare the results of the VLCFA analyses with those of molecular studies performed in women from X-ALD families, describing sensitivity and specificity of VLCFA analysis; (b) Methods: women from 18 families where X-ALD was segregating, were recruited. VLCFA analyses had been done before DNA investigation was available, and during genetic counseling. After consent, blood was collected and DNA was extracted. All samples were screened for the specific mutation found in the family, by automated DNA sequencing; (c) Results: Fifty women were analyzed: 39/50 were either obligate carriers or were molecularly confirmed as heterozygous, and 11/50 had normal molecular analyses.   Thirty-three women had normal VLCFA profiles. Twenty-four of the 33 women with normal VLCFA levels were actually heterozygotes, corresponding to a rate of 72.7% of false-negative results. Two women with previous high levels of VLCFA had actually normal genotype, corresponding to 11.8% of false-positive results; (d) Discussion: We have detected unexpectedly high rates of false-negative results and, surprisingly, women with false-positive results. This study shows the importance of the molecular diagnosis for the identification of heterozygous females and recommends the avoidance of VLCFA as a method to help women at risk for X-ALD in genetic counseling.

Dr. Laura Vilarinho is a biochemist with great experience in Inborn Errors of Metabolism in general, and in X-ALD in particular.

Cover Letter

To
Peter Kavsak,
Editor-in-Chief,
Clinical Biochemistry

Dear Peter Kavsak,

I am sending enclosed the manuscript entitled "Molecular analyses versus very long chain fatty acids profile in women from X-linked Adrenoleukodystrophy families", to be considered for publication in *Clinical Biochemistry*.

All co-authors have approved the publication of the present manuscript, which has never been published before, in whole or in part. We also warrant that the present work has been approved by the Ethics Committees from the institutions at which the work was performed, which follow the Code of ethics of the World Medical Association (Declaration of Helsinki) and the standards established by the author's Institutional Review Board and granting agency.

Yours sincerely,

Laura B. Jardim, MD PhD
Medical Genetics Service
Hospital de Clínicas de Porto Alegre
Rua Ramiro Barcelos 2350
90035-003 Porto Alegre
Brazil
Phone: + 55 51 3359 8011
Telefax: + 55 51 3359 8010
e-mail: ljardim@hcpa.ufrgs.br

Highlights:

X-ALD in women is currently diagnosed by mutational analysis.

We correlated VLCFA serum levels with mutational analysis in women from X-ALD families.

We detected high rates of both false-negative using VLCFA levels.

Surprisingly, false-positive results were also found using this approach.

We suggest not using VLCFA for genetic counseling purposes.

Molecular analyses versus very long chain fatty acids profile in women from X-linked Adrenoleukodystrophy families.

Fernanda dos Santos Pereira [a,c], Clarissa Toller Habekost [b,d], Ursula Matte [a,d], Daniella Coelho [b], Marion Deon [b,e], Angela Sitta [b], Carmen Regla Vargas [b,e,f], Laura Bannach Jardim[b,c,d,g]

[a] Gene Therapy Center, and [b] Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Brazil.
Post-Graduation Programs in [c] Medical Sciences, in [d] Genetics and Molecular Biology, and in [e] Pharmaceutical Sciences; and Departments of [f] Analysis, and [g] of Internal Medicine, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

Corresponding author:
Laura Bannach Jardim, MD, PhD
Medical Genetics Service,
Hospital de Clinicas de Porto Alegre
Rua Ramiro Barcelos 2350
90.035-903, Porto Alegre, Brazil
Phone: 55 51 3359 8011
Fax: 55 51 3359 8010
Email: ljardim@hcpa.ufrgs.br

Abstract

X-linked adrenoleukodystrophy (X-ALD) is caused by a defect in the gene encoding the adenosine triphosphate (ATP)-binding cassette *ABCD1*. The gene defect is related to an increase of completely saturated, very long chain fatty acids (VLCFA) in body fluids. Former studies in large samples have shown that VLCFA is abnormal in 99% of males with X-ALD, and in 80-85% of affected females. **(a) Aims:** to compare the results of the VLCFA analyses with those of molecular studies performed in women from X-ALD families, describing sensitivity and specificity of VLCFA analysis; **(b) Methods:** women from 18 families where X-ALD was segregating, were recruited. VLCFA analyses had been done before DNA investigation was available, and during genetic counseling. After consent, blood was collected and DNA was extracted. All samples were screened for the specific mutation found in the family, by automated DNA sequencing; **(c) Results:** Fifty women were analyzed: 39/50 were either obligate carriers or were molecularly confirmed as heterozygous, and 11/50 had normal molecular analyses. Thirty-three women had normal VLCFA profiles. Twenty-four of the 33 women with normal VLCFA levels were actually heterozygotes, corresponding to a rate of 72.7% of false-negative results. Two women with previous high levels of VLCFA had actually normal genotype, corresponding to 11.8% of false-positive results; **(d) Discussion:** We have detected unexpectedly high rates of false-negative results and, surprisingly, women with false-positive results. This study shows the importance of the molecular diagnosis for the identification of heterozygous females and recommends the avoidance of VLCFA as a method to help women at risk for X-ALD in genetic counseling.

Key-words: X-linked adrenoleukodystrophy, X-ALD, very long chain fatty acids, VLCFA, predictive value, specificity, sensitivity, heterozygous women.

Highlights: X-ALD in women is currently diagnosed by mutational analysis.
We correlated VLCFA serum levels with mutational analysis in women from X-ALD families.
We detected a high rate of false-negative results using VLCFA levels.
Surprisingly, false-positive results were also found using this approach.
We suggest not using VLCFA for genetic counseling purposes.

1. Introduction

X-linked adrenoleukodystrophy (X-ALD, OMIM ID: 300100) is the most common peroxisomal disorder, with an incidence of approximately 1 in 17,000 male newborns [1,2]. This neurodegenerative disease is characterized by great clinical expression variability. According to the age of onset, organs affected and the rate of the progression of neurologic symptoms, hemizygotes may present the cerebral form (CALD), adrenomyeloneuropathy (AMN), Addison disease only, or may be asymptomatic for a long period of time [3]. Female carriers might be asymptomatic or present with mild to severe myeloneuropathy that resembles AMN.

X-ALD is caused by a defect in the gene encoding the adenosine triphosphate (ATP)-binding cassette, subfamily D, member 1 located on Xq28 (*ABCD1*), also called ALD protein (ALDP) [3,4,5]. *ABCD1* has been proposed to play a crucial role transporting unbranched saturated very long chain fatty acids (VLCFA), or their CoA derivatives, into peroxisomes in humans.

The main biochemical abnormality associated with X-ALD is the accumulation of VLCFA in plasma and tissues, due to an impaired β-oxidation in the peroxisomes. The increase in VLCFA levels provides a reliable diagnostic tool for prenatal and postnatal identification of affected males [3]. In females, however, VLCFA levels present false-negative results in around 20% of the obligate carriers [3,6]. No references to false-positive results were found in the literature. When molecular analysis is unavailable, the correct genetic status of women is uncertain, and most of the genetic counseling rests on the risk obtained by VLCFA analysis.

This study aimed to compare the previous results of the VLCFA analyses obtained in women at risk for X-ALD, to their final genetic status, determined either by molecular analysis or by the identification of an obligate carrier. This study also described parameters such as sensitivity and specificity of the VLCFA analyses, by using the final genetic status as the gold-standard.

Abbreviations: X-ALD: X-linked adrenoleukodystrophy; *ABCD1*: adenosine triphosphate ATP-binding cassette; VLCFA: very long chain fatty acids; SSCP: single strand conformational polymorphism; AUC: area under the curve; CALD: cerebral X-ALD; HSCT: hematopoietic stem cell transplantation; ERNDIM: "External Quality Assurance Programme for Amino Acids, Quantitative Organic Acids, Purines and Pyrimidines, Special Assays in Serum and Urine, Cystine in White Blood Cells and Lysosomal Enzymes" (www.erndimqa.nl); GC-MS: gas chromatography-mass spectrometry.

2. Material and methods

The present study began in 2008, when all families with X-ALD started to be invited to perform molecular analyses in our lab [7], in order to improve their genetic counseling. After the identification, in each family, of the ABCD1 mutation in a hemizygote or in an obligate carrier, all the other relatives were invited to confirm their genetic status through molecular studies. This study only briefly mentioned male results, since the main focus was on the female results.

The invited women had been tested for VLCFA analysis in our lab in the last 18 years. To be included in the present molecular study, women should have 18 years of age or more, and should agree to perform a second blood collect for DNA extraction. The present work has been approved by the Ethics Committee of our institution, and we have obtained written informed consent from all participants.

Plasma VLCFAs were analyzed, in duplicate, in the past, according to the technique of Moser [8]. This laboratorial procedure consisted of the preparation of total lipid extract a treatment of this extract with methanolic HCl (3 N) for the formation of fatty acid methyl esters, which were then purified by thin-layer chromatography. The fatty acid methyl esters purified were extracted with hexane and analyzed by gas chromatography. In each battery of tests, as an internal control, one positive and one negative sample were included. A Varian gas chromatographer with an HP-5 column (5% methylphenyl silicone, 0.33 µm film thickness, 0.2 mm inner diameter and 25 m in length), a flame ionization detector, a split/splitless injector, and helium as the mobile phase was utilized. $C_{22:0}$ (docosanoic acid) [reference range in normal controls: 40.7 – 118.9; median value in heterozygotes: 57.2] and $C_{26:0}$ (hexacosanoic acid) [reference range 0.78 – 1.54; median value in heterozygotes: 1.42] were expressed in µmol/L and the $C_{26:0}/C_{22:0}$ ratio was calculated [reference range 0.01 – 0.03; median value in heterozygotes: 0.04]. Heptacosanoic acid ($C_{27:0}$) was used as internal standard.

The present results on $C_{22:0}$, $C_{24:0}$ and $C_{26:0}$ were related to samples collected and measured since 1993. During all this period, a female was considered to be at high risk of being a carrier if the following two abnormalities were found: a $C_{26:0}$ level of 1.42 µM/L or more and a $C_{26:0}/C_{22:0}$ ratio of 0.04 or more. If $C_{26:0}$ level and/or the $C_{26:0}/C_{22:0}$ ratio were normal, the woman was informed that she was at low risk to be a carrier, and was also remembered that at least 20% of the carriers would present these normal

results. Both the cutoff values and the measurements were in agreement with the external quality assurance scheme for VLCFA analysis.

When the invited subject came to the interview, she (he) was informed that molecular studies were now available and that she (he) could collect a DNA sample in order to confirm the previous biochemical diagnosis. After consent, blood was collected and DNA was extracted by the salting out procedure [9]. Using 10 PCR reactions it was possible to screen the entire coding sequence of the *ABCD1* gene and intron-exon boundaries using the protocol described previously [10]. All samples were screened by single strand conformational polymorphism (SSCP) analysis followed by automated DNA sequencing to establish the specific mutation in each family. The fragments obtained were purified with Exo-SAP (GE Healthcare) and submitted to automated sequencing on ABI 3100 Genetic Analyzer using BigDye v3.0 (Life Technologies). Mutations were confirmed by reverse strand PCR sequencing. More details were described elsewhere [7].

Previous VLCFA levels were then compared to the final genetic status. A woman was categorically defined as a heterozygote if she carried the causal mutation of her family or if she was an obligate carrier; or as a normal homozygote, if her molecular analysis resulted normal. Proportions of true positive, true negative, false positive and false negative VLCFA results were then obtained. Sensitivity and specificity values were calculated from the contingence table obtained. To confirm if simultaneous alterations in both parameters ($C_{26:0}$ levels and $C_{26:0}/C_{22:0}$ ratio) are more sensitive and/or specific to identify a carrier than any of them alone, the same tests were done individually. Roc curves were obtained for each one, and analysed according to the area under the curve (AUC): the best discriminant test for diagnosis is that whose AUC would be near 100%, whereas a total non-discriminant test (results obtained at random) would have an AUC of near 50%.

Age distributions showed a normal distribution on Kolmogorov–Smirnov test, and were compared with two-tailed unpaired Student's t test. Statistical significance was defined as $p < 0.05$.

3. Results

From 2007 to 2011, thirty-eight families had a molecular diagnosis of X-ALD in our lab; detailed analyses were described elsewhere [7]. All molecular diagnosis matched perfectly with previous VLCFA analysis, in males.

Thirty-three Brazilian women from 14 identified families, had been tested for their plasmatic levels of VLCFA, and also for their gene sequencing (Table 1). Seventeen obligate carriers from these and other four families, whose DNA has not been tested, were included in the study since they had performed VLCFA analysis. Therefore, 50 women comprised the present sample of women with defined genetic status.

According to the molecular results and to the pedigree analysis, thirty-nine women (or 78%) were defined as mutation carriers, whereas eleven (or 22%) were defined as normal homozygotes.

The mean (±sd; range) age at the time of VLCFA analyses of the mutation carriers and of the normal homozygotes were respectively of 36.9 (±13.4; 21 to 91 years of age) and 35.6 (± 11.5; 20 to 52 years of age) (ns, t test).

$C_{26:0}$ values and $C_{26:0}/C_{22:0}$ ratios did not correlate with age (ns, Spearman) nor varied during the period in which they had been tested (ns, Kruskal-Wallis test).

3.1 VLCFA criteria for a high risk of heterozygosis

Thirty-three women had received, in the past, a possible diagnosis of normal homozygosis by VLCFA double criteria (high levels of $C_{26:0}$ and $C_{26:0}/C_{22:0}$ ratio), whereas seventeen had received a possible diagnosis of a heterozygote state due to altered results (Table 2).

Normal results assigned by VLCFA were confirmed by the present molecular studies in only 27.3%: in other words, there were 72.3% of false negatives, in the present series (Table 2). Altered results on VLCFA were confirmed in 88.2% of the present cases. In two cases (or 11.8%), these were false-positive results. These results were related to a sensitivity of 38% and a specificity of 82%, of VLCFA criteria.

In order to test if the association of the two criteria (high levels of $C_{26:0}$ and of $C_{26:0}/C_{22:0}$ ratio) produced such a low sensitivity, we analyzed the diagnostic power of each individual criteria, separately.

When only high levels of $C_{26:0}$ were used, there were sixteen (or 72.7%) false-negative cases out of 22 normal results, and five (or 17.9%) false-positive cases out of 28 women with altered results (Table 2). The area under the obtained ROC curve was of 0.59.

When only high levels of $C_{26:0}/C_{22:0}$ ratio were used as a diagnostic criteria, there were eleven (or 55%) false-negative cases out of 20 normal results, and two (or 6.7%) false-positive cases out of 30 women with altered results (Table 2). The area under the obtained ROC curve was of 0.82, and the distribution of $C_{26:0}$ results between mutation carriers and normal homozygotes were depicted in Figure 2. These results were related to a sensitivity of 72% and a specificity of 82%.

3.2 Clinical description of the two false-positive women by VLCFA analysis found in this series

Two women previously characterized as heterozygotes, based on their VLCFA analyses, have been proved to be have a normal ABCD1 gene sequence ((Table 3).

C.R.O.L., DOB 03/09/1969, was the mother of a CALD patient (B.L.S.), the index-case of family 20, who started with cerebral symptoms at 10 years of age, being deceased at 16 years. There was no recurrence in the family, whose pedigree has been depicted in the Figure 2B of Pereira et al [7]. B.L.S. had neuroimaging and VLCFA compatible with CALD. A DNA sample was stored and his molecular study, performed in 2010, disclosed a new mutation on exon 6, p.Arg538fs. This is a frameshift mutation, related to a 27 amino acids insertion between residues 538 and 539 of the wild peptide (to a complete description, see Pereira et al [7]). The first VLCFA analysis of the mother C.R.O.L. was performed in 2006, and presented a pattern compatible with the heterozygote state: a $C_{26:0}$ level of 2.42 and a $C_{26:0}/C_{22:0}$ ratio of 1.16. She was informed at that time to have a high probability of being a carrier. The VLCFA profile of her mother, sister and niece gave normal results. In 2010, molecular studies were performed in C.R.O.L. and her three female relatives, and none of them carried the p.Arg538fs mutation. The negative result of sequencing was confirmed in a second, independent DNA sample. A second VLCFA analysis was performed in C.R.O.L. in 2010, and gave

doubtful results (a $C_{26:0}$ level of 1.49 and a $C_{26:0}/C_{22:0}$ ratio of 0.03). C.R.O.L. had undergone tubal ligation in 2000, years before the X-ALD diagnosis. She presented migraine since 2006, and Graves disease since 2009. No other co-morbidities were detected.

M.M.L, DOB 11/08/1957, was aunt of another CALD patient, the index and unique case of family 8, called F.M.L. F.M.L., DOB 06/01/1987, started with motor symptoms with 11 years of age; his VLCFA and neuroimaging was compatible with the diagnosis of CALD. In 2000, he received an unrelated donor HSCT and since them, his neurological and cognitive examinations are normal [11]. Given the HSCT performed in the index case, the molecular analysis started with her mother C.C.M.L., who presented the new mutation p.Glu577X that creates an early stop codon at exon 7 (to a complete description, see Pereira et al [7]). C.C.M.L had four sisters (aunts of the index-case): all but one had normal VLCFA profiles. The forth sister (aunt of the index-case) was M.M.L. M.M.L. performed the first VLCFA analysis in 1998 in our lab and received the diagnosis of being probably a carrier, since she presented a $C_{26:0}$ level of 3.8 and a $C_{26:0}/C_{22:0}$ ratio of 0.06. In 1999 she repeated the analysis in another lab, and although actually not completely clear - there was an increased level of $C_{26:0}$ (1.56) and a normal $C_{26:0}/C_{22:0}$ ratio (0.03) –, the pattern was considered "consistent with a X-ALD carrier". Her molecular study proved that she was not a carrier of p.Glu577X. A second sample was collected and the *ABCD1* gene was entirely sequenced, with normal results. M.M.L. had no comorbidities.

4. Discussion

The importance of diagnosis of a carrier state in X-linked disorders is paramount, especially in such a dramatic disease such as X-ALD. Unfortunately, the molecular investigation is not available in most parts of the world. Therefore, VLCFA might continue to be the only tool to help physicians and genetic counselors in some communities. Such was our former scenario, changed after the introduction of molecular studies for X-ALD. We decided to describe our local rates of VLCFA sensitivity and specificity in order to better understand the usefulness of VLCFA analysis for women belonging to X-ALD families.

Our strategy was commonsense and portrayed the usual way a genetic counseling process is carried out. After identification of the primary DNA defect among index cases, we studied all the family members who wanted to be tested. As expected, we confirmed by mutation analysis all X-ALD hemizygous subjects previously diagnosed by VLCFA measurement in serum. Heterozygous detection resulted in a diverse panorama.

In the past 18 years, we have used the previous, traditional criteria for a woman to be considered at risk for X-ALD: to having elevated $C_{26:0}$ blood levels and also elevated $C_{26:0}/C_{22:0}$ ratio. By performing the present study, we now know that these traditional criteria produced the impressive rate of 72.3% of false-negative results (Table 2), much higher than those described in literature, of 20%. At first look, these results could suggest that our cutoff values were very stringent. However, Figure 1 showed that either $C_{26:0}$ levels and $C_{26:0}/C_{22:0}$ ratio obtained in mutation carriers and normal homozygotes overlapped completely, preventing to choose a better cutoff value. It is important to emphasize that we have been certificated by an international quality control programme (ERNDIM) since 1999.

We have then analysed $C_{26:0}$ blood levels and $C_{26:0}/C_{22:0}$ ratio separately, and have found that results of $C_{26:0}/C_{22:0}$ ratio gave less false positives and false negatives than the combined criteria, when relative numbers were analyzed. The ROC curve obtained for $C_{26:0}/C_{22:0}$ ratio also showed a better response, with an area of 82% under the curve, suggesting that the accuracy of this ratio alone is better than the absolute levels of $C_{26:0}$, in women. Actually, the area under the ROC curve for $C_{26:0}$ plasmatic levels, of 59%, means that these levels were almost randomly found in carriers and non-

carriers. Adding $C_{26:0}$ to the ratio, in order to interpret VLCFA results in women, have been therefore misleading, in our experience.

Our data may be related to general characteristics of the present population of Brazilian females. For instance, there may be some undetected nutritional characteristic that might remove the power of VLCFA relationship with the genetic state. For instance, food containing large amounts of VLCFAs, in particular peanuts, may have a considerable impact on $C_{26:0}$ levels [12]. Although these women were instructed not to eat before three hours before blood collection, one cannot rule out the possible long-lasting effect of some dietetic peculiarity. Alternatively, the high rate of false-negative results might suggest that our females would have unexpected inactivation patterns towards the normal chromosome. The relationship between inactivation patterns of X chromosome and phenotype among heterozygotes for X-ALD have been raised for a long time [13,14], although no relation could be clearly found between VLCFA levels and inactivation patterns. All these are conjectural and epidemiological studies are necessary to support these hypotheses.

However, the most unexpected finding was actually the two molecularly normal women with previous elevated VLCFA profiles. False-positive results in females received no previous mention in literature [6,12,15]. We have reviewed these two women, and found no other explanation to these abnormal profiles. Both women presented one abnormal and one doubtful VLCFA profile: and in both cases, we and others have concluded towards a heterozygote diagnosis in the past. Fortunately, both women were not in the reproductive period by the time of their mistaken diagnosis. In the first case described, patient C.R.O.L (family 20), we have actually detected a *de novo* mutation: his son carried a deleterious new mutation whereas C.R.O.L did not, at least in her peripheral blood. It is possible that C.R.O.L could be a mosaic and, therefore, could present ambiguous or even altered VLCFA profile. This explanation is not applicable to the second false positive case, M.M.L., however.

These false-positive results in females should raise our concern about the other women, who were not obligate carriers and who were previously investigated and characterized as biochemical heterozygotes, whose DNA has not been tested subsequently. Our present results reinforce the notion that VLCFA determined in blood plasma cannot be the only approach for women, but actually contraindicate them for the diagnosis of heterozygous status in females. If there is no other way to study a female, choosing only the $C_{26:0}/C_{22:0}$ ratio as a criteria might give less ambiguous results - 55%

false-negative and 6.7% false-positive results, with a sensitivity of 72% and a specificity of 82%.

Other diagnostic, biochemical algorithms have been presented, such as performing three analyses in positive cases – repeating the plasmatic analysis twice and after that, measuring VLCFA in fibroblasts -, or using gas chromatography–mass spectrometry (GC–MS) to analyze $C_{22:0}$, $C_{24:0}$, and $C_{26:0}$ in white blood cells [12]. Given that these alternative methods are time-consuming, expensive, and probably do not achieve total accuracy, we still believe that mutation analysis is the best approach in order to improve genetic counseling to the X-ALD families in general, and to females in particular. It is not only the gold-standard but the only reliable option.

5. Acknowledgements

6. References

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7. List of Tables

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C$_{26:0}$ levels

C$_{26:0}$/C$_{22:0}$ ratio

ROC Curve

Diagonal segments are produced by ties.

Table(s)

Table 1: *ABCD1* mutations and families of the women under study.

Families and mutations found	Number of women	Genetic status	
		Mutation carriers, (detected by mutation analysis) *	Normal homozygotes, all by mutation analysis
p.Tyr33_Pro34fsX34, Family 11	1	1 (1)	
p.Gly266Arg, Family 12	1	1 (1)	
p.Arg401Gly, Family 32	1	1 (1)	
p.Ile481Phe, Family 24	3	2	1
p.Gly512Ser, Family 1	4	2 (2)	2
p.Arg518Gln, Family 10	15	12 (8)	3
p.Arg538fs , Family 20	1	0	1
p.Pro545fs, Family 29	1	1 (1)	
p.Arg554His, Family 9	1	1 (1)	
p.Glu577X, Family 8	5	2 (2)	3
p.Trp601X, Family 3	6	5 (1)	1
p.Ser606Leu, Family 2	2	2 (2)	
p.Arg617His, Family 4	1	1 (1)	
p.Leu628Glu, Family 27	1	1 (1)	
Family 6 (without molecular diagnosis)	2	2	
Family 13 (without molecular diagnosis)	1	1	
Family 15 (without molecular diagnosis)	1	1	
Family 16 (without molecular diagnosis)	1	1	
Family 21 (without molecular diagnosis)	1	1	
Family 22 (without molecular diagnosis)	1	1	
Total	50	39	11

* The remainder women were obligate carriers.

Table 2

Table 2: Comparison between different VLCFA criteria for heterozygous diagnosis, and final genetic status on 50 women of the present series.

Double criteria for high risk of heterozygosity: $C_{26:0}$ level and $C_{26:0}/C_{22:0}$ ratio raised in plasma					
			Genetic status		Total
			Carrier	Non-Carrier	Total
Altered VLCFA profile (high $C_{26:0}$ and $C_{26:0}/c_{22:0}$ ratio)	Yes		15	2	17
		%	88.2%	11.8%	100%
	No		24	9	33
		%	72.7%	27.3%	100%
Total			39	11	50
		%	78%	22%	100%

Simplified VLCFA criteria for high risk of heterozygosity: only $C_{26:0}$ raised.

			Genetic status		Total
			Carrier	Non-Carrier	Total
Only $C_{26:0}$ Raised	Yes		23	5	28
		%	82.1%	17.9%	100%
	No		16	6	23
		%	72.7%	27.3%	100%
Total			39	11	50
		%	78%	22%	100,0%

Simplified VLCFA criteria for high risk of heterozygosity: only $C_{26:0}/C_{22:0}$ ratio raised.

			Genetic status		Total
			Carrier	Non-Carrier	Total
$C_{26:0}/C_{22:0}$ ratio raised	Yes		28	2	30
		%	93.3%	6.7%	100%
	No		11	9	20
		%	55%	45%	100%
Total		Count	39	11	50
		%	78%	22%	100%

Table 3

Table 3: The false-positive women by VLCFA analysis found in this series.

	ABCD1 mutation found in the family	Age at VLCFA analyses (in years)	$C_{26:0}$	$C_{26:0}/C_{22:0}$ ratio	Interpretation of VLCFA profile
			Values found in previous obligate carriers; cutoff		
			m = 1,42 -1sd to + 1 sd = 0,66 to 2,20 Cutoff $\geq$ 1.42	m = 0,05 -1sd to + 1 sd = 0,04 to 0,06 Cutoff $\geq$ 0.04	
C.R.O.L.	p.Arg538fs (family 20)	37	2.42	1.16	Consistent with the heterozygous state *
		41	1.49	0.03	Doubtful **
M.M.L.	p.Glu577X (family 8)	40	3.8	0.06	Consistent with the heterozygous state *
		41	1.56	0.03	Doubtful **

* "Consistent with the heterozygous state": when both $C_{26:0}$ and $C_{26:0}/C_{22:0}$ ratio were higher.
** "Doubtful": when only one parameter was abnormally high.

Artigo 3 – a ser submetido

Copy Number Variation May Play a Role in Phenotypic Variability in X-linked Adrenoleukodistrophy

PEREIRA, Fernanda dos Santos[1,3]; RODRÍGUEZ-SANTIAGO, Benjamín[6,7]; PÉREZ-JURADO, Luiz A[6,7]; GORT, Laura[8]; NAVARRO-SASTRE, Aleix[8]; MATTE, Ursula[1,4]; JARDIM, Laura Bannach[2,3,4,5] , GIRÓS, Marisa[7,8]

[1]Gene Therapy Center, Experimental Research Center and [2]Medical Genetics Service, Hospital de Clínicas de Porto Alegre, Brazil; Post-Graduation Programs in [3]Medical Sciences and [4]Genetics and Molecular Biology, and [5]Department of Internal Medicine, Universidade Federal do Rio Grande do Sul, Brazil; [6] Dept. de Ciències Experimentals i de la Salut, UPF, Barcelona, Spain; [7]CIBER de Enfermedades Raras, CIBERER, Spain Pompeu Fabra University, Spain; [8]Institute of Clinical Biochemistry, Clinic Hospital Barcelona, Spain.

Corresponding author:

Laura Bannach Jardim, MD, PhD

Medical Genetics Service,

Hospital de Clinicas de Porto Alegre

Rua Ramiro Barcelos 2350

90.035-903, Porto Alegre, Brazil

Phone: 55 51 3359 8011

Fax: 55 51 3359 8010

Email: ljardim@hcpa.ufrgs.br

Short title: CNV in X-ALD

ABSTRACT

X-linked adrenoleukodystrophy (X-ALD) is a neurodegenerative disease associated with the accumulation of unbranched saturated very long chain fatty acids (VLCFA) in plasma and tissues. It is characterized by great clinical variability even within the same family. Clinical spectrum ranges from the rapidly progressive childhood cerebral form (CALD), which typically leads to severe disability and death during the first decade, to the milder adrenomyeloneuropathy (AMN) that manifests between 20-30 years of age with normal lifespan. To investigate if copy number variants (CNV) could influence X-ALD phenotypes, 29 candidate genes involved in inflammatory response, lipid metabolism and regeneration of the nervous system were investigated by Multiple Ligation-dependent Probe Amplification (MLPA) in a group of 85 Spanish and Brazilian patients. According to clinical characteristics, 39 patients were classified as AMN and 46 as CALD. Using a set of custom-designed probes for MLPA we were able to show copy number variation in nine genes. Possible interactions among these genes were analyzed using the web-based Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) version 9. Network analysis revealed three main interaction hubs, with POMC (Proopiomelanocortin) acting as a central protein, joining not only these three hubs but also linking other isolated proteins. Some genes described here present a potential to influence phenotype, although by mechanisms not completely understood. To the best of our knowledge, this is the first study to investigate CNV influence in phenotypic variability in X-ALD.

Key words: Adrenoleukodystrophy, copy number variation, adrenomyeloneuropathy.

1. INTRODUCTION

X-linked adrenoleukodystrophy (X-ALD, OMIM #300100) is a neurodegenerative disease characterized by great clinical variability even within the same family. Several phenotypes are recognized in males according to the age of onset, affected organs and rate of the progression of neurologic symptoms. The X-ALD clinical spectrum ranges from the rapidly progressive childhood cerebral form (CALD), which typically leads to severe disability and death during the first decade, to the milder adrenomyeloneuropathy (AMN) that usually manifests between 20-30 years of age with normal lifespan, to pure Addison's disease (Jardim *et al*., 2010). Whereas AMN is characterized mainly by a noninflammatory ''dying-back'' axonopathy involving the long spinal tracts, the inflammatory nature of the demyelinating lesion in CALD resembles those found in multiple sclerosis (MS), the most common central demyelinative disease. It is postulated that modifier genes or environmental factors are involved in the pathogenesis of these highly variable phenotypes (Moser *et al.,* 2001; Heinzer *et al*., 2003; Singh & Pujol, 2010).

The main biochemical abnormality associated with X-ALD is the accumulation of unbranched saturated very long chain fatty acids (VLCFA) in plasma and tissues, due to impaired β-oxidation in peroxisomes (Moser *et al*., 2001). This is caused by defects in the ALD protein (ALDP), a structural protein related to the transport of VLCFA across peroxisomal membranes (Kemp & Wanders, 2010), that is coded by the adenosine triphosphate (ATP)-binding cassette protein, subfamily D, member 1 gene (*ABCD1*), located on Xq28 (Moser *et al*., 2001).

Clinical and molecular studies of X-ALD patients show no genotype-phenotype correlation (Berger *et al*., 2006). The lack of genotype-phenotype correlation is stressed by the fact that in the same family (and therefore the same mutation) one can identify all the clinical phenotypes of X-ALD. Moreover, mutations known to cause complete loss of ALDP activity, such as large deletions, may be associated with all phenotypes, including the late onset form of AMN, and there are reports of monozygotic twins with discordant clinical phenotypes (Korenke *et al*., 1996; Di Rocco *et al*., 2001). These data suggest that additional factors, such as modifier genes, environmental or stochastic events may be acting alone or in conjunction to modulate the expression of different phenotypes observed.

Copy number variants (CNV) are defined as heritable losses and gains of DNA segments of a size greater than 1 kb within the human genome (Feuk *et al*., 2006). Some studies suggest that around 0.2 per cent of the human genome varies in copy number among individuals (McCarroll *et al*., 2008). CNV can originate from a number of mutational mechanisms, such as non-homologous end joining, retrotransposition and non-allelic homologous recombination, which seems to be the most usual in humans (Schrider & Hahn, 2010).

CNV can influence phenotypes by different molecular mechanisms, such as gene dosage, unmasking of recessive alleles or functional polymorphism, position effects, gene interruption and gene fusion (Zhang *et al*., 2009). Their role has been reported in a number of complex diseases, such as schizophrenia (Xu *et al*., 2008), celiac disease (N. Fernandez-Jimenez et al. 2010), diabetes (Grayson *et al*., 2010) and cancer (de Leeuw *et al*., 2011; Jin *et al*., 2011). Since *de novo* CNV have been observed between identical twins (Bruder *et al*., 2008), CNV remains as a possible explanation to the phenotypic variations between monozygotic twins in general, and, in particular, to the phenotypic heterogeneity found in X-ALD.

The objective of this study was to investigate CNV in 29 candidate genes involved in inflammatory response, lipid metabolism and regeneration of the nervous system that could be involved in the phenotypic expression of X-ALD.

2. MATERIALS AND METHODS

2.1 Patients

DNA was extracted from peripheral blood, after informed consent, from a total of 85 samples selected among Spanish and Brazilian X-ALD patients diagnosed by increased plasma VLCFA. According to clinical characteristics, 39 patients were classified as AMN (31 from Spain and 8 from Brazil). The remaining 46 patients were CALD, 28 from Spain and 18 from Brazil. Clinical and molecular characteristics of these Brazilian and Spanish patients were described elsewhere (Pereira et al., submitted; Coll *et al*., 2005). DNA samples from 22 healthy subjects from Spain were used as controls to calculate relative peak height. This study was approved by the local Ethics Committees and all subjects gave their informed written consent.

2.2 CNV detection

Copy number variation was analyzed by Multiple Ligation-dependent Probe Amplification (MLPA), which has been used to detect alterations of gene dosages due to its more limited DNA consumption and its single-tube assay approach (Sellner & Taylor, 2004; Kozlowski *et al*., 2008). No commercial kits were used and 46 sets of probes were designed according to MRC-Holland (MRC-Holland, Netherlands) recommendations. Probes had between 2 to 3 bases difference in size so as not to overlap and their final size ranged from 87 pb to 124 pb for probes labeled with FAM and 84 pb to 125 pb for probes labeled with HEX. Four control probes, chosen based on their low variability in the control population, were used for each dye (Supplementary table 1). Probes were purchased from MRC-Holland (MRC-Holland, Netherlands) and reagents from Invitrogen (Carlsbad, CA, USA).

Capillary electrophoresis was performed on ABI 3130XL Genetic Analyser (Life Technologies, USA) and peak height was determined using GenMapper v.3.2 software. The Relative Peak Height (RPH) method recommended by MRC-Holland was calculated using Excel 97-2003 (Microsoft, USA). Samples were normalized by the mean height of control genes (Ratio 1) and then by the average height of control samples for each gene of interest (Ratio 2). Values between <0.7 and >1.25 were considered normal, whereas values <0.7 were considered deletions and values >1.25 were considered duplications of the investigated genes.

Statistical analysis between different phenotypes and the control population were performed using Pearson's Chi-square test. Statistical significance was defined as p < 0.05. Since this is an exploratory study, aiming to detect possible CNV candidates related to phenotypic differences, correction for multiple tests was not performed.

2.3 Network analysis

Possible interactions among genes with copy number variation in AMN and CALD phenotypes were analyzed using the web-based Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) version 9, available at http://string-db.org (Szklarczyk *et al*., 2011).

3. RESULTS

Of the 29 genes analyzed, 9 showed statistically significant difference in copy number between CALD and AMN phenotypes (table 1). Four genes (*CHID1, PLD5, PNPLA2,* and *SLC25A22*) presented a gain in copy number in samples with the AMN phenotype compared to CALD phenotype. *ABCA2, AGPAT2, ATP8B4,* and *HLA-DRB5* presented gain of copy number in individuals with CALD phenotype. *ABCA2* also showed an increased loss of copies in AMN individuals, whereas *NDUFS7* only showed a loss of copy number in AMN individuals.

The analysis of these nine genes (*CHID1, PLD5, PNPLA2, SLC25A22, ABCA2, AGPAT2, ATP8B4, HLA-DRB5,* and *NDUFS7)* revealed three main interaction hubs (figure 1). One was related to NDUFS7 and other proteins related to NAD complex in mitochondrial respiratory chain. The other hub was related to IGF1 (Insulin-like Growth Factor) and its receptors and binding proteins, whereas a smaller node was represented by melanocortin receptors. Interestingly, POMC (Adrenocorticotropin) acted as a central hub, joining not only these three hubs but also linking other isolated proteins. These proteins, related to genes *PLD5, PNPLA2, SLC25A22, ABCA2, AGPAT2* and *HLA-DRB5* showed only weak interactions with the remaining network. Proteins related to *CHID1* and *ATP8B4* did not show any interaction with the remaining network at the parameters set for analysis.

4. DISCUSSION

Several distinguishing features of CNVs support their role in disease pathogenesis (Ionita-Laza *et al*., 2009). Genome-wide studies demonstrated that CNVs can be implicated in central nervous systems diseases, such as autism spectrum disorder (Sebat *et al*., 2007) and schizophrenia (Walsh *et al*., 2008). Using a set of custom-designed probes for MLPA we were able to show copy number variation in selected genes in patients with X-ALD.

Out of the 29 candidate genes analyzed, 9 showed CNV. Some of these genes are close located in their chromosomes, which suggest that CNV can be co-inherited as a single block. This is the case for *CHID1, PNPLA2,* and *SLC25A22,* which are all located on chromosome 11, region 11p15.5. The same happens to *ABCA2* and *AGPAT2,* both mapped to 9q34.3. Thus, a hitchhiking effect of some of these genes cannot be ruled out, as this phenomenon has already been described in other conditions (Chun & Fay, 2011; Huff et al., 2001). *NDUFS7* (19p13.3), *HLA-DRB5* (6p21.3), *PLD5* (1q43), and *ATP8B4* (15q21.2) are all located on different chromosomes.

Four genes presented a gain in copy number in samples with the AMN phenotype compared to CALD phenotype. *CHID* encodes for a Saccharide and LPS-binding protein with possible roles in pathogen sensing and endotoxin neutralization (Meng *et al*., 2009). *PLD5* encodes for member 5 of phospholipase D family and has been implicated in autism spectrum disorder (Anney *et al*., 2010) and multiple sclerosis (McCauley *et al*., 2009). *PNPLA2* encodes an enzyme which catalyzes the first step in the hydrolysis of triglycerides in adipose tissue. Genetic variations in PNPLA2 may be associated with plasma free fatty acids, triglycerides levels, and fasting glucose concentrations (Campagna *et al*., 2008). *SLC25A22* encodes a mitochondrial glutamate carrier and mutations in this gene were associated with early infantile epileptic encephalopathy (Molinari *et al*., 2009).

Another five genes presented gain of copy number in individuals with CALD phenotype and one a loss of copy number in AMN individuals. *ABCA2* is a member of the superfamily of ATP-binding cassette (ABC) transporters, highly expressed in brain tissue and may play a role in macrophage lipid metabolism and neural development. Single nucleotide polymorphisms (SNPs) in this gene have been associated to susceptibility to Alzheimer's disease (Macé *et al*., 2005), although with contradictory

results (Minster *et al*., 2009). *AGPAT2* encodes a member of the 1-acylglycerol-3-phosphate O-acyltransferase family. The protein is located within the endoplasmic reticulum membrane and converts lysophosphatidic acid to phosphatidic acid, the second step in de novo phospholipid biosynthesis (Solanki *et al*., 2008). *ATP8B4* is a member of the phospholipid-transporting ATPase family. An intergenic SNP located near *ATP8B4* has been associated to Alzheimer's disease (Li *et al*., 2008). *HLA-DRB5* belongs to the HLA class II beta chain family, playing a central role in the immune system, and has been implicated in multiple sclerosis (Caillier *et al*., 2008). *NDUFS7* encodes a subunit of complex I of the mitochondrial respiratory chain (Lebon *et al*., 2007).

No clear direct interaction among these genes was observed on network analysis. It is noteworthy that the main protein represented in this analysis was Proopiomelanocortin (POMC), a polypeptide hormone precursor that undergoes extensive, tissue-specific, post-translational processing yielding as many as ten biologically active peptides involved in diverse cellular functions. Some of its derived peptides localize in the peroxisome in cells expressing high levels of ALDP, although this protein is not required for peroxisomal targeting of these peptide hormones (Höftberger *et al*., 2010). Although the observed relation of POMC with genes showing CNV in different X-ALD phenotypes may be spurious, further investigation of POMC activity and modulation in X-ALD patients may be interesting, giving its close relationship to adrenal function.

To the best of our knowledge, this is the first study to investigate CNV influence in phenotypic variability in X-ALD. Brønstad *et al*. (2011) investigated the influence of CNV in patients with autoimmune Addison's disease and found that low copy number of *UGT2B28* and high copy number of *ADAM3A* were significantly more frequent in Addison's disease patients compared to controls. Although the mechanism by which these variations confer susceptibility to Addison's disease remains unclear, it may involve steroid inactivation for *UGT2B28* and T cell maturation for *ADAM3A*.

In our study, we chose to investigate genes related to lipid metabolism and inflammation due to clinical characteristics of the disease, especially on its cerebral, demyelinating form. Some genes described here present a potential to influence phenotype, although by mechanisms not completely understood. More studies on the

metabolic pathways involved and in CNV variation across different tissues are needed to establish which, if any, of these genes is a modifier gene in X-ALD.

5. ACKNOWLEDGEMENTS

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Table 1: Number of individuals presenting copy number variation at the corresponding phenotypes.

Supplementary Table 1: Design and characteristics of the MLPA probes used in this study.

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Figure 1. Predicted protein interaction among genes with copy number variation in AMN and CALD phenotypes.

Table 1: Number of individuals presenting copy number variation at the corresponding phenotypes. Bold characters represent difference from expected values (shown in parenthesis). Only genes with significant differences in copy number are shown.

Gene	CALD (n=46) *versus* AMN (n=39)				
	Loss of copies		Gain of copies		p value
	CALD	AMN	CALD	AMN	
ABCA2	**2 (6)**	**9 (5)**	**6 (3)**	**0 (3)**	0.004
AGPAT2	0 (1)	1 (0)	**11 (6)**	**1 (5)**	0.012
ATP8B4	1 (0)	0 (0)	**19 (14)**	**7 (11)**	0.039
CHID1	0 (0)	0 (0)	**17 (24)**	**28 (20)**	0.006
HLA-DRB5	18 (21)	20 (17)	**22 (16)**	**8 (13)**	0.028
NDUFS7	**1 (9)**	**15 (7)**	5 (5)	4 (4)	0.000
PLD5	1 (1)	0 (0)	**13 (18)**	**21 (15)**	0.043
PNLPA2	0 (0)	0 (0)	**24 (33)**	**37 (27)**	0.000
SLC25A22	0 (1)	2 (1)	**13 (18)**	**21 (15)**	0.010

Figure 1: Predicted protein interaction among genes with copy number variation in AMN and CALD phenotypes.
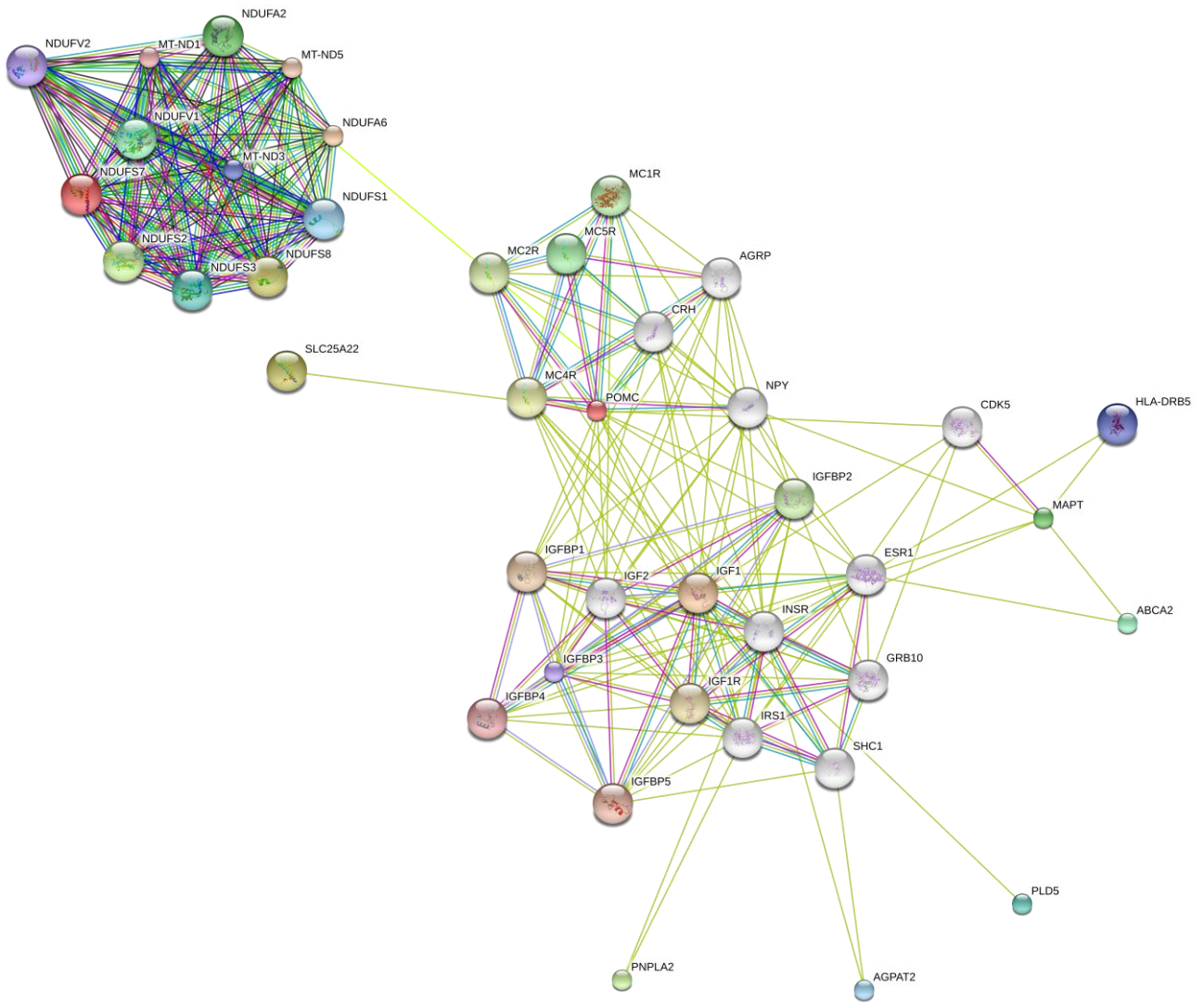
Supplementary table 1: Design and characteristics of the MLPA probes used in this study.

Gene	Location	Sequence	Lenght (bp)
FAM-labeled probes			
PCDHA1	5q31	LPO-GGGTTCCCTAAGGGGTTGGAGAATCTAGACTCCTGAATTCGCGTTTTCCGATAGAAGGAGCTGCT	116
		RPO-GATGCAGACATTGGTGCTAACGCTCTTCTAACGTACGTCTAGATTGGATCTTGCTGGCAC	
PRKRIP1*	7q22.1	LPO-GGCCGCGGGAATTCGATTGGAGTTCCACGTGTACAGACATCTGCGCCGGA	124
		RPO-GAGAATATCAGCGACAGGACTACATGGATGCCATGGCTGAGCACTAGTGAATTCGCGGC	
HLA-DRB5	6p21.3	LPO-GGGTTCCCTAAGGGGTTGGAcgatcCTACCGGTTCAGCAACTCTGCAGAAAATGTCC	126
		RPO-ATCCTTGTGGCTTCCTCAGCTCCTGCCCTTGGCCTGAAGTCTAGATTGGATCTTGCTGGCAC	
SH2B2	7q22	LPO-GGGTTCCCTAAGGGGTTGGAGTACAGCATCTGTGGTTCCAGTCTGTGCTTG	99
		RPO-ACATGCTCCGCCACTTCCACACACACTCTAGATTGGATCTTGCTGGCAC	
ORM1	9q31-q32	LPO-GGGTTCCCTAAGGGGTTGGAGAATTGGAACTCCAGGCTGGCTTCAGATCTGCCTCTCTCTCAT	118
		RPO-GCCCTCTTTAAGATCCTTTGCAAACCAATGGTAGAAGCCTGTCTAGATTGGATCTTGCTGGCAC	
ORM2	9q32	LPO-GGGTTCCCTAAGGGGTTGGAGTACAATAAGTCGGTTCAGGAGATCCAAGCAACCTTCTTTTACTTT	120
		RPO-ACCCCCAACAAGACAGAGGACACGATCTTTCTCAGAGAGTACTCTAGATTGGATCTTGCTGGCAC	
AGPAT2	9q34.3	LPO-GGGTTCCCTAAGGGGTTGGACATCATCGGCTGGTTCGTGCGAAGCTTCA	101
		RPO-AGTACTTTTACGGGCTCCGCTTCGAGGTGCTCTAGATTGGATCTTGCTGGCAC	
ABCA2	9q34	LPO-GGGTTCCCTAAGGGGTTGGAGAGGAGAGCATTGTCAACTACACCCTCAAC	103
		RPO-CAGGCCTACCAGGACAACGTCACTGTTTTTGTCTAGATTGGATCTTGCTGGCAC	
EHMT1	9q34.3	LPO-GGGTTCCCTAAGGGGTTGGCCTCTTTCTTTCTCGGGATTCAGATGTCACCTTAAAG	98
		RPO-AACAAGGAAGGAGAGACGCCCCTGCAGTGTGTCTAGATTGGATCTTGCTGGCAC	
GPRIN2	10q11.22	LPO-GGGTTCCCTAAGGGGTTGGAGAGCCTGGCTCTAGGACCAAAGA	91
		RPO-TGTGTGGACCATGACCTCAGCCAATGTCTAGATTGGATCTTGCTGGCAC	
PCDH20	13q21	LPO-GGGTTCCCTAAGGGGTTGGAGGAGTCCCACAAGCTCACCATCCTTG	93
		RPO-CTAATGGACCAGGCTGCATCCCTGCTCTAGATTGGATCTTGCTGGCAC	
PCDH17	13q21.1	LPO-GGGTTCCCTAAGGGGTTGGAGATCAGGCTGACAGTGACCAAGACACTA	95
		RPO-ACAAAGGCTCCTGCTGTGACATGTCTCTAGATTGGATCTTGCTGGCAC	

SLC27A2	15q21.2	LPO-GGGTTCCCTAAGGGGTTGGAGGTAACGTTCTGATCACTCATCAGCGCATATGGTATGGAA	130
		RPO-CTGGCCTCACTTTTGTAAGCGGATTGAAGGGGTTCAGGTCGTCTAGATTGGATCTTGCTGGCAC	
RAI 1*	17p11.2	LPO-GGGTTCCCTAAGGGGTTGGGCAAGGAAAAGTGGCCTTTTGCGTGACA	122
		RPO-TCTAGATTGGATCTTGCTGGCACCAGAAGTGGACACATCATTCTGATCTCAGGGC	
GPX4*	19p13.3	L-GGGTTCCCTAAGGGGTTGGAGTGCATCGTCACCAACGTGGCCTCCCAGTGAG	107
		R-GCAAGACCGAAGTAAACTACACTCAGCTCGTCGTCTAGATTGGATCTTGCTGGCAC	
GRIN3B*	19p13.3	L-GGGTTCCCTAAGGGGTTGGACTCAACGCCTTCATCATGGACAAGTCGCTCCTG	109
		R-GACTACGAGGTCTCCATCGACGCCGACTGCAAACTCTAGATTGGATCTTGCTGGCAC	
CIRBP	19p13.3	LPO-GGGTTCCCTAAGGGGTTGGACTTCTGACGCCGGGATGGCCTCGTTACTAGACTTTTCTTTT	114
		RPO-TAAGGAAGTGCTGTTTTTTTTTGAGGGTTTTCAAAACTCTAGATTGGATCTTGCTGGCAC	
NDUFS7	19p13.3	LPO-GGGTTCCCTAAGGGGTTGGAGCTCACATGTATGGACAGATGTGTACACGGACCACACGCAC	112
		RPO-ACTCACGCACACAATGCACATATGCACACTCGCTCTAGATTGGATCTTGCTGGCAC	
ABCA7	19p13.3	LPO-GGGTTCCCTAAGGGGTTGGAGTCTCACCATGGCCTTCTGGACACAGCTGATGCTG	105
		RPO-CTGCTCTGGAAGAATTTCATGTATCGCCTCTAGATTGGATCTTGCTGGCAC	

HEX-labeled probes

PLD5	1q43	LPO-GGCCGCGGGAATTCGATTCCTCCTCCTTCTGGATCGTGGACAA	87
		RPO-ACAGCACGTGTATATCGGCAGTGCCGCACTAGTGAATTCGCGGC	
BCL2L11*	2q13	L-GGCCGCGGGAATTCGATTCTGCTAGTCGTCGTCCGAATGGTTATCTTACGACTGTTACGTTACAT	127
		R-TGTCCGCCTGGTGTGGAGAATGCATTGACAGGCCTAGTATGTCCCACTAGTGAATTCGCGGC	
ATP6V1G1	9q32	L-GGCCGCGGGAATTCGATTCCTTGTCCTGGGACAGCCTGGGGCAAGTTATT	98
		R-AAGCATATTTCCTTCCTGGTGGGCTAGATGCACTAGTGAATTCGCGGC	
IRF7	11p15.5	LPO-GGCCGCGGGAATTCGATTGTCTAATAAAAAGAACTCCAGAACACGTACTGGC	100
		RPO-ATCTTGGCTGGTGGGGAATTGGGTCCAGGCCACTAGTGAATTCGCGGC	
SLC25A22	11p15.5	LPO-GGCCGCGGGAATTCGATTGCCCCACCCTCACTCATTCCCATTCCCTCATCC	102
		RPO-TTCTAGGACAGAAGTGGCGGTTGCTGACGCCTGCACTAGTGAATTCGCGGC	
TSPAN4	11p15.5	LPO-GGCCGCGGGAATTCGATTCTTAATAAAGTGTGAGCAGCAACCTTGCGTCTAT	104
		RPO-TGCTCGTCTGCCTTCCCTACCCTATCTTCCCTTCCACTAGTGAATTCGCGGC	
CHID1	11p15.5	LPO-GGCCGCGGGAATTCGATTCGATGTCACCAAGGTCTTTGGGAGCAAGTTCACAC	106

		RPO-AGATCTCACCCGTCTGGCTGCAGCTGAAGAGACGTCACTAGTGAATTCGCGGC	
LRDD	11p15.5	LPO-GGCCGCGGGAATTCGATTGTGAGCAACAAAACTGCACTGTTTCTTTCACCTCAGAG	110
		RPO-CTTGATTTATTTGTTGGATGGAGAAGCCGTTGGAGGCACTAGTGAATTCGCGGC	
PNPLA2	11p15.5	LPO-GGCCGCGGGAATTCGATTGAGTGACATCTGTCCGCAGGACAGCTCCACCAACATCCAC	112
		RPO-GAGCTGCGGGTCACCAACACCAGCATCCAGTTCAACCACTAGTGAATTCGCGGC	
AP2A2	11p15.5	LPO-GGCCGCGGGAATTCGATTGTGTGGTAACTGCAGCCACAAGTCTGATCACCACTTTAGC	116
		RPO-ACAGAAGAACCCAGAAGAGTTTAAAACCTCCGTGTCTCTGCACTAGTGAATTCGCGGC	
CD151*	11p15.5	L-GGCCGCGGGAATTCGATTCAGGATGGGTGAGTTCAACGAGAAGAAGACAACATGTGGCAC	114
		R-CGTTTGCCTCAAGTACCTGCTGTTTACCTACAATTGCACTAGTGAATTCGCGGC	
ATP8B4	15q21.2	LPO-GGCCGCGGGAATTCGATTCCTCCTTGACCTGGTTTACCACCATTGTGCCTTTGGTCCTGGTGA	125
		RPO-TAACTATGACAGCTGTCAAAGATGCCACAGATGACTATGTGAGCCACTAGTGAATTCGCGGC	
BECN1	17q21	LPO-GGCCGCGGGAATTCGATTGGATGACAGTGAACAGTTACAGATGGAGC	91
		RPO-TAAAGGAGCTGGCACTAGAGGAGGAGCACTAGTGAATTCGCGGC	
SBNO2	19p13.3	LPO-GGCCGCGGGAATTCGATTCAGCGACTTCAACTCCTCCCCCGAGTCCCTGGTGGATGACGACGTT	118
		RPO-GTCATCGTTGATGCAGTCGGGCTCCCCAGTGACGACCACTAGTGAATTCGCGGC	
BCL2L12	19q13.3	LPO-GGCCGCGGGAATTCGATTCTAGCTAGTACGTACCCTCATGTCCCTGCCCTCTTCGTGTGCTTTTCC	129
		RPO-AAGTCTTCCTATTCCACTCAGGGCTGTGGAAACGGGTTACCGTCCCACTAGTGAATTCGCGGC	
GNB1L	22q11.2	LPO-GGCCGCGGGAATTCGATTGATCCTAAGTTAACCGTCGAAGGTGTGCAGCCGCATCGCCTGCCATGA	123
		RPO-GGAGCCCGTCATGGACCTTGACTTTGACTCGAGTCATGGCACTAGTGAATTCGCGGC	
IL13RA2*	Xq13.1-q28	LPO-GGCCGCGGGAATTCGATTCTCCATTGTGAACCAGTAAGAACACTCTC	93
		RPO-GTGAGTCTAACGGTCTTCCGGATGAAGGCACTAGTGAATTCGCGGC	
PLS3*	Xq23	L-GGCCGCGGGAATTCGATTCTTTAGATCTTTAAATGGATGAGATGGCTACCACTCAGATTTCCAA	120
		R-AGATGAGCTTGATGAACTCAAAGAGGCCTTTGCAAAAGCACTAGTGAATTCGCGGC	

6. CONSIDERAÇÕES FINAIS

Esta tese teve como objetivo geral estudar os aspectos moleculares da X-ALD, relacionando-os com dados epidemiológicos, métodos diagnósticos e potenciais genes modificadores.

Os resultados relacionados aos objetivos específicos 1, 2, 3 e 4 desta tese foram descritos no primeiro artigo "Mutations, clinical findings and survival estimates in South American patients with X-linked adrenoleukodystrophy". Este artigo descreveu provavelmente a primeira série de casos de pacientes sul-americanos com X-ALD nos quais foram pesquisadas mutações no gene *ABCD1*. Os resultados enfatizam a heterogeneidade molecular da X-ALD, pois dentre as 38 famílias estudadas, apenas três apresentavam a mesma mutação, sendo as demais exclusivas de uma única família. Além disso, doze novas mutações foram identificadas e aparentemente há um possível *cluster* de mutações nos éxons 2 e 8-9. A proporção de mutações de sentido trocado, sem sentido e de mudança de quadro de leitura foi similar à encontrada em outras populações e a taxa de mutação *de novo* foi de 10%, o dobro da encontrada por (Jardim *et al*., 2010).

A proporção fenotípica dos homens analisados neste estudo também difere um pouco da encontrada na literatura. Foram identificados 87 homens afetados em 38 diferentes genealogias (2,2 homens/família), uma taxa similar a outras grandes séries estudadas (Bezman *et al*., 2001). Contudo, na presente amostra, a proporção do fenótipo CALD (62%) foi maior do que a esperada (45-57%) (Moser *et al*., 2001; Dubey *et al*., 2005; Raymond *et al*., 2010; http://www.x-ald.nl). É possível que este número seja o reflexo de um subdiagnóstico dos demais fenótipos, até porque somente 6% da presente série apresentavam Addison-only, enquanto outros estudos na literatura relatam freqüências entre 8 e 20%. Se esta explicação fosse correta, entretanto, esperaríamos encontrar mais hemizigotos entre os familiares afetados do que o esperado – e isso não aconteceu. Portanto, permanece plausível a hipótese de que tenhamos mais formas cerebrais do que os países temperados ou desenvolvidos.

Dentre as mulheres analisadas por métodos moleculares, 49% eram heterozigotas, o que está de acordo com o risco *a priori* para doenças mendelianas. Este achado nos instigou a comparar o resultado molecular dessas mulheres com os resultados bioquímicos (dosagem de VLCFA), o que resultou no segundo artigo apresentado nesta

tese, "Molecular analyses versus very long chain fatty acids profile in women from X-linked Adrenoleukodystrophy". Neste artigo foram descritos os resultados relacionados ao objetivo específico 5 desta tese.

Como em muitos locais, o acesso à análise molecular do gene *ABCD1* não está disponível, é importante conhecer as taxas de sensibilidade e especificidade da dosagem de VLCFA. Utilizando o critério clássico que considera como em risco para X-ALD uma mulher com altos níveis de $C_{26:0}$ e razão $C_{26:0}/C_{22:0}$ também elevada, foi encontrada uma taxa de 72,7% de resultados falso-negativos. Esse valor é muito maior do que o descrito na literatura, que fica em torno de 20%. Analisando cada um desses parâmetros separadamente, verificou-se que a razão $C_{26:0}/C_{22:0}$ apresenta maior acurácia. Esses achados podem ser devidos a alguma característica presente na população brasileira, como um fator nutricional que interfira nos níveis de $C_{26:0}$. Também é possível, que esses achados estejam relacionados a um padrão de inativação não-aleatório do cromossomo X nesta amostra.

Porém, o resultado mais inesperado deste artigo foi a identificação de duas mulheres com diagnóstico molecular negativo para X-ALD, mas com níveis elevados de VLCFA. Não há relatos na literatura de resultados falso-positivos em mulheres (Moser *et al*., 1999; Unterberger *et al*., 2007; Jangouk *et al*., 2012). Dados clínicos, bioquímicos, moleculares e familiares foram revisados, porém não encontramos nenhuma explicação para esses perfis alterados. Estes casos reforçam a necessidade de que a análise molecular deve ser realizada em mulheres para determinação do *status* de heterozigota, contra-indicando a análise de VLCFA como único método diagnóstico.

Os resultados relacionados ao objetivo específico 6 desta tese foram relatados no terceiro artigo "Copy Number Variation May Play a Role in Phenotypic Variability in X-linked Adrenoleukodistrophy". Como descrito anteriormente, diversos estudos têm estabelecido que não há correlação de mutações no gene *ABCD1* e a heterogeneidade clínica dos fenótipos X-ALD. Assim, a razão para o desenvolvimento de diferentes formas clínicas da X-ALD pode ser pela influência de fatores ambientais, genéticos ou ambos. Além disso, análises de segregação genética fornecem suporte para a hipótese de que pelo menos um gene autossômico tenha papel na manifestação clínica da X-ALD (Maestri *et al*., 1992). Assim, apesar da X-ALD ser claramente uma doença monogênica herdada, determinantes ambientais e genéticos da doença podem apresentar-se com etiologia muito heterogênea e complexa.

Uma alternativa para identificar possíveis genes modificadores é a análise de variação de número de cópias (CNV). No terceiro artigo foi apresentada a análise de CNVs para genes envolvidos na resposta inflamatória, metabolismo de lipídios e regeneração do sistema nervoso em pacientes com os fenótipos AMN e CALD. Dos 29 genes estudados, 9 apresentaram diferenças estatisticamente significativas entre esses fenótipos.

Os genes *CHID1*, *PLD5*, *PNPLA2* e *SLC25A22* apresentaram aumento no número de cópias nos indivíduos AMN. Já os genes *ABCA2*, *AGPAT2*, *ATP8B4* e *HLA-DRB5* também apresentaram aumento no número de cópias, porém no fenótipo CALD. Curiosamente, o gene *ABCA2* além de apresentar aumento no número de cópias entre indivíduos CALD, também apresentou menor número de cópias entre indivíduos AMN, enquanto o gene *NDUFS7* apresentou menor número de cópias somente entre indivíduos AMN.

Através de análise computacional utilizando Biologia de Sistemas, foram identificadas três principais vias de interação entre esses genes. De maneira interessante, *POMC* (Proopiomelanocortina), que não era um dos genes estudados para variação de CNV, demonstrou ser um eixo central na união, não somente dessas 3 vias, mas também unindo outras proteínas isoladas. Alguns dos peptídeos derivados da modificação pós-traducional dos transcritos de *POMC* localizam-se nos peroxissomas de células que expressam altos níveis de ALDP (Höftberger *et al*., 2010). Uma análise mais detalhada do papel deste gene na variabilidade fenotípica da X-ALD deve ser realizada, dado sua relação com a função adrenal.

Pela revisão bibliográfica realizada até janeiro de 2012, este parece ser o primeiro estudo a avaliar CNV na variabilidade fenotípica da X-ALD. Considerando que interferência do ambiente e a possibilidade de que mais de um gene ou mais de uma determinada região possam estar influenciando na variabilidade fenotípica da X-ALD, este estudo contribuiu com a identificação de regiões e genes que, por sua variabilidade, podem estar envolvidos neste evento. O fato de um gene ou uma determinada região apresentar diferenças de CNV, não necessariamente significa que esteja diretamente relacionado à variabilidade. Estudos mais complexos, abrangendo a quantificação da expressão desses genes e as interações de seus produtos com os demais genes, suas vias e rotas devem ser realizados. Também não se pode descartar a possibilidade de que esses fenômenos atuem simultaneamente, o que implica na participação de mais de um

gene na variabilidade fenotípica da X-ALD, inclusive por mecanismos outros que não CNV.

Em resumo, os dados apresentados nessa tese descrevem características clínicas de pacientes sul-americanos com X-ALD e demonstram uma maior incidência da forma cerebral nessa população. As mutações identificadas reforçam a heterogeneidade genotípica da doença. A correlação dos níveis plasmáticos de VLCFA com a análise molecular reforça a importância desta última para a avaliação de mulheres em risco de serem heterozigotas. Por fim, a análise de genes candidatos ou por *array* demonstrou que CNV pode ser um mecanismo envolvido na variabilidade fenotípica da X-ALD e revelou alguns genes candidatos a atuarem nesse sentido. Entre as perspectivas geradas por este trabalho, destaca-se o estudo mais aprofundado destes genes, suas vias e mecanismos de ação na modulação fenotípica da X-ALD. Por outro lado, uma análise mais detalhada das causas que podem interferir no diagnóstico bioquímico de mulheres, incluindo inativação do X e variáveis alimentares que possam influenciar os níveis de VLCFA. Estas últimas poderiam inclusive levantar hipóteses para uma maior prevalência da forma cerebral em nossa população.

7. ANEXOS

7.1 Resposta da revista PLoS ONE, referente à submissão do artigo 1 desta tese.

-----Mensagem original-----

De: em.pone.0.28cd21.d9e3aedc@editorialmanager.com [mailto:em.pone.0.28cd21.d9e3aedc@editorialmanager.com] Em nome de PLoS ONE

Enviada em: quarta-feira, 1 de fevereiro de 2012 10:23

Para: Laura Bannach Jardim

Assunto: PLoS ONE Decision: Revise [PONE-D-11-26023]

PONE-D-11-26023

Mutations, clinical findings and survival estimates in South American patients with X-linked adrenoleukodystrophy

PLoS ONE

Dear Prof. Jardim,

Thank you for submitting your manuscript for review to PLoS ONE. After careful consideration, we feel that your manuscript will likely be suitable for publication if it is revised to address the points below. Therefore, my decision is "Minor Revision."

We invite you to submit a revised version of the manuscript that addresses the points made by the two reviewers.

We encourage you to submit your revision within sixty days of the date of this decision.

When your files are ready, please submit your revision by logging on to http://pone.edmgr.com/ and following the Submissions Needing Revision link. Do not submit a revised manuscript as a new submission.

Please also include a rebuttal letter that responds to each point brought up by the academic editor and reviewer(s). This letter should be uploaded as a Response to Reviewers file.

In addition, please provide a marked-up copy of the changes made from the previous article file as a Manuscript with Tracked Changes file. This can be done using 'track changes' in programs such as MS Word and/or highlighting any changes in the new document.

If you choose not to submit a revision, please notify us.

Yours sincerely,

Mathias Toft, MD, PhD

Academic Editor

PLoS ONE

Reviewers' comments:

Reviewer #1:

This paper is clearly written. It describes a cohort study of X-linked adrenoleukodystrophy (X-ALD) in South America, which extends the spectrum of mutations in X-ALD and benefits genetic counseling for X-ALD families in this region.

I have two major concerns, which should be addressed by the authors before the paper is accepted for publication.

1.   A total of twelve new sequence variations are reported, eight of which are considered pathogenic because of the creation of premature stop codon. The pathogenicity of the remaining four (i.e. p.Pro623Leu, p.Leu628Glu, p.Ile481Phe and p.Arg401Gly, all being missense base changes) should be evaluated at least in silico (using SIFT or/and PolyPhen). Further characterization could be performed by detecting each base change in at least 100 normal controls from the same population as the patients. (Lines 183-188 on Page 7)

2.   From the data provided by the authors, it is difficult to conclude that the four de novo mutations have occurred in two men and two women. For X-linked diseases, de novo mutations usually arise in the germline cells (or the precursor of these cells) of the patient's mother. The situation in families 20, 31 and 33 belongs most likely to such a case. In family 1, it is possible that the de novo mutation has occurred in the germline cells of maternal grandfather of the index case. It is recommended that the authors refer to the paper by Wang et al. (X-linked adrenoleukodystrophy: ABCD1 de novo mutations and mosaicism, published in Mol Genet Metab). (Lines 197-199 on Page 8 and Lines 268-275 on Page 10)

Some minor issues:

1.   Line 90 on Page 3: the word "this" should be changed to "these"

2.   Line 91 on Page 3: "other parts of " should be inserted after "throughout"

3.   Line 109 on Page 4: "aimed " should be changed to "aims"

4.   Line 115 on Page 5: "Committees" should be changed to its singular form, since only one institution is mentioned. Accordingly, "follow" in Line 117 should be changed to "follows"

5.   Line 122 on Page 5: the subheading "Population" should best be changed to "Patients"

6.   Line 137 on Page 5: change "Single Strand Conformational Polymorphism" to "single strand conformational polymorphism"

7. Line 175 on Page: a space should be inserted between "amino" and "acid"

8. Line 188 on Page 7: "last" should be changed to "remaining"

9. Line 215 on Page 8: "Figures 3" should be changed to "Figure 3"

10. Lines 191-192 on Page 8: "8,4%" should be changed to "8.4%" and "5,5%" to "5.5%". Similar cases can be seen elsewhere.

11. Line 230 on Page 9: Change "completed" to "completely"

12. References: one single style should be observed throughout the whole.

13. Table 2 can be improved by removing the subcolumn denoting "mean,CI 95% (range)" while introducing another footnote.

Reviewer #2:

The manuscript adds to the published data on the incidence, phenotypic proportions, and ABCD1 mutations of patients with X-linked adrenoleukodystrophy (X-ALD). The main strengths of the paper are the descriptions of the mutations identified in all 38 families, the documentation of the 4 families where de novo mutations were found, the report of a X-ALD heterozygote with skewed X inactivation, and the phenotypes and survival of the 87 males identified through extended family screening. The ethical issues, methods, statistics, Kaplan-Meyer curves and tables are described, referenced and are scientifically acceptable for the studies reported.

This is a clearly written manuscript which can be accepted for publication as the first large well-documented study on X-linked adrenoleukodystrophy extended family screening in South America.

Comments and possible changes to address in this manuscript:

It is curious that of the 87 males identified through extended family screening, the proportion of the childhood cerebral phenotype was higher than in previously reported studies. This may be due to the smaller number of X-ALD patients diagnosed in Brazil

as the males and females with adult onset symptoms of X-ALD may not be recognized. Another difference from previous literature is the lower number of symptomatic heterozygotes in this study. Were neurological studies done on all of the X-ALD female carriers as a part of the extended family screening? If so, the female X-ALD carrier phenotypic data should be added to this manuscript.

[NOTE: If reviewer comments were submitted as an attachment file, they will be accessible only via the submission site. Please log into your account, locate the manuscript record, and check for the action link "View Attachments". If this link does not appear, there are no attachment files to be viewed.]

7.2 Técnicas utilizadas nos artigos apresentados nesta tese

7.2.1 PCR (*Polymerase Chain Reaction*)

Para a análise molecular foram utilizados *primers* específicos do gene *ABCD1* que permitiram o estudo de toda a sequência codificante e das junções íntron-éxon utilizando o protocolo descrito por Boehm *et al.*, 1999 (tabela 1).

Tabela 1: Seqüência dos primers utilizados para amplificação de todos os exons do gene *ABCD1* (modificado de Boehm *et al.*, 1999).

Exon	Primer	Seqüência 5'-3'	Fragmento
1A	ALDe1A-F	ACAACAGGCCCAGGGTCAGA	422 pb
	ALDe1A-R	AGGAAGGTGCGGCTCACCA	
1B	ALDe1B-F	AACCGGGTATTCCTGCAGCG	385 pb
	ALDe1B-R	ACTGGTCAGGGTTGCGAAGC	
1C	ALDe1C-F	CCACGCCTACCGCCTCTACTT	484 pb
	ALDe1C-R	AGACTGTCCCCACCGCTC	
2	ALDe2-F	GGCACTGGGAGACCCTG	332 pb
	ALDe2-R	TCAGCACCCAGCGGTATGG	
3-4	ALDe3/4-F	GCAGAAGAGCCTCGCCTTTC	570 pb
	ALDe3/4-R	GCAGCAGGTCAGCACCTGCA	
5	ALDe5-F	CTGCCAGGGATGGGAATGAG	337 pb
	ALDe5-R	TCTCACCTTGACCTTGGCCC	
6	ALDe6-F	GCCATAGGGTACGGGAAGGG	276 pb
	ALDe6-R	GCCTCTGCAGGAAGCCATGT	
7	ALDe7-F	CGATCCACTGCCCTGTTTTGG	491 pb
	ALDe7-R	CTTCCCTAGAGCACCTGG	
8 -9	ALDe8/9-F	CTGAGCCAAGACCATTGCCCCCG	471 pb
	ALDe8/9-R	TGCTGCTGCCGGGCCCGC	
10	ALDe10-F	GAGGGGAGGAGGTGGCCTGGC	427 pb
	ALDe10-R	GCGGGGTGCGTGCATGGGTGG	

A reação de PCR utilizada para a obtenção de cada amplicon e os ciclos de temperaturas seguem descritos abaixo, respectivamente:

MIX PCR

Água	32,8
Tampão	5,0
dNTP 2mM	5,0
MgCl$_2$	2,0
DMSO	2,0
Primer +	1,0
Primer -	1,0
Taq DNA Polimerase	0,2

Reação de PCR

Temperatura	Tempo
95°C	5 min
95°C	30 seg
60°C	30 seg
72°C	45 seg
72°C	10 min

30 ciclos (aplicado aos passos 95°C 30 seg, 60°C 30 seg, 72°C 45 seg)

7.2.2 SSCP (*Single Strand Conformational Polymorphism*)

A técnica de SSCP normalmente foi utilizada como método de triagem para identificar a presença de possíveis mutações em amplicons obtidos pela PCR. Esta técnica é relativamente barata, laboriosa e apresenta uma sensibilidade de cerca de 70%, sendo afetada por fatores como umidade e temperatura do ambiente.

Nesta tese, a técnica de SSCP foi inicialmente aplicada em pacientes X-ALD de sexo masculino para a identificação de possíveis mutações. Considerando a baixa sensibilidade do SSCP, aleatoriamente escolhemos quatro diferentes condições de eletroforese na tentativa de aumentar ao máximo a possibilidade de identificarmos fragmentos alterados. As condições de eletroforese escolhidas foram: gel de poliacrilamida (PA) 6% à temperatura ambiente, gel de PA 8% à temperatura ambiente, gel de PA 8% à 4°C e gel de PA 10% à 4°C.

Cada gel foi preparado no mesmo dia em que seria realizada a eletroforese, conforme descrito abaixo:

Gel PA 6%	Gel PA 8%	Gel PA 10%
8,5mL TBE 1X	8,0mL TBE 1X	7,5mL TBE 1X
1,5mL PA	2,0mL PA	2,5mL PA
200uL APS 10%	200uL APS 10%	200uL APS 10%
20uL TEMED	20uL TEMED	20uL TEMED

As condições de eletroforese para cada éxon foram, respectivamente:

Gel 6% - TA	Voltagem	Miliamperagem	Tempo (hs)
Éxon 1A	250V	300mA	2:00
Éxon 1B	250V	300mA	2:00
Éxon 1C	250V	300mA	2:00
Éxon 2	250V	300mA	1:40
Éxon 3-4	250V	300mA	2:50
Éxon 5	250V	300mA	2:00
Éxon 6	250V	300mA	2:00
Éxon 7	250V	300mA	2:00
Éxon 8-9	250V	300mA	2:30
Éxon 10	250V	300mA	2:00

Gel 8% - TA	Voltagem	Miliamperagem	Tempo (hs)
Éxon 1A	250V	300mA	3:30
Éxon 1B	250V	300mA	3:30
Éxon 1C	250V	300mA	3:30
Éxon 2	250V	300mA	1:40
Éxon 3-4	250V	300mA	4:00
Éxon 5	250V	300mA	3:00
Éxon 6	250V	300mA	3:00
Éxon 7	250V	300mA	3:30
Éxon 8-9	250V	300mA	3:30
Éxon 10	250V	300mA	4:00

Gel 8% - 4°C	Voltagem	Miliamperagem	Tempo (hs)
Éxon 1A	250V	300mA	4:00
Éxon 1B	250V	300mA	4:00
Éxon 1C	250V	300mA	4:00
Éxon 2	250V	300mA	4:00
Éxon 3-4	250V	300mA	4:00
Éxon 5	250V	300mA	4:00
Éxon 6	250V	300mA	4:00
Éxon 7	250V	300mA	4:00
Éxon 8-9	250V	300mA	4:00
Éxon 10	250V	300mA	4:00

Gel 10% - 4°C	Voltagem	Miliamperagem	Tempo (hs)
Éxon 1A	250V	300mA	4:00
Éxon 1B	250V	300mA	4:00
Éxon 1C	250V	300mA	4:00
Éxon 2	250V	300mA	4:00
Éxon 3-4	250V	300mA	4:00
Éxon 5	250V	300mA	4:00
Éxon 6	250V	300mA	4:00
Éxon 7	250V	300mA	4:00
Éxon 8-9	250V	300mA	4:00
Éxon 10	250V	300mA	4:00

Após verificação da PCR em gel de agarose 1% corado com brometo de etídio, os amplicons obtidos foram analisados por SSCP, juntamente com uma amostra controle.

Misturamos 8uL de PCR com 4uL de corante (feito conforme protocolo abaixo) e desnaturamos em banho-seco à 95°C por 5 minutos.

Corante para SSCP (volume final = 10mL)

9,5 mL formamida deionizada

0,05 g azul de bromofenol

0,05g xileno cyanol

5,0 mL EDTA pH8,0

Imediatamente após colocamos as amostras no gelo para manter a dupla-fita de DNA desnaturada. Aplicamos as amostras no respectivo gel de PA e seguimos uma das quatro condições de eletroforese descritas acima.

Terminada a eletroforese, o gel foi submetido ao processo de coloração, que consta dos seguintes passos:

1. Fixação: incubar o gel em etanol 10% por 5 minutos. Retirar o etanol 10%.

2. Oxidação: incubar o gel em ácido nítrico 1% por 3 minutos. Lavar com água destilada.

3. Marcação com nitrato de prata 0,012M: incubar o gel em solução de nitrato de prata por 20 minutos, deixando-o no escuro. Lavar duas vezes com água destilada.

4. Coloração do gel: carbonato de sódio 0,28M com formaldeído37%: incubar o gel até que as bandas apareçam. Descartar a solução.

5. Parada do processo: incubar o gel em solução de ácido acético 10% por 2 minutos. Lavar o gel com água destilada.

6. Fixação das bandas: incubar o gel em solução de etanol 50% por 10 minutos.

7. Secar o gel em máquina de vácuo para poder guardá-lo.

Ao final desse processo, as amostras eram visualmente comparas à amostra controle presente no mesmo gel e, uma vez que apresentavam padrão de migração alterado, eram submetidas ao sequenciamento automatizado (figura 1). Se o padrão de migração era igual ao da amostra controle, esse mesmo amplicon era submetido às outras 3 condições de SSCP. Amostras que não apresentaram padrão de migração alterado, em relação à amostra controle em nenhuma das quatro diferentes condições de eletroforese tiveram seus 10 éxons sequenciados de maneira automatizada.
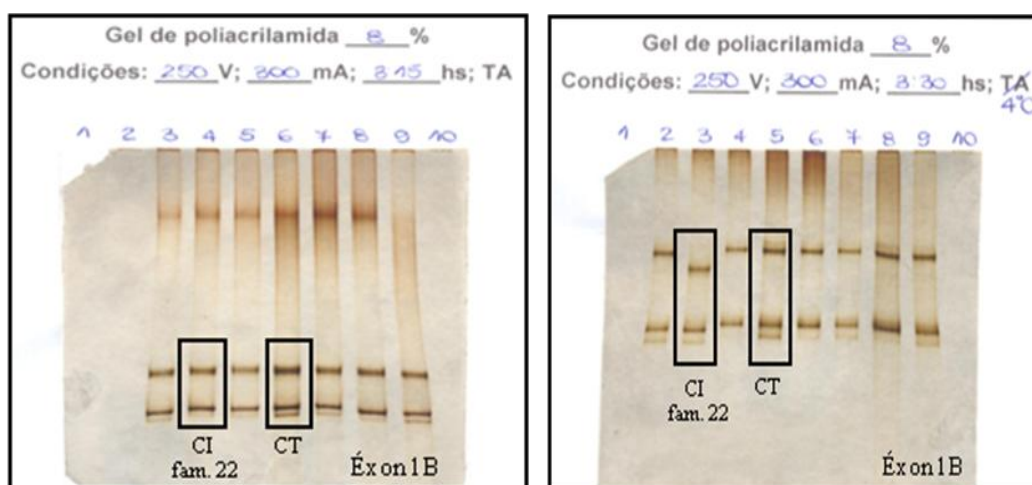

Figura 1: Dois géis de SSCP em duas diferentes condições de eletroforese do mesmo amplicon, demonstrando a sensibilidade quanto à temperatura. Após sequenciamento automatizado, foi identificada a mutação p.Trp137fsX57. CI = caso-índice; CT = controle.

7.2.3 MLPA (*Multiplex Ligation-dependent Probe Amplification*)

A técnica de MLPA foi utilizada nesta tese para a análise de CNV de possíveis genes modificadores de fenótipo na X-ALD (artigo 3).

MLPA é um tipo de PCR multiplex fluorescente, que pode detectar número alterado de cópias de cerca de 50 diferentes sequências de DNA ou RNA simultaneamente, permitindo a identificação de até um nucleotídeo apenas de diferença. A possibilidade de utilização de diferentes fluoróforos permite que mais de uma reação seja realizada no mesmo tubo de ensaio. Para tanto, necessita-se de um aparelho termociclador e um equipamento de eletroforese capilar.

O objetivo principal desta técnica não é a amplificação de sequências-alvo, mas sim a hibridização de sondas específicas à essas sequências. Cada sonda de MLPA consiste de duas sequências de oligonucleotídeos separadas, cada um contendo uma sequência de primers para a PCR.

Ao contrário da PCR multiplex convencional, um único par de *primers* é utilizado para a amplificação por MLPA. Os amplicons resultantes da amplificação podem variar entre 90 e 480 nucleotídeos de tamanho e podem ser analisados por eletroforese capilar. A comparação do padrão obtido no pico controle com a amostra de interesse indica quais sequências apresentam número de cópias alterado.

Para a análise dos possíveis genes modificadores de fenótipo na X-ALD, sondas específicas foram desenhadas de acordo com as recomendações do fabricante (MRC-Holland, Netherlands) (tabela 2). Para não haver sobreposição dos picos dos amplicons obtidos, as sondas desenhadas tinham entre 2 e 3 nucleotídeos de diferença.

Tabela 2: Desenho e características das sondas de MLPA utilizadas nesse estudo.

Gene	Localização	Sequencia	Tamanho (bp)
sondas marcadas FAM			
PCDHA1	5q31	LPO-GGGTTCCCTAAGGGGTTGGAGAATCTAGACTCCTGAATTCGCGTTTTCCGATAGAAGGAGCTGCT RPO-GATGCAGACATTGGTGCTAACGCTCTTCTAACGTACGTCTAGATTGGATCTTGCTGGCAC	116
PRKRIP1*	7q22.1	LPO-GGCCGCGGGAATTCGATTGGAGTTCCACGTGTACAGACATCTGCGCCGGA RPO-GAGAATATCAGCGACAGGACTACATGGATGCCATGGCTGAGCACTAGTGAATTCGCGGC	124
HLA-DRB5	6p21.3	LPO-GGGTTCCCTAAGGGGTTGGAcgatcCTACCGGTTCAGCAACTCTGCAGAAAATGTCC RPO-ATCCTTGTGGCTTCCTCAGCTCCTGCCCTTGGCCTGAAGTCTAGATTGGATCTTGCTGGCAC	126
SH2B2	7q22	LPO-GGGTTCCCTAAGGGGTTGGAGTACAGCATCTGTGGTTCCAGTCTGTGCTTG RPO-ACATGCTCCGCCACTTCCACACACACTCTAGATTGGATCTTGCTGGCAC	99
ORM1	9q31-q32	LPO-GGGTTCCCTAAGGGGTTGGAGAATTGGAACTCCAGGCTGGCTTCAGATCTGCCTCTCTCTCAT RPO-GCCCTCTTTAAGATCCTTTGCAAACCAATGGTAGAAGCCTGTCTAGATTGGATCTTGCTGGCAC	118
ORM2	9q32	LPO-GGGTTCCCTAAGGGGTTGGAGTACAATAAGTCGGTTCAGGAGATCCAAGCAACCTTCTTTTACTTT RPO-ACCCCCAACAAGACAGAGGACACGATCTTTCTCAGAGAGTACTCTAGATTGGATCTTGCTGGCAC	120
AGPAT2	9q34.3	LPO-GGGTTCCCTAAGGGGTTGGACATCATCGGCTGGTTCGTGCGAAGCTTCA RPO-AGTACTTTTACGGGCTCCGCTTCGAGGTGCTCTAGATTGGATCTTGCTGGCAC	101

Gene	Locus	Sequences	Size
ABCA2	9q34	LPO-GGGTTCCCTAAGGGGTTGGAGAGGAGAGCATTGTCAACTACACCCTCAAC	103
		RPO-CAGGCCTACCAGGACAACGTCACTGTTTTTGTCTAGATTGGATCTTGCTGGCAC	
EHMT1	9q34.3	LPO-GGGTTCCCTAAGGGGTTGGCCTCTTTCTTTCTCGGGATTCAGATGTCACCTTAAAG	98
		RPO-AACAAGGAAGGAGAGACGCCCCTGCAGTGTGTCTAGATTGGATCTTGCTGGCAC	
GPRIN2	10q11.22	LPO-GGGTTCCCTAAGGGGTTGGAGAGCCTGGCTCTAGGACCAAAGA	91
		RPO-TGTGTGGACCATGACCTCAGCCAATGTCTAGATTGGATCTTGCTGGCAC	
PCDH20	13q21	LPO-GGGTTCCCTAAGGGGTTGGAGGAGTCCCACAAGCTCACCATCCTTG	93
		RPO-CTAATGGACCAGGCTGCATCCCTGCTCTAGATTGGATCTTGCTGGCAC	
PCDH17	13q21.1	LPO-GGGTTCCCTAAGGGGTTGGAGATCAGGCTGACAGTGACCAAGACACTA	95
		RPO-ACAAAGGCTCCTGCTGTGACATGTCTCTAGATTGGATCTTGCTGGCAC	
SLC27A2	15q21.2	LPO-GGGTTCCCTAAGGGGTTGGAGGTAACGTTCTGATCACTCATCAGCGCATATGGTATGGAA	130
		RPO-CTGGCCTCACTTTTGTAAGCGGATTGAAGGGTTCAGGTCGTCTAGATTGGATCTTGCTGGCAC	
RAI 1*	17p11.2	LPO-GGGTTCCCTAAGGGGTTGGGCAAGGAAAAGTGGCCTTTTGCGTGACA	122
		RPO-TCTAGATTGGATCTTGCTGGCACCAGAAGTGGACACATCATTCTGATCTCAGGGC	
GPX4*	19p13.3	LPO-GGGTTCCCTAAGGGGTTGGAGTGCATCGTCACCAACGTGGCCTCCCAGTGAG	107
		RPO-GCAAGACCGAAGTAAACTACACTCAGCTCGTCGTCTAGATTGGATCTTGCTGGCAC	
GRIN3B*	19p13.3	LPO-GGGTTCCCTAAGGGGTTGGACTCAACGCCTTCATCATGGACAAGTCGCTCCTG	109
		RPO-GACTACGAGGTCTCCATCGACGCCGACTGCAAACTCTAGATTGGATCTTGCTGGCAC	
CIRBP	19p13.3	LPO-GGGTTCCCTAAGGGGTTGGACTTCTGACGCCGGGATGGCCTCGTTACTAGACTTTTCTTTT	114
		RPO-TAAGGAAGTGCTGTTTTTTTTTGAGGGTTTTCAAAACTCTAGATTGGATCTTGCTGGCAC	
NDUFS7	19p13.3	LPO-GGGTTCCCTAAGGGGTTGGAGCTCACATGTATGGACAGATGTGTACACGGACCACACGCAC	112
		RPO-ACTCACGCACACAATGCACATATGCACACTCGCTCTAGATTGGATCTTGCTGGCAC	
ABCA7	19p13.3	LPO-GGGTTCCCTAAGGGGTTGGAGTCTCACCATGGCCTTCTGGACACAGCTGATGCTG	105
		RPO-CTGCTCTGGAAGAATTTCATGTATCGCCTCTAGATTGGATCTTGCTGGCAC	

sondas marcadas HEX

Gene	Locus	Sequences	Size
PLD5	1q43	LPO-GGCCGCGGGAATTCGATTCCTCCTCCTTCTGGATCGTGGACAA	87
		RPO-ACAGCACGTGTATATCGGCAGTGCCGCACTAGTGAATTCGCGGC	
BCL2L11*	2q13	LPO-GGCCGCGGGAATTCGATTCTGCTAGTCGTCGTCCGAATGGTTATCTTACGACTGTTACGTTACAT	127
		RPO-TGTCCGCCTGGTGTGGAGAATGCATTGACAGGCCTAGTATGTCCCACTAGTGAATTCGCGGC	
ATP6V1G1	9q32	LPO-GGCCGCGGGAATTCGATTCCTTGTCCTGGGACAGCCTGGGGCAAGTTATT	98
		RPO-AAGCATATTTCCTTCCTGGTGGGCTAGATGCACTAGTGAATTCGCGGC	
IRF7	11p15.5	LPO-GGCCGCGGGAATTCGATTGTCTAATAAAAAGAACTCCAGAACACGTACTGGC	100
		RPO-ATCTTGGCTGGTGGGGAATTGGGTCCAGGCCACTAGTGAATTCGCGGC	
SLC25A22	11p15.5	LPO-GGCCGCGGGAATTCGATTGCCCCACCCTCACTCATTCCCATTCCCTCATCC	102
		RPO-TTCTAGGACAGAAGTGGCGGTTGCTGACGCCTGCACTAGTGAATTCGCGGC	
TSPAN4	11p15.5	LPO-GGCCGCGGGAATTCGATTCTTAATAAAGTGTGAGCAGCAACCTTGCGTCTAT	104
		RPO-TGCTCGTCTGCCTTCCCTACCCTATCTTCCCTTCCACTAGTGAATTCGCGGC	
CHID1	11p15.5	LPO-GGCCGCGGGAATTCGATTCGATGTCACCAAGGTCTTTGGGAGCAAGTTCACAC	106
		RPO-AGATCTCACCCGTCTGGCTGCAGCTGAAGAGACGTCACTAGTGAATTCGCGGC	
LRDD	11p15.5	LPO-GGCCGCGGGAATTCGATTGTGAGCAACAAAACTGCACTGTTTCTTTCACCTCAGAG	110
		RPO-CTTGATTTATTTGTTGGATGGAGAAGCCGTTGGAGGCACTAGTGAATTCGCGGC	
PNPLA2	11p15.5	LPO-GGCCGCGGGAATTCGATTGAGTGACATCTGTCCGCAGGACAGCTCCACCAACATCCAC	112
		RPO-GAGCTGCGGGTCACCAACACCAGCATCCAGTTCAACCACTAGTGAATTCGCGGC	
AP2A2	11p15.5	LPO-GGCCGCGGGAATTCGATTGTGTGGTAACTGCAGCCACAAGTCTGATCACCACTTTAGC	116
		RPO-ACAGAAGAACCCAGAAGAGTTTAAAACCTCCGTGTCTCTGCACTAGTGAATTCGCGGC	
CD151*	11p15.5	LPO-GGCCGCGGGAATTCGATTCAGGATGGGTGAGTTCAACGAGAAGAAGACAACATGTGGCAC	114
		RPO-CGTTTGCCTCAAGTACCTGCTGTTTACCTACAATTGCACTAGTGAATTCGCGGC	
ATP8B4	15q21.2	LPO-GGCCGCGGGAATTCGATTCCTCCTTGACCTGGTTTACCACCATTGTGCCTTTGGTCCTGGTGA	125
		RPO-TAACTATGACAGCTGTCAAAGATGCCACAGATGACTATGTGAGCCACTAGTGAATTCGCGGC	
BECN1	17q21	LPO-GGCCGCGGGAATTCGATTGGATGACAGTGAACAGTTACAGATGGAGC	91
		RPO-TAAAGGAGCTGGCACTAGAGGAGGAGCACTAGTGAATTCGCGGC	
SBNO2	19p13.3	LPO-GGCCGCGGGAATTCGATTCAGCGACTTCAACTCCTCCCCCCGAGTCCCTGGTGGATGACGACGTT	118
		RPO-GTCATCGTTGATGCAGTCGGGCTCCCCAGTGACGACCACTAGTGAATTCGCGGC	
BCL2L12	19q13.3	LPO-GGCCGCGGGAATTCGATTCTAGCTAGTACGTACCCTCATGTCCCTGCCCTCTTCGTGTGCTTTTCC	129
		RPO-AAGTCTTCCTATTCCACTCAGGGCTGTGGAAACGGGTTACCGTCCCACTAGTGAATTCGCGGC	
GNB1L	22q11.2	LPO-GGCCGCGGGAATTCGATTGATCCTAAGTTAACCGTCGAAGGTGTGCAGCCGCATCGCCTGCCATGA	123
		RPO-GGAGCCCGTCATGGACCTTGACTTTGACTCGAGTCATGGCACTAGTGAATTCGCGGC	
IL13RA2*	Xq13.1-q28	LPO-GGCCGCGGGAATTCGATTCTCCATTGTGAACCAGTAAGAACACTCTC	93
		RPO-GTGAGTCTAACGGTCTTCCGGATGAAGGCACTAGTGAATTCGCGGC	

| PLS3* | Xq23 | LPO-GGCCGCGGGAATTCGATTCTTTAGATCTTTAAATGGATGAGATGGCTACCACTCAGATTTCCAA | 120 |
| | | RPO-AGATGAGCTTGATGAACTCAAAGAGGCCTTTGCAAAAGCACTAGTGAATTCGCGGC | |

*: sondas controle

Para a reação de MLPA fizemos uma mistura de todas as sondas (senso e antisenso) marcadas com o mesmo fluoróforo (FAM ou HEX), em um único tubo.

A reação de MLPA pode ser dividida em 5 passos principais (figura 1):

1) Desnaturação do DNA e hibridização das sondas:

Diluir a amostra de DNA (100 ng) em água, em um volume final de 5uL.

Incubar o DNA por 5 minutos à 98°C.

Preparar a PROBEMIX (mistura das sondas) misturando com tampão de MLPA.
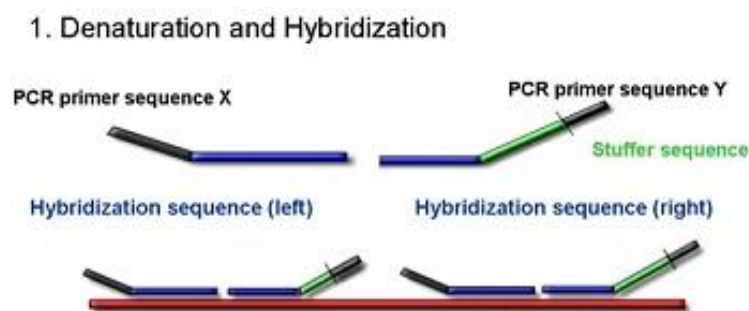
<div align="center">

MIX PROBEMIX 1X

1,5uL de PROBEMIX + 1,5uL de MLPA buffer

</div>

Com o termociclador à 25°C, adicionar 3uL do MIX PROBEMIX a cada amostra previamente desnaturada.

Incubar por 1 minuto à 95°C e iniciar a incubação por 16 horas à 60°C.

Durante estas horas, as as duas partes da sonda se hibridizam imediatamente de maneira adjacente à sequência alvo.

2) Reação de ligação:

Após as 16 horas de incubação, a temperatura do termociclador foi reduzida à 54°C. Mantendo o termociclador nesta temperatura, adicionamos 32uL do MIX LIGASE-65 a cada amostra e misturamos com a pipeta.

MIX LIGASE 1X

(realizar esta mistura menos de 1 hora antes do seu uso e mantê-lo no gelo)

3uL LIGASE-65 BUFFER A

3uL LIGASE-65 BUFFER B

25uL de água (misturar)

1uL LIGASE-65 (misturar novamente)

Incubar por 15 minutos à 54°C e, em seguida, incubar 5 minutos à 98°C para inativar a LIGASE-65. Guardar à 4°C (até uma semana) ou à -20°C (para períodos maiores).

Somente quando ambos oligonucleotídios estão hibridizados ao DNA é que eles podem ser ligados, durante a reação de ligação.

2. Ligation

3) Reação de PCR:

Como somente as sondas ligadas serão exponencialmente amplificadas durante a PCR, o número de produtos de ligação das sondas é uma medida para o número de sequências alvo na amostra.

Preparar, em tubos novos, a seguinte reação:

Sample mix 1X PCR MLPA sondas

(fazer o mix de PCR menos de 1 hora antes do uso e manter no gelo)

13uL água

2uL 10X SALSA PCR

Com os tubos abertos no termociclador à 60°C, adicionar 5uL do MIX POLIMERASE a cada novo tubo e iniciar a reação de PCR.

MIX POLIMERASE 1X MLPA

1,8uL de água

1uL Enzyme Dilution buffer

1uL PCR-primes MLPA-FAM

1uL PCR-primers MLPA-HEX

0,2uL Taq Polymerase (3U)

(misturar bem)

Iniciar o programa da PCR:

95°C – 30''
60°C – 30'' } 32 ciclos
72°C – 60''

72°C – 22''

3. PCR with universal primers X and Y
exponential amplification of ligated probes only

4) Separação dos amplicons em eletroforese capilar:

Os produtos amplificados são separados por eletroforese capilar. Para tanto, faz-se a seguinte reação:

160uL formamida

6,4uL ROX 500

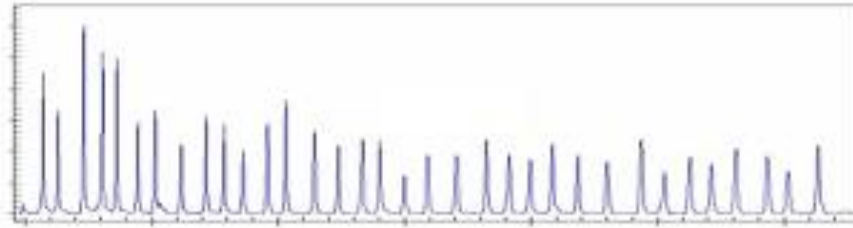Diluir o produto de PCR da seguinte maneira:

2,0uL PCR

10uL de água

Em tubos novos, preparar:

10uL (formamida + ROX)

2uL PCR diluído

4. Fragment analysis

5) Análise dos dados:

Para a análise dos resultados, primeiramente fizemos uma normalização somando a altura relativa de cada pico controle (regiões que sabidamente não variam ou variam muito pouco na população em geral) e dividimos essa soma pelo número de regiões controle (MÉDIA).

Após, realizamos a divisão da altura relativa de cada pico pela MÉDIA para todas as regiões de cada amostra (RADIO1). A seguir calculamos a média e o desvio padrão das amostras controle. Por fim, calculamos a média entre RADIO 1 pela média das amostras controle, para cada amostra de interesse (RADIO 2).

Amostras que apresentaram valor menor que 0,7 foram consideradas com menos cópias (deleção) desta região, amostras que apresentaram valores maiores que 1,25 foram consideradas com mais cópias (duplicação) desta região.