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# Evidence that oxidative stress is increased in patients with X-linked adrenoleukodystrophy

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**Abstract**

X-linked adrenoleukodystrophy (X-ALD) is a hereditary disorder of peroxisomal metabolism biochemically characterized by the accumulation of very long chain fatty acids (VLCFA), particularly hexacosanoic acid (C26:0) and tetracosanoic acid (C24:0) in different tissues and in biological fluids. The disease is clinically characterized by central and peripheral demyelination and adrenal insufficiency, which is closely related to the increased concentrations of these fatty acids. However, the mechanisms underlying the brain damage in X-ALD are poorly known. Considering that free radical generation is involved in various neurodegererative disorders, like Parkinson disease, multiple sclerosis and Alzheimer's disease, in the present study we evaluated various oxidative stress parameters, namely chemiluminescence, thiobarbituric acid reactive species (TBA-RS), total radical-trapping antioxidant potential (TRAP), and total antioxidant reactivity (TAR) in plasma of X-ALD patients, as well as the activities of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in erythrocytes and fibroblasts from these patients. It was verified a significant increase of plasma chemiluminescence and TBA-RS, reflecting induction of lipid peroxidation, as well as a decrease of plasma TAR, indicating a deficient capacity to rapidly handle an increase of reactive species. We also observed a significant increase of erythrocytes GPx activity and of catalase and SOD activities in fibroblasts from the patients studied. It is therefore proposed that oxidative stress may be involved in pathophysiology of X-ALD.


*Keywords:* X-linked adrenoleukodystrophy; Oxidative stress; Free radical

## 1. Introduction

X-linked adrenoleukodystrophy (X-ALD) is the most frequent peroxisomal disorder, with an estimated frequency of 1:25,000 in males [1–3]. It should be differentiated clinically and biochemically from neonatal ALD, an autosomal recessive disorder of peroxisome biogenesis in which the function of at least five peroxisomal enzymes is impaired. Tissue accumulation of very long chain fatty acids (VLCFA), mainly hexacosanoic ($C_{26:0}$) and tetracosanoic acids ($C_{24:0}$), is the biochemical hallmark of X-ALD, which is caused by a defect in very long chain acyl-CoA synthetase transport [1,5,6]. Fatty acids accumulation seems responsible for adrenal cortex malfunction and nervous system demyelination [7,8]. Known clinical forms are classical (or childhood) cerebral X-ALD (cALD), adrenomyeloneuropathy (AMN) and isolated Addison's disease (AD). Patients may be completely asymptomatic and several phenotypes may occur in the same family [7,12]. cALD and AMN are the two most frequent clinical forms of X-ALD [7,10]. AMN may be confounded with multiple sclerosis. Patients with classical X-ALD usually develop the symptoms from age 4 to 8, including visual and auditory disturbances,

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decreased school performance, adrenal insufficiency, walking difficulties, demyelinization and leukodystrophy. The disease progresses rapidly and untreated patients usually die approximately 2 to 5 years after symptom onset [10,11]. In contrast, AMN appears around age 20–30 [10,11] and is characterized by progressive paraparesis and sphincter disturbances because the spinal cord is affected. Two-thirds of the X-ALD patients have adrenal insufficiency. Treatment consists of a diet restricted in saturated fatty acids, combined with the use of glycerotrioleate/glyceroltrierucate (GTO/GTE), known as Lorenzo's oil [2,9,13,14]. The objective is to normalize VLCFA concentrations, but favourable responses apparently occur only in patients beginning treatment before the appearance of neurological symptoms [1,2,9,13,15]. Bone marrow transplantation and immunosuppression are also considered therapeutic alternatives [10]. Lovastatin and sodium phenylacetate were tested as therapeutic drugs for normalizing VLCFA levels in plasma and skin fibroblasts of X-ALD patients [16,17].

The peroxisome is a small cellular organelle involved in both oxygen and lipid metabolism. Defects in peroxisomal functions are associated with major, and often fatal, changes at the neurological level during human development. The neurological damage that occurs in X-ALD could be mediated by astrocyte activation and cytokine pro-inflammatory induction (tumoral necrose factor α, interleucine 1β and interleucine 6) [3,4]. However, the exact mechanisms underlying the brain damage in X-ALD are poorly known. Lorenzo's oil treatment is not directed to the neuroinflamatory aspects of X-ALD.

It has been shown in animal models that lovastatin (3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitor) and sodium phenylacetate (pyrophosphate mevalonate decarboxilase inhibitor) inhibit nitric oxide synthetase induction and the cytokine pro-inflammatory discharge involved in pathogenesis of the neurological damage in X-ALD [16]. It was verified that these drugs normalize the VLCFA levels in skin fibroblast of X-ALD patients with a concomitant decrease in VLCFA tissue levels [17]. Therefore, it is possible that lovastatin could be promissory by inhibiting the neuroinflammatory process characteristic of X-ALD.

Free radicals seem to be involved in a large number of human diseases. Increasing evidence shows that damage caused by free radicals is an important contributing factor in neurodegenerative, chronic-inflammatory, vasculary and neoplasic diseases [18]. Brain has relatively low levels of antioxidant defenses and a high lipid content with great quantities of unsaturated fatty acids and cathecolamines, which are highly susceptible to reactive oxygen species attack. The involvement of free radicals in the pathophysiology of various common neurological diseases has been described, including Parkinson's disease, Alzheimer's disease, strokes, multiple sclerosis and epilepsy [19–21].

Our objective in the present study was to determine various parameters of oxidative stress in plasma, erythrocytes and cultured fibroblasts from X-ALD patients in order to verify whether free radicals could be involved in the pathophysiology of the tissue damage in these patients.

## 2. Materials and methods

### 2.1. Patients and controls

Plasma, erythrocytes and fibroblasts from 19 X-ALD patients (13 cases of cALD and 6 cases of AMN) aged between 5 and 38 years (mean ± S.D.: 15.1 ± 11 years old) were used to evaluate the different parameters of oxidative stress. The mean age for cALD and AMN were, respectively, 8.38 ± 2.5 and 29.7 ± 9.5 years old. Clinical symptoms of the cALD patients included leukodystrophy, learning difficulty, seizures, hyperactivity and of AMN patients were leukodystrophy, limb weakness, paraparesis, behavioral and sphincter disturbances. Patients were diagnosed in our laboratory by increased plasma levels of VLCFA detected by gas chromatography [22]. The period between plasma collection and analysis was always less than 2 weeks. Plasma, erythrocytes and fibroblasts from controls were obtained from healthy age-matched individuals (21.1 ± 8.1 years old).

### 2.2. Reagents

All chemicals were of PA purity and were purchased from Sigma (St. Louis, MO) except TBA, which was from Merck (Darmstadt, Germany). Chemiluminescence, total antioxidant reactivity (TAR) and total radical-trapping antioxidant potential (TRAP) were assayed using a beta liquid scintillation spectrometer (Tricarb 2100 and Wallac model 1409, respectively) and TBA-RS was measured with a double-bean spectrophotometer with temperature control (Hitachi U-2001).

### 2.3. Preparation of erythrocytes, plasma and fibroblasts

Erythrocytes and plasma were prepared from whole blood samples obtained from fasting individuals (controls and X-ALD) by venous puncture with heparinized vials. Whole blood was centrifuged at 3000 rpm, and the plasma was immediately removed by aspiration and frozen at − 80 °C until determination. Erythrocytes were washed three times with cold saline solution (sodium chloride 0.153 mol/l). Lysates were prepared by addition of 100 μl of washed erythrocytes in 1 ml of distilled water and frozen at − 80 °C until determination of the antioxidant enzyme activities.

Skin biopsies were taken and placed in polyethylene sterile vials (type Falcon T25) for culture cells. The primary and secondary cultures were carried out in HAM-F10 culture medium supplemented with 20% of fetal bovine

serum. After approximately 4 weeks, when cell confluence was achieved, the fibroblasts were collected and frozen in liquid nitrogen until enzymatic activity determination, which was performed in less than 2 weeks [23].

For antioxidant enzyme activity determination, erythrocytes were frozen/thawed three times and centrifuged at 9200 rpm for 10 min. The supernatants were diluted to approximately 0.5 mg/ml of protein. Fibroblast extracts were resuspended with 20 mM sodium phosphate buffer containing 140 mM KCl and 0.1 M phenylmethasulfonyl fluoride, pH 7.4; the pellet was measured and buffer was added in a volume 10 times higher. The cells were frozen in liquid nitrogen and thawed in water at 37 °C. This process was repeated twice. The suspension was centrifuged at 3000 rpm for 10 min and the supernatant was used for the tests.

### 2.4. Chemiluminescence

Samples were assayed for chemiluminescence in a dark room by the method of Gonzalez-Flecha et al. [24]. Incubation flasks contained 3.5 ml of medium consisting of 20 mM sodium phosphate, pH 7.4, and 140 mM KCl. The background chemiluminescence was measured and 0.5 ml of plasma, at a protein concentration of 0.5 mg/ml, was immediately added to the incubation medium. Chemiluminescence was measured for 10 min at room temperature. The background chemiluminescence was subtracted from the total value. Chemiluminescence was calculated as cpm/mg protein.

### 2.5. Thiobarbituric acid reactive species (TBA-RS)

TBA-RS were determined according to the method described by Esterbauer and Chesseman [25]. Briefly, 300 μl of 10% trichloroacetic acid was added to 150 μl of plasma and centrifuged at $300 \times g$ for 10 min at 4 °C. Three hundred microliters of the supernatant was transferred to a vial and incubated with 300 μl 0.67% thiobarbituric acid (in 7.1% sodium sulfate) at 100 °C for 25 min. The mixture was allowed to cool on water for 5 min. The resulting pink-stained TBA-RS were determined in a spectrophotometer (Hitachi U-2001) at 535 nm. The acid did not produce color when tested without the addition of the sample, demonstrating the absence of a direct reaction to TBA. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and each curve point was subjected to the same treatment as that of the supernatants. TBA-RS were calculated as nmol TBA-RS/mg protein.

### 2.6. Total radical-trapping antioxidant potential (TRAP)

TRAP, representing the total antioxidant capacity of the tissue, was determined by measuring the luminol chemiluminescence intensity induced by 2,2′ -azo-bis-(2-amidinopropane) (ABAP) according to the method of Lissi et al. [26]. The background chemiluminescence was measured by adding 4 ml of 10 mM ABAP (in 0.1 M glycine buffer, pH 8.6) into a glass scintillation vial. Ten microliters of luminol was added to each vial and the chemiluminescence was measured. This was considered to be the initial value. Ten microliters of 300 μM Trolox or plasma was added and the chemiluminescence was measured until it reached the initial levels. The addition of Trolox or supernatant to the incubation medium reduces the chemiluminescence. The time necessary for the chemiluminescence intensity to return to the initial value was considered to be the induction time (IT). IT is directly proportional to the antioxidant capacity of the tissue and the IT of each sample was compared with the IT of Trolox. TRAP values are expressed as nM Trolox/mg protein.

### 2.7. Total antioxidant reactivity (TAR)

TAR, which represents the quality of the tissue antioxidants, was determined by measuring the luminol chemiluminescence intensity induced by ABAP according to the method of Lissi et al. [27]. The background chemiluminescence was measured by adding 4 ml of 2 mM ABAP (in 0.1 M glycine buffer, pH 8.6) into a glass scintillation vial. Fifteen microliters of luminol (4 mM) was added to each vial and the chemiluminescence was measured. This was considered to be the basal value. Ten microliters of 10 μM Trolox or plasma (prepared in the same buffer as for TRAP) was then added and the chemiluminescence was measured during 60 s. The Trolox or supernatant addition reduces the chemiluminescence. The rapid reduction in luminol intensity is considered as a measure of the TAR capacity. TAR measurement was calculated as nmol Trolox/mg protein.

Table 1
Chemiluminescence and TBARS parameters in plasma of X-ALD patients

| Assays | Control | | X-ALD patient | | Phenotype |
|---|---|---|---|---|---|
| | Mean ± S.D. | Range | Mean ± S.D. | Range | |
| Chemiluminescence ($n=3$) | 104 ± 56.1 | 47.5–160 | 272 ± 69.9* | 202–342 | Cerebral ALD |
| TBA-RS ($n=7$) | 0.15 ± 0.08 | 0.07–0.23 | 0.32 ± 0.08** | 0.24–0.40 | 5 Cerebral ALD 2 AMN |

Plasma chemiluminescence from adrenoleukodystrophy patients and controls. Chemiluminescence was calculated as cpm/mg protein. Data represent the mean ± S.D. ($n=3$). Difference from control, $^*P<0.05$ (non-paired Student's $t$ test).
Plasma TBA-RS from adrenoleukodystrophy patients and controls. TBA-RS were calculated as nmol TBA-RS/mg protein. Data represent the mean ± S.D. ($n=7$). Difference from control, $^{**}P<0.01$ (non-paired Student's $t$ test).

Table 2
TAR and TRAP parameters in plasma of X-ALD patients

| Assays | Control | | X-ALD patient | | Phenotype |
|---|---|---|---|---|---|
| | Mean ± S.D. | Range | Mean ± S.D. | Range | |
| TRAP ($n=4$) | 1053 ± 379.48 | 674–1433 | 992 ± 384 | 608–1376 | Cerebral ALD |
| TAR ($n=8$) | 90.3 ± 38.3 | 51.9–129 | 49.7 ± 24.8** | 24.8–74.5 | 4 Cerebral ALD 4 AMN |

Plasma TRAP from adrenoleukodystrophy patients and controls. TRAP values are expressed as nM Trolox/mg protein. Data represent the mean ± S.D. ($n=4$). No significance was detected (non-paired Student's $t$ test).
Plasma TAR from adrenoleukodystrophy patients and controls. TAR measurement was calculated as nmol Trolox/mg protein. Data represent the mean ± S.D. ($n=8$). Difference from control, $^{**}P<0.01$ (non-paired Student's $t$ test).

### 2.8. Antioxidant enzyme activities

#### 2.8.1. Catalase assay (CAT)

CAT activity was assayed by the method of Aebi [28] by measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM $H_2O_2$, 10 mM potassium phosphate buffer, pH 7.0, and 0.1–0.3 mg protein/ml. One unit of the enzyme is defined as 1 μmol of $H_2O_2$ consumed per minute and the specific activity is reported as units per milligram of protein.

#### 2.8.2. Glutathione peroxidase (GPx)

GPx was measured by the method of Wendel [29] using *tert*-butyl-hydroperoxide as substrate. The activity was determined monitoring the NADPH disappearance at 340 nm in a medium containing 2 mM glutathione, 0.15 U/ml glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl-hydroperoxide and 0.1 mM NADPH. One GPx unit is defined as 1 μmol of NADPH consumed per minute and the specific activity is represented as units per milligram of protein.

#### 2.8.3. Superoxide dismutase (SOD)

SOD activity was determined using the RANSOD kit (Ransox, Antrim, United Kingdom). The method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and superoxide radical (produced in the incubation medium from the xanthine–xanthine oxidase reaction system), which is assayed spectrophotometrically at 505 nm. The inhibition of the produced chromogen is proportional to the activity of the SOD present in the sample. A 50% inhibition is defined as 1 U of SOD and the specific activity is represented as units per milligram of protein.

### 2.9. Protein determination

Protein concentrations were determined by the method of Lowry et al. [30], using bovine serum albumin as standard.

### 2.10. Statistical analysis

Data are expressed as mean ± standard deviation. The Student's $t$ test for non-paired samples was used to compare results from controls and X-ALD patients. A $P$ value of less than 0.05 was considered to be significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer.

## 3. Results

The lipid peroxidation parameters chemiluminescence and TBA-RS were determined in plasma of X-ALD patients. Table 1 shows that chemiluminescence was significantly increased by 264% (from 104 to 272) [$t(4)=8.053$, $P<0.05$] in plasma of cALD patients, as compared to controls. Furthermore, TBA-RS measurement was also significantly increased by 215% (from 0.15 to 0.32) [$t(12)=3.913$, $P<0.01$] in plasma from these patients (5 cALD and 2 AMN) (Table 1). These results strongly indicate that lipid peroxidation is stimulated in X-ALD.

We also observed that TRAP measurement, which is indicative of the tissue nonenzymatic antioxidant defenses, was not altered in plasma of cALD patients [$t(6)=0.181$, $P>0.05$] (Table 2). However, TAR measurement, which is a measure of the tissue capacity to react with free radicals, was markedly reduced (43%) [$t(14)=2.515$; $P<0.01$] (90.3 to 49.7) (Table 2). These results suggest that the overall content of nonenzymatic antioxidants was not significantly altered in plasma of X-ALD patients, in contrast with the antioxidant reactivity (TAR), which was significantly decreased, indicating a deficient capacity of the tissue to modulate the damage associated with the enhanced production of reactive species.

Table 3
Antioxidant enzyme activities in erythrocytes of X-ALD patients

| Antioxidant Enzyme | Control | | X-ALD patient | | Phenotype |
|---|---|---|---|---|---|
| | Mean ± S.D. | Range | Mean ± S.D. | Range | |
| Cat ($n=4$) | 3.13 ± 0.33 | 2.80–3.46 | 2.57 ± 0.57 | 2.00–3.14 | Cerebral ALD |
| GPx ($n=4$) | 0.83 ± 0.12 | 0.71–0.95 | 0.94 ± 0.12* | 0.82–1.06 | Cerebral ALD |
| SOD ($n=4$) | 1.03 ± 0.42 | 0.61–1.45 | 1.11 ± 0.41 | 0.70–1.52 | Cerebral ALD |

CAT: catalase (pmol/mg prot); GPx: glutathione peroxidase (mU/mg prot); SOD: superoxide dismutase (U/mg prot). One unit is defined as 1 μmol of NADPH consumed per minute for GPx and 50% of produced chromogen inhibition for SOD. Data represent the mean ± S.D. ($n=4$). Difference from control, $^{*}P<0.05$ (non-paired Student's $t$ test).

Table 4
Antioxidant enzyme activities in fibroblasts of X-ALD patients

| Antioxidant enzyme | Control | | X-ALD patient | | Phenotype |
|---|---|---|---|---|---|
| | Values | Range | Values | Range | |
| Cat | 0.053 0.113 | 0.05–0.11 | 1.45 | 1.45–1.73 | Cerebral |
| ($n=2$) | | | 1.73 | | ALD |
| SOD | 0.40 0.58 | 0.40–0.58 | 1.70 | 1.70–1.85 | Cerebral |
| ($n=2$) | | | 1.85 | | ALD |

CAT: catalase (pmol/mg prot); SOD: superoxide dismutase (U/mg prot). Data represent the mean ± S.D. One unit is defined as 50% of produced chromogen inhibition for SOD.

Next, we examined the activities of the antioxidant enzymes CAT, GPx and SOD in erythrocytes and fibroblasts. It can be seen in Tables 3 and 4 that these activities were altered in both tissues from X-ALD patients. A moderate but significant increase (13%) of GPx activity [$t(6)=4.599$, $P<0.05$] was detected in erythrocytes (Table 3). It can be also seen that catalase and SOD activities were much greater (1887% and 262% increase, respectively) in fibroblasts from two cALD patients, as compared to controls (Table 4).

## 4. Discussion

Neurological symptoms and brain abnormalities are characteristic of patients affected by X-ALD. However, very little is known about the pathomechanisms involved in the tissue damage of this disorder. In the present study we investigated various parameters of oxidative stress in plasma, erythrocytes and cultured fibroblasts from X-ALD patients. We verified a significant increase of chemiluminescence and TBA-RS in the plasma from these patients. Considering that light emitted in the chemiluminescence assay usually arises from peroxidizing lipids due to an increase in reactive oxygen or nitrogen species production and that TBA-RS reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation [31], our data indicate that lipid peroxidation is induced in plasma of X-ALD, which probably occurs due to increased free radical generation.

It was also demonstrated that TRAP measurement was not altered in the patients plasma, indicating no alteration in the content of nonenzymatic antioxidant defenses. Since the major contributors of TRAP value in plasma are urate, plasmatic proteins, ascorbic acid and vitamin E, it is feasible that the concentrations of these substances are not modified in plasma from X-ALD patients. This should be, however, tested in future studies. In contrast, a significant decrease of TAR measurement was found in plasma of the affected patients, reflecting a deficient capacity to rapidly handle an increase of reactive species. It should be noted that TAR corresponds to a useful index of the capacity of a given tissue to modulate the damage associated with an increased production of free radicals and reflects not only the quantity of antioxidants (given by TRAP), but also, and particularly, its quality (given by its reactivity) [26]. Given the above results of increased lipid peroxidation, which indirectly reflects greater reactive species formation, it may be presumed that TAR measurement is a more sensitive marker of free radical production than TRAP in vivo. We have previously observed that TRAP measurement was not altered by in vitro 3-hydroxy glutaric acid in rat cerebral cortex whereas TAR was significantly decreased by the same doses of the metabolite [32], a fact that agrees with this hypothesis.

In this study we also demonstrated an increase of erythrocyte GPx activity and of catalase and SOD activities in fibroblasts from patients, as compared to the controls. The significant increase of these antioxidant enzyme activities may have been a response to high sustained levels of reactive species in X-ALD patients. The increase of catalase and GPX activity suggests that oxygen peroxide is formed in excess, whereas the increase of SOD suggests that superoxide is formed in excess. Peroxisomes contain many of the cellular enzymes that generate $H_2O_2$, such as glycolate oxidase, urate oxidase and flavoprotein dehydrogenases involved in the β oxidation of fatty acids, a metabolic pathway that operates in mitochondria and peroxisomes of animal tissues [33]. In peroxisomes the flavoproteins react with $O_2$ to give rise to $H_2O_2$. It seems logical therefore that these enzymes are packaged into an organelle with high capacity to destroy $H_2O_2$.

On the other hand, taken together our present data showing a significant increase of chemiluminescence and TBA-RS levels and a diminution of TAR in plasma, and considering that an unbalance between the total antioxidant defenses and the reactive species formed in the tissues is indicative of oxidative stress [34], it is proposed that free radical generation is involved in the pathophysiology of the tissue damage found in X-ALD. Our results on the alterations of the antioxidant enzymatic activities in erythrocytes and fibroblasts strongly corroborate with this presumption.

It should be pointed out that most assays were performed using specimens exclusively from cALD patients, so that conclusions from the results of these assays should be directed to this phenotype. However, as far as TAR and TBA-RS measurements are concerned, we used samples from cALD and AMN patients. In this case, although the individual values do not appear in the tables, there was no visual difference between these values obtained from the two distinct phenotypes, suggesting that oxidative stress also occurs in the AMN phenotype. However, further experiments should be conducted to clarify this matter.

At this point, it should be emphasized that the brain has low antioxidant defenses compared with other tissues [35], a fact that makes this tissue more vulnerable to increased reactive species. Mounting evidence has shown that oxidative stress has been implicated in the pathophysiology of common neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease, multiple sclerosis as well as in

epileptic seizures and demyelination [19,35–37]. Therefore, considering that free radicals are also produced in the brain similarly to what occurs in plasma, as here demonstrated, and that the antioxidant defenses are reduced in this tissue as occurred in fibroblasts and erythrocytes, it may be presumed that oxidative stress may compromise the brain, in X-ALD similarly to what occurs in the above mentioned neurodegenerative disorders.

Our results should be taken with caution and confirmed with a higher number of patients and with other techniques to measure oxidative stress since we used specimens from only a few patients. In this context, CSF specimens maybe useful to evaluate whether brain is also a target for reactive species. If they are confirmed, we may conclude that oxidative stress contributes at least in part to the pronounced neurological dysfunction found in X-ALD. In this scenario, it has been proposed that the neurological damage that occurs in X-ALD could be mediated by astrocyte activation and cytokine pro-inflammatory induction. Since free radicals are also involved in inflammatory response, our findings and these observations may reflect the same phenomenon. Finally, since so far no therapy for X-ALD has been proven to be successful, it may also be presumed that the administration of antioxidants should be considered as a potential therapy for the patients affected by X-ALD.

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## References

[1] H.W. Moser, Lorenzo's oil, Lancet 341 (1993) 544.
[2] G.C. Korenke, D.H. Hunneman, J. Kohler, S. Stöcker, K. Landmark, F. Hanefeld, Glyceroltrioleate/glyceroltrierucate therapy in 16 patients with X-chromosomal adrenoleukodystrophy/adrenomyeloneuropathy: effect on clinical, biochemical and neurophysiological parameters, Eur. J. Pediatr. 154 (1995) 64–70.
[3] C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, The Metabolic and Molecular Bases of Inherited Disease, 8th ed., McGraw-Hill, New York, 2001.
[4] H.W. Moser, Peroxisonal diseases, in: H. Harris, K. Hirschhorn (Eds.), Advances in Human Genetics, vol. 21, Plenum, New York, 1993, pp. 1–106.
[5] R.J.A. Wanders, C.W.T Van Roermund, W. Lageweg, B.S. Jakobs, R.B.H. Schutgns, A.A. Nijenheis, J.M. Tager, X-linked adrenoleukodystrophy: biochemical diagnosis and enzyme defect, J. Inherit. Metab. Dis. 15 (1992) 634–644.
[6] R.J.A. Wanders, H.S. Heymans, R.B.H. Schutgens, H. Van Den Bosch, J.M. Tager, Peroxisomal disorders in neurology, J. Neurol. Sci. 88 (1988) 1–39.
[7] R.J.A. Wanders, R.B.H. Schutgns, P.G. Barth, Peroxisomal disorders, in: N. Blau, M. Duran, M.E. Blaskovics (Eds.), Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases, Chapman & Hall, London, 1996, pp. 359–376.
[8] H.W. Moser, A. Bergin, S. Naidu, P.W. Ledenson, Adrenoleukodystrophy: news aspects of adrenal cortical disease, in: D.H.E. Nelson (Ed.), Endocrinology and Metabolism Clinics of North America: New Aspects of Adrenal Cortcal Disease, vol. 20, Saunders, Philadelphia, 1991, pp. 297–318.
[9] R. Koike, S. Tsuji, T. Ohno, Y. Suzuki, T. Orri, T. Miyatake, Physiological significance of fatty acid elongation system in adrenoleukodystrophy, J. Neurol. Sci. 103 (1991) 188–196.
[10] H.W. Moser, A.B. Moser, K.D. Smith, A. Bergin, A.J. Borel, J. Stine, O.C. Stine, C. Merette, J. Ott, W. Krivit, E. Shapiro, Adrenoleukodystrophy: phenotypic variability and implications for therapy, J. Inherit. Metab. Dis. 15 (1992) 645–664.
[11] R.J.A. Wanders, P.G. Barth, R.B.H. Schutgens, J.M. Tager, Clinical and biochemical characteristics of peroxisomal disorders: an update, Eur. J. Pediatr. 153 (Suppl 1) (1994) S44–S48.
[12] R.J.A. Wanders, R.B.H. Schutgens, P.G. Barth, Peroxisomal disorders: a review, J. Neuropathol. Exp. Neurol. 17 (1995) 726–739.
[13] H.W. Moser, Adrenoleukodystrophy: natural history, treatment and outcome, J. Inherit. Metab. Dis. 18 (1994) 435–447.
[14] M. Ruiz, T. Pampols, M. Girós, Glycerol trioleate/glycerol trierucate therapy in X-linked adrenoleukodystrophy: saturated and insaturated fatty acids in blood cells. Implications for the follow-up, J. Inherit. Metab. Dis. 19 (1996) 188–192.
[15] H.W. Moser, G.V. Raymond, W. Köhler, P. Sokolowski, A. Moser, G.C. Korenke, A. Greene, D.J. Loes, D.F. Hunnemann, R.O. Jones, S.E. Lu, G. Uziel, F. Roels, Evaluation of the preventive effect of glyceryl trioleate-trierucate (''Lorenzo's Oil'') therapy in X-linked adrenoleukodystrophy, Results of two concurrent studies, United Leukodystrophy Foundation News 19 (2002) 3–4.
[16] I. Singh, M. Khan, L. Key, S. Pai, Lovastatin for X-linked adrenoleukodystrophy, N. Eng. J. Med. 339 (10) (1998) 702–703.
[17] I. Singh, K. Pahan, M. Khan, Lovastatin and sodium phenylacetate normalise the levels of very long chain fatty acids in skin fibroblasts of X-adrenoleukodystrophy, FEBS Lett. 426 (1998) 342–346.
[18] B. Halliwell, Free radicals, antioxidants, and human disease: curiosity, cause or consequence? Lancet 344 (1994) 721–724.
[19] A.Z Reznick, L. Packer, Free radicals and antioxidants in muscular neurological diseases and disorders, in: G. Poli, E. Albano, M.U. Dianzani (Eds.), Free Radicals: from Basic Science to Medicine, Birkhäuser, Basel, 1993, pp. 425–437.
[20] S. Przedborski, D.B.S. Donaldson, M. Jakowec, J.S. Kish, M. Guttman, G. Rosoklija, A.P. Hays, Brain superoxide dismutase, catalase and glutathione peroxidase activities in amyotrophic lateral sclerosis, Ann. Neurol. 39 (1996) 158–165.
[21] E. Bem-Menachem, R. Kyllerman, S. Markleind, Superoxide dismutase and glutathione peroxidase function in progressive myoclonus epilepsies, Epilepsy Res. 40 (2000) 33–39.
[22] H.W. Moser, A.B. Moser, Measurement of saturated very long chain fatty acid in plasma, in: F.A. Hommes (Ed.), Techniques in Diagnostics Human Biochemical Genetics, Wiley-Liss, New York, 1991, pp. 177–191.
[23] J.A Coelho, R. Giugliani, Fibroblasts of skin fragments as a tool for the investigation of genetic diseases: technical recommendations, Genet. Mol. Biol. 23 (2) (2000) 269–271.
[24] B. Gonzalez-Flecha, S. Llesuy, A. Boveris, Hydroperoxide-initiated chemiluminescence: an assay for oxidative stress in biopsies of heart, liver and muscle, Free Radic. Biol. Med. 10 (1991) 93–100.
[25] H. Esterbauer, K.H. Cheeseman, Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal, Methods Enzymol. 186 (1990) 407–421.
[26] E. Lissi, M. Salim-Hanna, C. Pascual, M.D. Del Castillo, Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements, Free Radic. Biol. Med. 18 (1995) 153–158.
[27] E. Lissi, C. Pascual, M.D. Del Castillo, Luminol luminescence induced by 2,2′ -azo-bis-(2-amidinopropane) thermolysis, Free Radic. Res. Commun. 17 (1992) 299–311.
[28] H. Aebi, Catalase in vitro, Methods Enzymol. 105 (1984) 121–126.

[29] A. Wendel, Glutathione peroxidase, Methods Enzymol. 77 (1981) 325–332.
[30] O.H. Lowry, N.J. Rosebrough, A. Lewis-Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[31] B. Halliwell, J.M.C Gutteridge (Eds.), Free radicals in Biology and Medicine, 3rd ed., Oxford University Press, Oxford, 2001.
[32] A. Latini, R.B. Rosa, K. Scussiato, S. Llesuy, A. Belló-Klein, M. Wajner, 3-Hydroxyglutaric acid induces stress and decreases the antioxidant defences in cerebral cortex of young rats, Brain Res. 956 (2002) 367–373.
[33] B. Halliwell, J.M.C. Gutteridge, Antioxidant defenses: glutathione metabolism, in: B. Halliwell, J.M.C. Gutteridge (Eds.), Free radicals in Biology and Medicine, Oxford University Press, Oxford, 2001, pp. 146–161.
[34] J.B Schulz, J. Lindenau, J. Seyfried, J. Dichgans, Glutathione, oxidative stress and neurodegeneration, Eur. J. Biochem. 267 (2000) 4904–4911.
[35] B. Halliwell, J.M.C. Gutteridge, Oxygen radicals and nervous system, Trends Neurosci. 8 (1996) 22–26.
[36] E. Méndez-Álvarez, R. Soto-Otero, A. Hermida-Aeijeiras, A.M. López-Real, J.L. Labandeira-García, Effects of aluminium and zinc on the oxidative stress caused by 6-hydroxydopamine autoxidation: relevance for the pathogenesis of Parkinson's disease, Biochim. Biophys. Acta 1586 (2001) 155–168.
[37] E. Karelson, N. Bogdanovic, A. Garlind, B. Winblad, K. Zilmer, T. Kullisaar, T. Vihalemm, C. Kairane, M. Zilmer, The cerebrocortical areas in normal brain aging and in the Alzheimer's Disease: noticeable difference in the lipid peroxidation level and in antioxidant defense, Neurochem. Res. 26 (2001) 353–361.