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SHORT COMMUNICATION

Progression rate of myelopathy in X-linked adrenoleukodystrophy heterozygotes

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Abstract X-linked adrenoleukodystrophy heterozygote women can present adult onset myeloneuropathy and little is known about its natural history. We aimed to describe the progression rate of the neurological impairment in the prospective follow-up of our cohort and to look for prognostic factors. The neurological scales Japanese Orthopaedic Association (JOA) and Severity Score System for Progressive Myelopathy (SSPROM) were applied at baseline in 29 symptomatic carriers and in follow-up visits. Age at onset, disease duration, X inactivation pattern, determination of the allele expressed, plasma levels of the very long chain fatty acids and of the neuron-specific enolase, and somatosensory evoked potentials, were taken at baseline. The slope of the linear regression of both JOA and SSPROM versus disease duration since the first symptom was estimated using mixed modeling. JOA and SSPROM decreased 0.42 and 1.87 points per year, respectively ($p<0.001$). None of the parameters under study influenced these rates. We estimated that the number of carriers per arm needed in a future 12 month trial with 80 % power and a 50 % reduction in disease progression would be 225 women for JOA and 750 for SSPROM. The progression rates of the studied neurological scales were small, did not depend on any modifier factor known, and reflected the characteristically slow worsening of symptoms in X-ALD heterozygotes. Better biomarkers are still necessary for future studies.

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Abbreviations

ABCD1	Gene for the adenosine triphosphate (ATP)-binding cassette protein, subfamily D
cDNA	Complementary DNA
HUMARA	Human androgen-receptor locus
JOA	Japanese Orthopaedic Association
NH	Natural history
NSE	Neuron-specific enolase
OMIM	Online Mendelian Inheritance in Man
SSEP	Somato-sensory evoked potentials
SSPROM	Severity Score System for Progressive Myelopathy
VLCFA	Very long chain fatty acids
X-ALD	X-linked adrenoleukodystrophy

Introduction

X-linked adrenoleukodystrophy (X-ALD, OMIM #300100) is a peroxisomal disorder caused by defects in the gene for the adenosine triphosphate (ATP)-binding cassette protein, subfamily D (*ABCD1*) (Moser et al. 2001; Jangouk et al. 2012; Engelen et al. 2012; Horn et al. 2013), leading to accumulation of unbranched saturated very long chain fatty acids (VLCFA) in plasma and tissues and to diverse clinical presentations in males and females.

Heterozygote women might be asymptomatic or present with mild/moderate myeloneuropathy (Moser et al. 2001; Jangouk et al. 2012; Engelen et al. 2012; Mosser et al. 1994; Garg et al. 1983; Restuccia et al. 1997; Schmidt et al. 2001). We and others have recently reported results from cross-sectional studies, where there was a higher than expected proportion of symptomatic women (63 to 87 %), disability was mild, and symptoms were clearly related to ageing (Horn et al. 2013; Habekost et al. 2014; Engelen et al. 2014). As symptomatic individuals are very common among female heterozygotes, pharmacological management of heterozygotes will be needed, and clinical trials are expected.

Clinical evaluations have been efficient to detect neurological impairment in females (Habekost et al. 2014; Engelen et al. 2014). The myelopathy scales Japanese Orthopaedic Association (JOA) (Yonenobu et al. 2001) and Severity Score System for Progressive Myelopathy (SSPROM) (Castilhos et al. 2012) differentiated symptomatic from asymptomatic heterozygote women, and were correlated with age and with disease duration(DD) (Habekost et al. 2014). Since there is a lack of longitudinal or natural history (NH) of X-ALD females, we aimed to describe the progression rate of JOA and SSPROM scales in this prospective follow-up of our cohort and to relate it to factors such as age at onset, X inactivation pattern, plasma levels of VLCFA, neuron-specific enolase (NSE), and somato-sensory evoked potentials (SSEP), obtained at baseline.

Methods

Population

This is a prospective follow-up cohort of X-ALD of women whose cross-sectional findings have already been described (Habekost et al. 2014). All symptomatic heterozygotes diagnosed by our center in South Brazil were invited to be screened for other causes of myeloneuropathy. The heterozygotes whose symptoms were only attributed to X-ALD were invited to come for follow-up visits, when new neurological assessments were performed. Detailed information about population of origin was presented by Jardim et al. (Jardim et al. 2010). Exclusion criteria and baseline findings have been previously described (Habekost et al. 2014). This study was approved by the Institutional Bioethics Commission (GPPG-HCPA-110308) and all subjects gave written consent to participate.

Neurological assessments

At the baseline, heterozygote women were classified as symptomatic if complaints of neuropathic pain, paresthesia, sphincter dysfunction or paresis were present or as asymptomatic if these complaints were absent. To describe the NH of JOA and SSPROM scales, new assessments for the symptomatic females were planned at 6 month intervals. JOA and SSPROM scores varies from −2 to 17 and from 0 to 100 points, respectively; the higher scores correspond to the asymptomatic state. Developed to evaluate patients with degenerative cervical compression, JOA includes questions about motor disability of upper and lower limbs, about sensory losses and about sphincter function. SSPROM includes questions about these same domains, plus questions about motor strength and tonus/reflexes (Supplemental File 1). All examinations were carried out by the same examiner (CTH).

Neurophysiological testing and biochemical studies

At baseline, SSEP of the posterior tibial nerves produced the potentials P40 (for details, see (Habekost et al. 2014). Blood collections to measure plasma levels of the VLCFA C22:0, C24:0 and C26:0, and NSE, were obtained under fasting conditions (at least 3 h). VLCFA values of C26:0, of C26:0/C22:0, and of discriminant factor 3.805(C24:0/C22:0) +

5.296(C26:0/C22:0) + 5.15(C26:0) (Moser and Moser 1991; Moser et al. 1999), were studied. Details of all these methods were described in (Habekost et al. 2014).

X chromosome inactivation and cDNA studies

At baseline, peripheral blood was collected to assess the pattern of X chromosome inactivation. Genomic DNA was obtained by the salting out procedure (Miller et al. 1988) and X-inactivation patterns were assessed with the human androgen-receptor locus (HUMARA) methylation assay (Habekost et al. 2014; Allen et al. 1992).

Complementary DNA (cDNA) was obtained from women with skewed inactivation patterns (equal or larger than 75:25), in order to determine which allele was being preferably expressed. Peripheral blood was collected in PAXgene Blood RNA tubes (PreAnalytix, BD) and mRNA was extracted using PAXgene Blood RNA Kit (Qiagen). Then, 5 μg of RNA were converted to cDNA using *SuperScript II Reverse Transcriptase* and RNaseOUT (Invitrogen). Primers for exons 3, 6, 7 and 8, where the mutations carried by those with skewed inactivation patterns - p.Arg401Trp, p.Arg518Gln, p.Glu577X and p.Arg617His - are located, were used to amplify the cDNA. Amplified fragments were purified with Exo I-SAP (GE Healthcare) and submitted to automate sequencing on ABI 3100 using BigDye v3.1 (Applied Biosystems). Peak height at the site of heterozigosity was used to infer which allele was predominant (Wang et al. 2013).

Statistical analysis and determination of the progression rate of disease

To assess the disease progression rate, JOA and SSPROM scores of symptomatic women were studied. The slope of the linear regression of JOA and SSPROM versus disease duration since the start of the first symptom was estimated using mixed modeling, adjusting for repeated measurements.

Random coefficient models were adjusted to fit the growth curves. Some advantages of this model are that it dispenses equal intervals between assessments and that it incorporates patients with single measures - although it is not possible to estimate a slope for these patients, they contribute to estimate the intercept of the curves. This model fits curves for each subject, and the parameters of the individual curves are used to estimate group curves. Models were adjusted to the following explanatory variables: age at onset, VLCFA, NSE, SSEP and X inactivation pattern, at baseline.

Each model resulted in a mean adjusted line, represented by a graphic, showing JOA and/or SSPROM progression. Adjusted lines represented the whole group or its subgroups produced in accordance to prognostic variables. Where prognostic variables were continuous, percentiles were calculated, and a line for each value was produced in order to see the changes in JOA and/or SSPROM progression.

Estimates obtained from growth curves without covariates were used to calculate sample size needed in a future 12 month trial with 80 % power, expecting to reduce the progression to 50 %. The Zhang and Wang SAS macro were used (Zhang and Wang 2009).

All tests were two-sided and $p<0.05$ was considered significant. The statistical analyses were done using the Statistical Analysis Software SAS version 9.3.

Results

Twenty-nine out of 33 heterozygote women were symptomatic. General characteristics of the study population are depicted in Table 1. Only the symptomatic women were analyzed. None of them were on chronic management such as drugs for spasticity, or bladder dysfunctions.

The 29 baseline evaluations were obtained before May, 2013. Several subjects did not come back in 6 months, as planned; some of them came back 3–4 years later. A total of 21 follow up evaluations of JOA and SSPROM were obtained in a mean (range) interval of 9±3 (6 to 48) months. Fourteen women were evaluated once, nine were evaluated twice, and six women were evaluated three times. To assess disease progression, we used these 50 JOA and SSPROM scores (29 baseline plus 21 follow up evaluations). Individual raw data were presented in Supplemental File 2.

From the 29 symptomatic women, 17 were identified as informative women by HUMARA locus and capillary electrophoresis, at baseline. The median ratio of X-inactivation was 66:33. The five women with less skewing (percentile 25) had ratios lower than 60.25:39.75; the five more skewed women (percentile 75) had ratios higher than 72.5:27.5 (Table 1). Among these five women, four showed ratios greater than 75:25, but none showed highly skewed patterns (of more than 90:10). The results for cDNA sequencing for these four women can be seen on Supplemental Figure 3, which shows preferential pattern of expression, consistent with HUMARA results.

Disease progression and sample size estimations

Age at onset, VLCFA, skewed patterns of inactivation, NSE and SSEP at baseline were not related to different JOA or SSPROM progression rates (Supplemental Figures 1, 2 and 3).

With the obtained growth curves, the estimated value of JOA score at the disease onset was of 16.6 points, and the mean (SE) annual decrease (worsening) of JOA was of 0.42 points (0.048) ($p<0.001$, Fig. 1a). The estimated value of SSPROM score at the disease onset was of 96.5 points, and

Table 1 Clinical, biochemical and molecular characteristics of the X-ALD women, at baseline

	Values obtained in symptomatic women	Values obtained in asymptomatic carriers
Number of subjects	29	4
Age at onset of the first symptom mean±sd (range)	39.4±10 (21–59)	
Age (years) at examination mean±sd (range)	43.9±10.2 (24–61)	
Disease duration (years) mean±sd (range)	4.5±3.3 (1–15)	
JOA mean±sd (range: −2 to 17)	14.5±1.7	16.6±0.2
SSPROM mean±sd (range: 0 to 100)	86.6±7.9	98.4±1.1
SSPROM Motor Disability (range: 0 to 30 – normal) *based on the* "*overall disability sum score*", *ODSS* (Merkies et al. 2002)	27.3±2.6	30±0
SSPROM Motor Strength (0 to 20 – normal) *based on The Medical Research Council scale – 0 to 5 – for each limb* (Medical Research Council 1976)	17.5±5	19.1±1.3
SSPROM sensory losses (0 to 20 – normal)	16.1±2.3	18.5±9
SSPROM spasticity/hyperreflexia (0 to 10 – normal) *Adapted from Ashworth scale in a descending order* (Bohannon and Smith 1987).	5.7±1.8	9.4±1.2
SSPROM sphincter control (0 to 30 – normal) *Based on Kurtzke scale* (Kurtzke 1983)	17.8±2.3	27.6±2.6
SSEP N20 (ms) Mean±sd (normal: up to 20.5) [a]	20.1±4.2	18±0.9
SSEP P40 (ms) mean±sd (normal: up to 41.5) [a]	55.3±32.8	38.9±3.8
VLCFA C26:0 (um/L) mean±sd (normal: 0.78–1.54) [a]	1.93±0.42	
VLCFA C26:0/C22:0 mean±sd (normal: 0.01–0.03) [a]	0.076±0.028	
VLCFA discriminant factor [b] mean±sd (normal: up to 10.86)[a]	14.28±2.55	
NSE (ng/mL) mean±sd	12.95±7.3	7.2±7
Inactivation pattern of X chromosome in leukocytes: median (range)	66:34 (50:50 to 85:15)	
Skewed inactivation (>75:25)	4 carriers	

[a] Reference range in normal females

[b] Discriminant factor = 3.805(C24:0/C22:0) + 5.296(C26:0/C22:0) + 5.15(C26:0), as described by (Moser and Moser 1991; Moser et al. 1999)

the mean (SE) annual decrease (worsening) of SSPROM was of 1.87 points (0.22) ($p<0.001$, Fig. 1b).

The number of patients needed per arm in a future 12 months trial with 80 % power and that expects to reduce the neurological progression to 50 % in comparison to NH, would be 225 per arm for JOA, and 750 per arm for SSPROM.

Discussion

We were able to give a comprehensive quantitative account of the neurologic deterioration in X-ALD heterozygote women. Progression rate of JOA and SSPROMM was slow as expected, presented small variances, and no modifier factors were identified.

We have chosen to follow the NH of JOA and SSPROM because these scales cover both pyramidal, sphincter and sensory manifestations of a myelopathy, and because they correlated with age and with disease duration in the cross-sectional segment of this study (Habekost et al. 2014). Although we did not obtain follow-up evaluations from all women – some of them live in remote regions and were lost -, the range of the disease duration covered by the present cohort allowed a statistical model for disease progression. The study design produced a linear progression of neurologic disability. A non linear progression could not be detected by this study: although possible, however, there is no reason to anticipate different progression rates for different phases of this disease. Due to these findings, we believe that the present data is the best evidence on the NH of X-ALD heterozygote women.

The slow progression of the neurological impairments in X-ALD women is in agreement with its under recognition in general (Jangouk et al. 2012), and with the mild disabilities detected in recent studies (Habekost et al. 2014; Engelen et al. 2014). These progression rates might raise some doubt on the applicability of these scales in future clinical trials due to the large number of patients or the long follow up duration necessary for a measurable effect. Therefore, biomarkers that make future clinical trials feasible are needed.

Age at onset, the amount of the neurological damage (measured by SSEP of lower limbs and by NSE, at baseline), and the X inactivation patterns (directly measured by the proportion of active alleles; but also roughly estimated by the serum VLCFA levels) were our first candidates as modifier factors for the progression rate of this disease. All gave negative results.

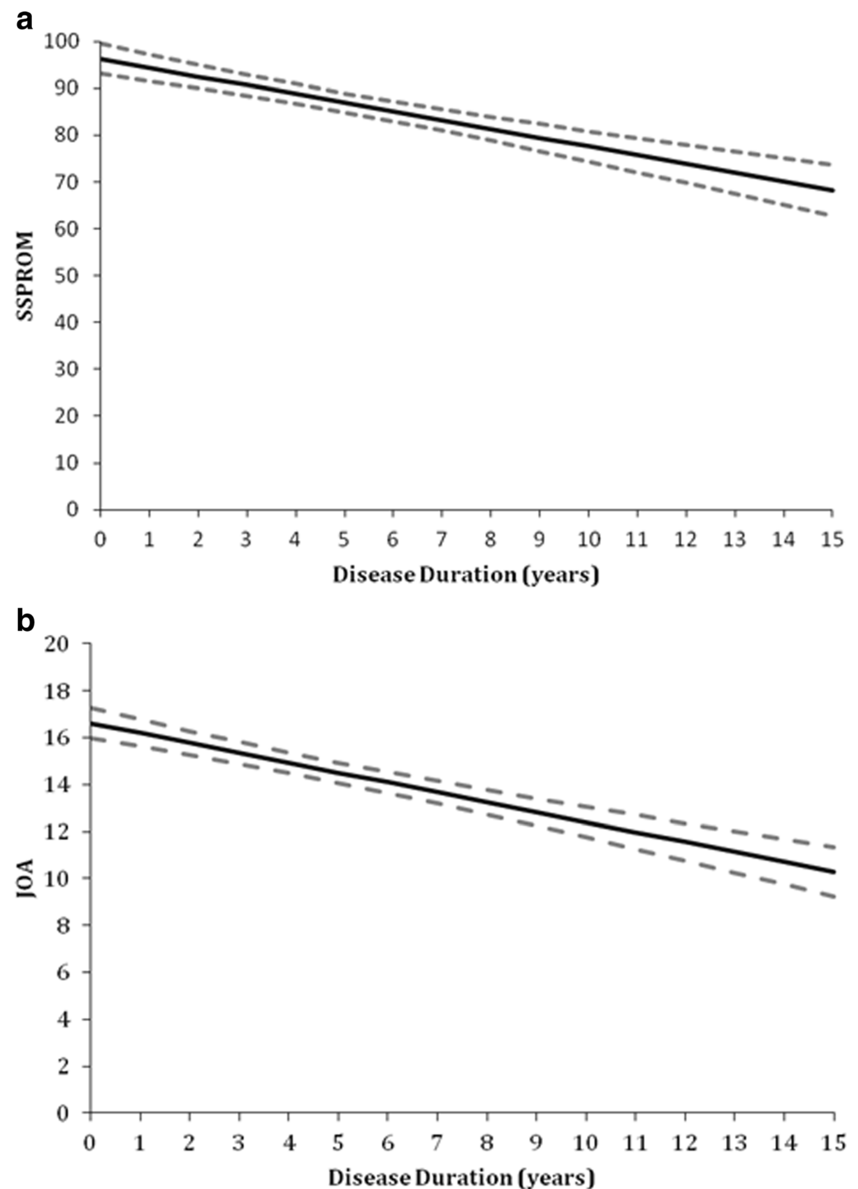

Fig. 1 The progression rate of (1A) JOA and (2) SSPROM scores of symptomatic women heterozygote for X-ALD, according to disease duration, in years. General growth curves (linear model)

We did not identify a factor that affects disease progression in females, but this should not discourage the search for better biomarkers in this rare disease, especially when facing the slow rate of neurologic progression. A traceable substance or biological variable that would change together with the neurological impairment, and whose responsiveness to disease progression and to a potential drug would be measurable, should be one of the important targets in the study of female carriers of X-ALD.

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Authors' contributions CTH participated in the design of the study, carried out the recruitment, interviews and clinical studies, and helped to draft the manuscript. FSP carried out the molecular genetic studies, participated in the analysis, and helped to draft the manuscript. PS carried out

the SSEP studies and participated in the analysis. PS and VTF carried out peripheral neurophysiology studies and participated in the analysis of the data. DMC performed VLCFA analyses and helped in the coordination of data. CRV performed VLCFA analyses and helped in the acquisition of funding. VT performed NSE analyses and participated in the analysis of data. LVP performed NSE studies and helped in the acquisition of funding. UM participated in the molecular analysis and in the coordination of data, and helped to draft the manuscript. VLT performed the statistical analyses, and helped to draft the manuscript. LBJ conceived the study, participated in the design and coordination of the study, helped to perform the statistical analysis, and drafted the manuscript. All authors read and approved the final manuscript.

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