Free Radical Biology and Medicine 88 (2015) 18–29

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Oxidative stress, mitochondrial and proteostasis malfunction in adrenoleukodystrophy: A paradigm for axonal degeneration

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ARTICLE INFO

Article history:
Received 2 February 2015
Received in revised form
7 April 2015
Accepted 26 May 2015
Available online 12 June 2015

Key words:
X-linked adrenoleukodystrophy
Oxidative damage
Mitochondrial dysfunction
Peroxisome
Very long-chain fatty acids
Proteasome
Autophagy

ABSTRACT

Peroxisomal and mitochondrial malfunction, which are highly intertwined through redox regulation, in combination with defective proteostasis, are hallmarks of the most prevalent multifactorial neurodegenerative diseases—including Alzheimer's (AD) and Parkinson's disease (PD)—and of the aging process, and are also found in inherited conditions. Here we review the interplay between oxidative stress and axonal degeneration, taking as groundwork recent findings on pathomechanisms of the peroxisomal neurometabolic disease adrenoleukodystrophy (X-ALD). We explore the impact of chronic redox imbalance caused by the excess of very long-chain fatty acids (VLCFA) on mitochondrial respiration and biogenesis, and discuss how this impairs protein quality control mechanisms essential for neural cell survival, such as the proteasome and autophagy systems. As consequence, prime molecular targets in the pathogenetic cascade emerge, such as the SIRT1/PGC-1α axis of mitochondrial biogenesis, and the inhibitor of autophagy mTOR. Thus, we propose that mitochondria-targeted antioxidants; mitochondrial biogenesis boosters such as the antidiabetic pioglitazone and the SIRT1 ligand resveratrol; and the autophagy activator temsirolimus, a derivative of the mTOR inhibitor rapamycin, hold promise as disease-modifying therapies for X-ALD.

Contents

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http://dx.doi.org/10.1016/j.freeradbiomed.2015.05.041

1. Introduction: Peroxisome biology

First described by Rhodin in 1954 and then isolated by de Duve in 1966, the peroxisome is an organelle derived from the endoplasmic reticulum [1,2]. In contrast to mitochondria, they have single rather than double membranes, and lack DNA. They are essential organelles of eukaryotic origin, ubiquitously distributed in cells and organisms, playing key roles in multiple metabolic pathways including degradation of long-chain and very long-chain fatty acids (LCFA, VLCFA ($C \geq 22{:}0$)) by α- or β-oxidation; hydrogen peroxide detoxification; degradation of amino acids, methanol and purines; or in the synthesis of bile acids and essential polyunsaturated fatty acids. Loss or malfunction of peroxisomes causes more than 20 fatal inherited conditions including Zellweger's syndrome and X-linked adrenoleukodystrophy (X-ALD) (See also www.peroxisomedb.org) [3,4]. Classical hallmarks of peroxisomal dysfunction with pathogenic implications are an excess of VLCFA, which generates oxidative stress and mitochondrial toxicity and will be addressed in detail in this review; a decrease of the ω3-polyunsuaturated fatty acid: C22:6ω3 (DHA), and a decrease in ether lipids called plasmalogens, both synthetized in peroxisomes, and bearing neuroprotective, neurotrophic and antioxidant functions [5–7]. Human peroxisomes contain 85 proteins, such as the peroxins (PEX) which are involved in peroxisome biogenesis and dynamics; three ATP-binding cassette transporters subfamiliy D or ABCD transporters (ABCD1, ABCD2 and ABCD3), which import fatty acids into the peroxisome which will undergo a process of synthesis or degradation through beta-oxidation; and several matrix enzymes which catalize these processes. A complex PEX interaction network controls peroxisome biogenesis (Pex23, Pex24, Pex25, Pex27, Pex28, Pex29, Pex30, Pex31 and Pex32); fission (Pex11,Pex25, Pex27 and Pex34); formation of peroxisomal membrane from the ER (Pex1, Pex3, Pex6, Pex16, Pex19, Pex23, Pex25 and Pex30); targeting of matrix proteins (Pex5, Pex7, Pex18, Pex20 and Pex21), matrix protein import (Pex1, Pex2, Pex4, Pex5, Pex6, Pex8, Pex10, Pex12, Pex13, Pex14, Pex15, Pex17, Pex22, Pex26 and Pex33) and direct targeting of peroxisomal membrane proteins (Pex3 and Pex19) [3,8,9]. The ABCD transporters serve as active uptake of mainly very long-chain fatty acids (VLCFA) and VLCFA-CoA for ABCD1 [10–12]; of long-chain saturated and omega9-monounsaturated fatty acids, and precursors of docosahexanoic acid (DHA) for ABCD2 [13,14]; and ofbranched-chain fatty acids and C27 bile acids for ABCD3 [15]. These transporters must form homo or heterodimers to function [16]. The high degree of homology of the three ABCD transporters accounts for overlapping fatty acid specificities when overexpressed [17,18]; Lately, interactions of ABCD1 with proteins involved in fatty acid synthesis (FASN, ACLY, ACC) and activation (FATP4) have been described, which suggests that a novel fatty acid synthesis-transport complex functions at the cytoplasmic side of the peroxisomal membrane [19].

2. X-linked adrenoleukodystrophy and redox homeostasis

2.1. The disease

X-ALD is the most common peroxisomal disorder and monogenic leukodystrophy (OMIM 300100). Patients suffer from central inflammatory demyelination in the brain and/or slowly progressing axonal degeneration of the corticospinal tracts, resulting in spastic paraparesis [20–22]. X-ALD is caused by mutations in the *ABCD1* gene (Xq28), which encodes the ATP-binding cassette transporter, an integral peroxisomal membrane protein. The ABCD1 protein imports very long-chain fatty acids and VLCFA-CoA esters into the peroxisome for degradation [11,12]. The defective function of the ABCD1 transporter leads to VLCFA excess due to decreased β-oxidation of these fatty acids in peroxisomes [12,13], leading to a disturbed fatty acid profile in tissues and plasma, particularly involving hexacosanoic acid (C26:0) and C26:0-containing lipids such as lysophosphatidilcholine. These are used as pathognomonic markers for the biochemical diagnosis of X-ALD [23,24].

Despite being a single-gene disease, X-ALD is a complex inherited syndrome in which the same mutation in the *ABCD1* gene can lead to clinically very distinct phenotypes [20–22,25]. Loss-of-function mutations in this protein are the cause of the disease, which is characterised by three main distinct phenotypes. Cerebral childhood ALD (cALD), which affects boys between 5 and 12 years of age, manifests in ~35% of all X-ALD patients and exhibits strong inflammatory demyelination with autoimmune components, leading to death within few years. AMN is the most frequent manifestation of X-ALD and affects adult patients. AMN patients present peripheral neuropathy and distal axonopathy involving corticospinal tracts of the spinal cord —but not brain neuroinflammation or major demyelination—with spastic paraparesis as major symptoms. Intermediate forms with non-inflammatory childhood cerebral demyelination occur, as do mixed forms, with around 20% of all AMN patients developing a cerebral inflammatory disease at later stages than in cALD. A majority of patients additionally suffer from adrenal insufficiency [20–22,25]. Interestingly, all clinical phenotypes can occur within the same family, indicating that there is no direct phenotype–genotype correlation [20–22,25]. Female carriers frequently develop AMN, with symptoms such as myelopathy and peripheral neuropathy [26].

To date, the only treatment is allogeneic bone marrow transplantation, which is associated with a high morbidity and mortality and is available only to nearly-asymptomatic X-ALD children [27]. A gene therapy approach using corrected hematopoietic stem cells CD34+, has proved to be successful and a good alternative to transplant as it is less invasive [28]. This alternative treatment has the same indications as bone marrow transplantation, meaning that it can be applied only to a small subset of patients with few to no symptoms [29]. A novel gene therapy strategy uses adeno-associated virus serotype 9 to express human *ABCD1,* which has yielded promising results in a mouse model of X-ALD [30]. However, a recent report suggests that allogeneic bone marrow transplant in cALD does arrest inflammatory progression and death, but does not prevent the adult AMN phenotype from developing AMN in adulthood [31].

Thus, for AMN patients there is no satisfactory treatment to date. Oral administration of "Lorenzo's oil" has been used, but its clinical efficacy and clinical indications have been controversial for more than 15 years [32]. Nonetheless, more recent successful pharmacological preclinical tests in the X-ALD mouse model warrant translation into the clinic [33–36].

2.2. Mouse models for X-linked adrenoleukodystrophy: Lessons learnt

The classical knockout of the murine *Abcd1* gene, the X-ALD mouse model, does not exhibit signs of demyelination in spite of

the accumulation of VLCFA in the brain [37]. This mouse model shows instead a phenotype resembling the late-onset axonal degeneration in spinal cords presented by most AMN patients, characterised by spastic paraparesis due to degeneration of corticospinal tracts, but without signs of active inflammatory demyelination in the brain. In the mouse, mainly axonal pathology is detectable in sciatic nerves and long tracts of spinal cords, but not in the brain [38]. This is correlated with slower motor nerve conduction, and with disability signs in rotarod, treadmill, barcross and clasping tests, of very late onset around 20 months of age [18,34–36,39]. Thus, the phenotype of *Abcd1*$^{-}$ mice mimics features of human AMN, providing a model for investigating disease pathogenesis. Also, this model provides evidences of axonal degeneration prior to myelin degeneration, an important finding that strongly suggests that the underlying phenotype in the human disease is AMN, with full penetrance at 50 years of age [40].

We generated double-mutants of *Abcd1* and its closest homolog, the *Abcd2* gene, which exhibits overlapping biochemical and physiological functions with *Abcd1* [13,41–44], and is able to compensate for *Abcd1* loss *in vivo* [40]. *Abcd2* is thus a prime therapeutic target [45–48]. These double mutant mice exhibited a more pronounced and earlier-onset axonal degenerative phenotype, manifesting at around 12 months of age, what makes them more suitable for therapeutic assays [18]. Both *Abcd1*$^{-}$ and *Abcd1*$^{-}$/*Abcd2*$^{-/-}$ mice are *bona fide* models of mild, late-onset axonopathy exhibited by AMN patients. These models are instrumental in dissecting pathomechanisms of AMN [49–53] and importantly, in pinpointing and evaluating tailored therapies [30,33–36,54].

It is worth mentioning that a non-negligeable degree of chronic neuroinflammation with astrocytosis and microgliosis is present in spinal cords of both, the *Abcd1-null* and more markedly, in the double *Abcd1/Abcd2* mouse model. Moreover, a functional genomics approach carried out on the *Abcd1 null* model, has unraveled a signature characterized by oxidative stress, mitochondrial dysregulation, adipocytokine signaling, and chronic inflammation routes, including NFκB activation with production of proinflammatory cytokines detected in spinal cords and plasma [53]. These findings challenge the notion that inflammatory reactions only occur in the most severe forms of X-ALD, and provide a basis to argue that a low-grade proinflammatory reaction initiated by VLCFA and present in the mouse models and in AMN patients, may adopt a more aggressive profile should a second (or third or several) hit appear [55]. These additional modifier genes or epigenetic, environmental or stochastic events that may ignite a full-blown cerebral inflammatory demyelination rassembling cALD or cAMN, have not been identified as of today. Thus, a suitable animal model that serves as a tool for the development of therapeutic strategies for the lethal stages of this disorder is still missing.

2.3. Oxidative damage and mitochondrial impairment in X-ALD

2.3.1. Early redox imbalance in X-ALD

Oxidative stress is defined as a pathological condition that results from a redox disequilibrium leading to an excess of ROS, which is not adequately neutralized by the cellular antioxidant defenses. A direct consequence of excessive ROS production is interaction with cellular biomolecules — in particular DNA, lipids, and proteins — which are then modified. Proteins can be directly damaged by ROS, or indirectly by reaction with active aldehyde products of lipid peroxidation — e.g. malondialdehyde (MDA) or hydroxynonenal — or as a result of alterations in membrane lipid microenvironment secondary to peroxidative processes. These changes are detrimental if the modified residues are essential for the protein's activity or turnover [56]. Moreover, oxidative lesions can also transform lipid properties and induce mutations in mitochondrial and nuclear DNA, causing mitochondrial dysfunction and pathology [57].

Taking advantage of the *Abcd1*$^{-}$ mouse model, and keeping track of the data obtained with expression microarrays [53], we uncovered an early-onset oxidative damage, which may be a major contributor to disease pathogenesis in the mouse [33,49,55,58,59]. This is a common feature to neurodegenerative diseases and aging, and is found in X-ALD patients in brain tissue as well as in peripheral cells or fluids such as fibroblasts, erythrocytes, peripheral mononuclear cells and plasma [46,58–62].

Abcd1^{-} mice present accumulation of MDAL (malonaldehydelysine), a consequence of lipoxidative damage to proteins, in spinal cords as early as 3.5 months of age. At 12 months, *Abcd1*$^{-}$ mice accumulate additional oxidative damage products arising from metal-catalyzed oxidation and glycoxidation/lipoxidation to proteins. We also detected several oxidatively modified proteins, mostly belonging to Krebs cycle, oxidative phosphorylation (OXPHOS) and to glycolysis [52,63]. This was associated with decreased activities of pyruvate kinase, and lowered levels of ATP and NADH, as well as reduced glutathione (GSH) [63]. Finally, a wide array of mitochondrial genes related to the pyruvate and oxoglutarate dehydrogenase complexes, tricarboxylic acid (TCA) cycle, OXPHOS system and antioxidant defenses were found dysregulated in the functional genomics analysis [53].

Thus, in X-ALD we find a prime example of intertwined redox and metabolic homeostasis in the nervous system. Diminished levels of ATP have also been described in AD and PD mouse models [64,65], although the ratios of NAD/NADH, of paramount importance, have not been systematically measured in most prevalent neurodegenerative diseases.

Importantly, we observed that the agent generating redox imbalance was the raised levels of VLCFA. Indeed, when in excess, C26:0 is able to generate ROS and decrease mitochondrial membrane potential in human X-ALD fibroblasts [49]. Similar studies showed that VLCFA was able to depolarize mitochondria and increase ROS in immortalized or primary neural cell cultures [66–69].

We thus tested a combination of carefully selected anti-oxidants — N-acetylcysteine (NAC), α-lipoic acid (LA) and vitamin E — which showed synergistic effects *in vitro*. Lipoic acid is of particular interest as it is at the same time a ROS scavenger [70], and a cofactor of the Krebs cycle enzyme α-keto-glutarate-dehydrogenase (αKGDH) [71], which is oxidized in X-ALD [63]. Lately, lipoic acid has been shown to rescue the hypometabolic state in a mouse model of AD, characterized by low levels of glutamine, glutamate, aspartate and N-acetylaspartate, metabolites of the TCA cycle [72]. We thus treated the X-ALD mice with the cocktail above mentioned, and obtained a neutralization of oxidative stress and lesions to proteins; preserved bioenergetic homeostasis maintaining ATP and NADH levels by recovering normal levels of oxidation on key proteins of Krebs cycle and glycolysis; halted signs of axonal degeneration in immunohistological stainings; and prevented locomotor impairment in bar-cross and treadmill tests [33–36].

These results provide conceptual proof of C26:0 –induced oxidative stress as a major causative disease-driving factor in X-ALD, thus warranting translation into clinical trials for X-AMN patients and inviting assessment of antioxidant strategies in other diseases with axonal degeneration in which oxidative damage may play a role. A word of caution is however in order as several trials with antioxidants have failed in AD, PD or ALS diseases [73,74]. Among the causes for this may be that the magnitude of neuronal loss and synaptic dysfunction present by the time the full clinical syndrome is evident, could overwhelm the functional benefits brought by the treatment. However, the treatment with idebenone, a synthetic analog of CoQ10, has shown promise in patients with Leber hereditary optic neuropathy (LHON) [75–78], mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS) [76,79], OPA1-mutant dominant optic atrophy [80] or Friedreich ataxia (FRDA) [81–85], constituting a good proof-of-principle accumulative evidence. Based on the results

with CoQ10 and idebenone, the novel mitochondrial antioxidant EPI-743 has been synthesized, which is 1000 to 10000 fold more potent than CoQ10 [73]. EPI-743 is a para-benzoquinone which acts augmenting the synthesis of glutathione and optimizing metabolic control by regulating electron transport at the mitochondria. It has been shown to be efficacious at ameliorating symptoms in several inherited mitochondria disease entities [86–88], and will be tested in a phase II clinical trial on AMN patients starting in 2015.

2.3.2. NOXIOUS eFFECTS ON MIToCHONDRIA exerTED BY eXCESS OF c26:0

A deficient redox homeostasis linked to mitochondrial dysfunction is at the core of axonal degeneration in several neurodegenerative diseases [89,90], with a strong correlation between the accumulation of damaged mitochondria with disease progression [91,92]. Mitochondria are both very sensitive to oxidative damage and the main producers of ROS, in particular when the mitochondrial electron transport chain (ETC) is malfunctioning. Although the major ROS producer in mitochondria are ETC (Complex I, and III) [92,93], other sites of mitochondrial ROS production have been identified. Among these are $p66^{Shc}$ and several enzymes such as NADPH oxidase, monoamine oxidase, uncoupled neuronal NO synthase, aconitase, electron transfer flavoprotein (ETF) and ETF quinone oxidoreductase (ETF dehydrogenase), dihydro-orotate dehydrogenase, α-glycerophosphate dehydrogenase, α-ketoglutarate dehydrogenase complex (KGDHC) and pyruvate dehydrogenase complex (PDHC) [92,93]. Notably, several subunits of complex III and IV [52], and of KGDHC and PDHC are oxidized [63] in the *Abcd1* null mouse, which presumably leads to malfunction and most likely, to elevated production of free radicals from these sites. In axons, mitochondria provide a substantial part of the energy required during neurotransmission, and their transport towards ATP-demanding areas is tightly regulated. Nonetheless, axonal mitochondria are more vulnerable than their cellular counterparts, as mitochondria require from an elongated shape to fit with the axon morphology, and thus, are more reliant on fusion-fission events to exchange material among them. An imbalance in the triad ROS/ATP/calcium that overcomes the homeostatic capacity of the mitochondria initiates a toxic cycle of events that will ultimately activate the axonal degeneration program. For instance, mitochondrial ROS may oxidize and inhibit the OXPHOS system, inducing a decline in the ATP production. This energetic failure, in its turn, impairs the ATP-dependent ionic pumps, triggering a massive calcium influx. Furthermore, ROS do not only impair the OXPHOS system but also promote the opening of the mtPTP leading to a subsequent efflux of calcium from the mitochondria to the cytosol. Calcium overload in the cytoplasm triggers then calcium-dependent activation of calpains. These proteases disassemble the microtubule structure and impair the correct transport along the axon. All these events will result in axonal swelling, loss of axonal continuity and eventually, axonal destruction [94].

In X-ALD, a very similar scenario takes place with unbalanced ROS/ATP/Ca^{2+} homeostasis [95]. Mitochondria appear to be the main source of ROS in X-ALD fibroblasts when cells are exposed to an excess of C26:0, as determined through measuring intramitochondrial ROS by MitoSOX or by using mitochondrial uncouplers. Excess of C26:0 also inhibits OXPHOS in fibroblasts [52]. Defective OXPHOS was also observed in both 158 N oligodendrocytal cells and SK-NB-E neuronal cells when exposed to a VLCFA excess [66,69], and in isolated brain mitochondria, which increase ROS production, and diminished Ca^{2+} uptake capacity [95]. However, we cannot rule out the intriguing possibility that the observed ROS production by mitochondria is provoked by signals originating in peroxisomes. Indeed, the redox status of mitochondria is modified in catalase-deficient cells and when oxidative stress is generated in peroxisomes, indicating a direct cross-talk between these two organelles on redox homeostasis, as discussed later [96–98]. Thus, a systematic assessment of additional cellular sources of ROS in X-ALD (peroxisomes, xanthine oxidase, NADPH oxidases) in relevant models for the disease, such as primary cultures of neural cells or, ideally, *in vivo* in the mouse nervous system, is warranted.

The molecular mechanisms by which the VLCFA excess triggers oxidative stress in mitochondria in X-ALD is far from clear. We hypothesize that excess of C26:0 could alter the permeability of the inner mitochondrial membrane, by replacing the lateral chains of phospholipid bilayers and physically interfering with the assembly or stability of OXPHOS system [52], or by increasing membrane microviscosity [99] and provoking its disruption [100]. This could elicit electron leakage, promoting ROS generation from the ETC. In the same manner, mitochondrial inner membrane instability could allow the proton flux into the matrix, decreasing mitochondrial inner membrane potential (ΔΨm) and possibly triggering OXPHOS dysfunction. Indeed, we revealed a defective respiration under uncoupled conditions both in X-ALD fibroblasts and in *Abcd1*$^-$ spinal cords, suggesting a specific impairment of the activity of complex V/H^+-ATP synthase, which correlates with the oxidation of several subunits of complex V and III of OXPHOS [52] (Fig. 1). A similar case has been recently reported for a mouse model of Zellweger syndrome, the PEX5 null mouse, which accumulates VLCFA as much as the X-ALD model does [101]. The authors show that assembly of OXPHOS is disturbed, along with membrane fluidity, leading to increased mitochondrial ROS production [102].

Recent advances in the study of the mitochondrial permeability transition pore (mPTP) indicate that dimeric ATP synthase is a *bona fide* component of the mPTP and interacts with the gate opener cyclophilin D (CypD) [103]. We have provided evidence of impaired mitochondrial metabolism in X-ALD fibroblasts, which cannot survive when forced to rely on mitochondrial energy production, i.e. on incubation in galactose [36,51,52]. Oxidative stress induced under galactose conditions leads to mitochondrial damage in the form of ΔΨm dissipation, ATP drop and necrotic cell death, together with increased expression and oxidative modifications in the CypD protein [51]. Thereby, we posit that in X-ALD, mPTP formation is favored by the increased amounts of oxidized CypD and Complex V/H^+-ATP synthase. Interestingly, inhibition of CypD genetically or pharmacologically with cyclosporine A prevents mitochondrial dysfunction in many age-related neurodegenerative disease models such as AD, PD, Amyotrophic Lateral Sclerosis (ALS) and MS, indicating that CypD is a prime therapeutic target for these disorders [104,105]. Notably, treatment with antioxidants normalizes the signs of mitochondrial damage in fibroblasts from X-ALD patients, including CypD oxidative modifications, and has reversed CypD induction in other neurodegenerative disorders *in vitro* and *in vivo* [51,52,104]. These findings provide mechanistic insight into the beneficial effects of antioxidants in neurodegenerative and non-neurodegenerative CypD-dependent disorders (Fig. 1).

2.3.3. Defective mitochondrial biogenesis in X-ALD: targeting the SIRT1/PGC-1α/PPARγ pathway

Besides malfunctioning of OXPHOS, significant mitochondrial depletion is found in the spinal cord of *Abcd1*$^-$ mice and in the affected white matter of X-ALD patients [35,53]. We unveil that *Abcd1*$^-$ mice show a 50% reduction in mitochondrial DNA copy number, concomitant with downregulation of the mitochondrial biogenesis pathway driven by PGC-1α/PPARγ, with diminished expression of the biogenesis factors NRF1 and TFAM, which negatively impact on the amounts of mitochondrial proteins such as cytochrome c, NDUFB8 and VDAC (Fig. 1). Moreover, an excess of VLCFA may play a

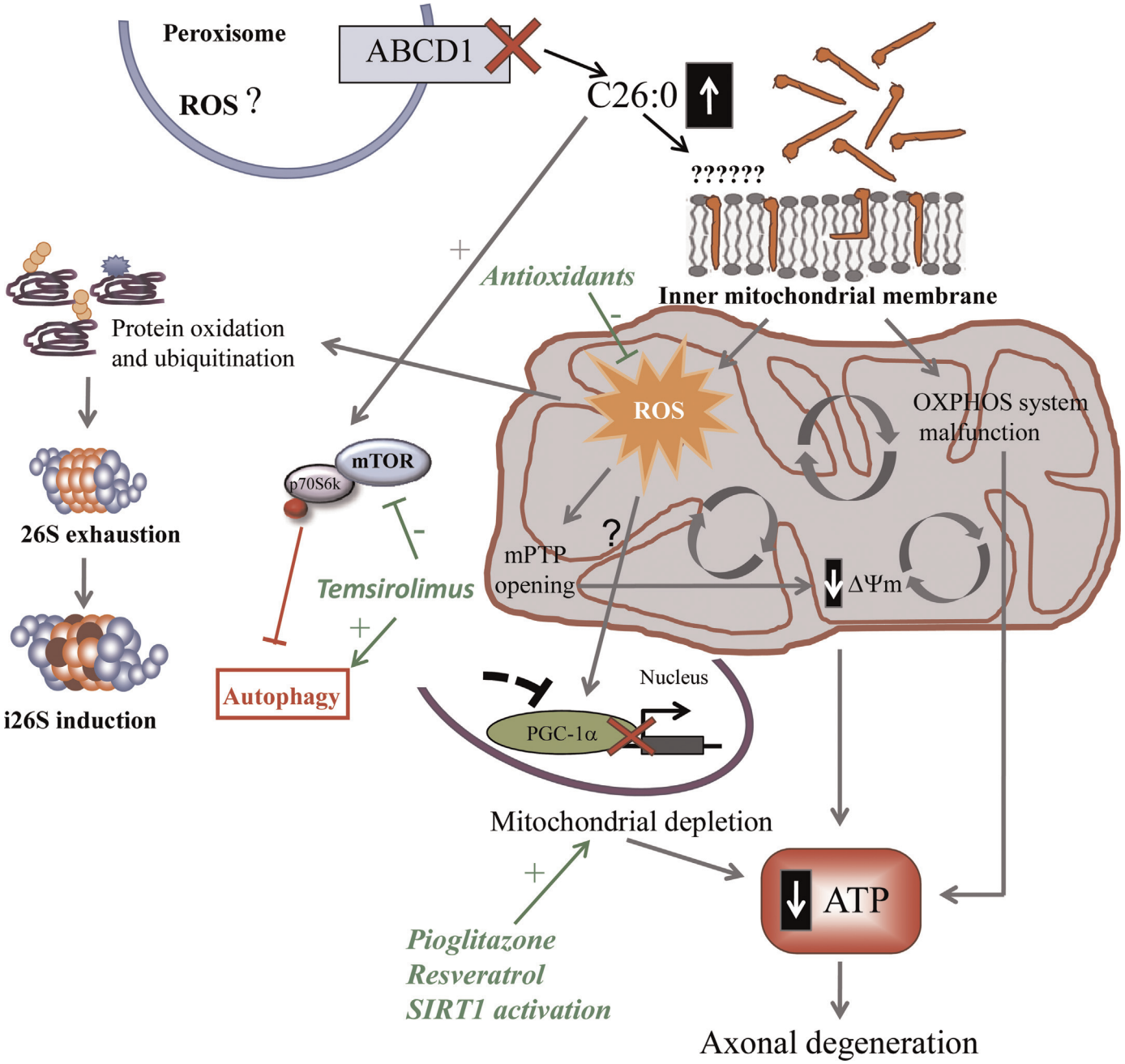

Fig. 1. Model recapitulating the noxious effects of C26:0 excess on mitochondria, redox homeostasis and proteolytic machineries in the X-ALD cell. In X-ALD, excess of C26: leads to the production of intramitochondrial ROS from the electron transport chain probably by an unknown mechanism. The consequences are mitochondrial dysfunction resulting in loss of ΔΨm and ATP production, mPTP opening, inhibition of mitochondrial biogenesis via the SIRT1/PGC-1α/PPARγ pathway, and ultimately axonal degeneration. VLCFA-dependent ROS inhibit UPS and autophagy process, with axonal degeneration as fatal outcome. The pathogenic cascade can be abrogated: i) by a combination of antioxidants; ii) by activating SIRT1 or boosting mitochondrial biogenesis with pioglitazone or resveratrol; iii) by activating autophagy *via* mTOR inhibition with temsirolimus. These results provide potential therapies to be translated into clinical trials for AMN patients.

role in this phenomenon, as subunits of complex III and IV of the ETC were decreased by C24:0 and C26:0, in the neuronal cell line SK-NB-E [69]. We believe that this reduction in mitochondrial amounts exerts an impact in the axonopathy exhibited in X-ALD for the reasons above mentioned. Indeed, deletion of TFAM is characterized by decrease of both mtDNA and OXPHOS activity, correlated to axonal degeneration and gliosis [106].

To compensate for the defective mitochondrial biogenesis, we sought to stimulate the PGC-1α/PPARγ axis with an agonist of PPARγ, thus treating the X-ALD mice with the thiazolidinedione pioglitazone, widely used as antidiabetic drug [107]. On a preclinical test, pioglitazone satisfactorily restored redox and bioenergetic homeostasis and halted axonal degeneration and associated locomotor disability [35] (Fig. 1). The data lend support to repurposing pioglitazone for clinical trials with AMN patients and reveal novel molecular mechanisms of action of pioglitazone in neurodegeneration. Pioglitazone has recently been granted orphan drug designation for the treatment of adrenoleukodystrophy (EU/3/14/1245), and holds great potential for clinical translation.

On a parallel approach, we targeted the sirtuin 1 gene (SIRT1), a protein deacetylase that activates PGC-1α, and thus controls mitochondrial content among other functions related to bioenergetics, redox homeostasis and inflammation [108,109]. We found that both transgenic overexpression of SIRT1 and a drug treatment by the

SIRT1 activator resveratrol, normalize mitochondrial content in spinal cords of *Abcd1-null* mice, along with restoring respiration, redox and bioenergetic homeostasis, while preserving axonal health and lomocotor capabilities [36] (Fig. 1).

The encouraging results obtained with pioglitazone and resveratrol do not however allow us to discriminate between the benefits of a purely antioxidant therapy and a second strategy combining both improved biogenesis and amelioration of oxidative damage, which are most likely directly linked in this model.

Alternative approaches to protect mitochondria may include the histone deacetylase inhibitor SAHA, which prevents energy failure, mitochondrial depolarization, decrease of mitochondrial amounts, along with normalizing antioxidant defences in astrocytes or oligodendrocytes where *ABCD1* has been silenced [110]. The mechanism presumably involves induction of *ABCD2*.

2.3.4. 2.3.4. Oxidative stress regulates proteostasis in X-ALD: UPS malfunction

The ubiquitin-proteasome system (UPS) is pivotal in the rapid clearance of damaged, misfolded or aggregated proteins in both healthy and diseased states, and has been shown to play a role in the degradation of oxidized proteins [111,112]. Conformational diseases involving proteasome malfunction such as polyglutamine diseases — Huntington's disease (HD) and several spinocerebellar ataxias, taupathies (e.g., AD and Pick's disease) and synucleinopathies (e.g., Lewy body disease) — are associated with oxidative stress, and in most cases oxidative damage arises very early in the course of the pathology [90,113]. Moreover, altered UPS activity has been observed in brain from AD, PD and ALS patients [114]. Further, selective lack of 26S proteasome subunits in sustantia nigra results in a PD-like phenotype, whereas mice with depletion of 26S in spinal motor neurons exhibit an ALS-like phenotype [114], underscoring the central role of proteasomes in neurodegeneration. In X-ALD, we have detected chronic and progressive malfunctioning of the ubiquitin-proteasome system resulting from the accumulation of oxidatively modified proteins, some involved in bioenergetic metabolism. Notably, the immunoproteasome (i-proteasome) machinery appears, in the absence of overt inflammation, upregulated in response to redox imbalance. I-proteasomes are recruited to mitochondria when fibroblasts are exposed to an excess of C26:0, as adaptive action to oxidative stress. Finally, antioxidant treatment regulates proteasome expression while normalizing its function, preventing i-proteasome induction and its translocation to mitochondria (Figs. 1 and 2). Our findings support a key role of i-proteasomes in quality control of mitochondria during oxidative damage in X-ALD [50,53]. This is consistent with data obtained from a mouse model with defects in the complex I subunit Ndufs4, the *Ndufs4*$^{-/-}$ mouse, which exhibits a decrease of UPS activity [115]. UPS function is also impaired in fibroblasts from patients with mutations in complex I and complex IV of OXPHOS [116]. Moreover, rotenone and antimycin, inhibitors of OXPHOS complex I and III respectively, repress proteasome

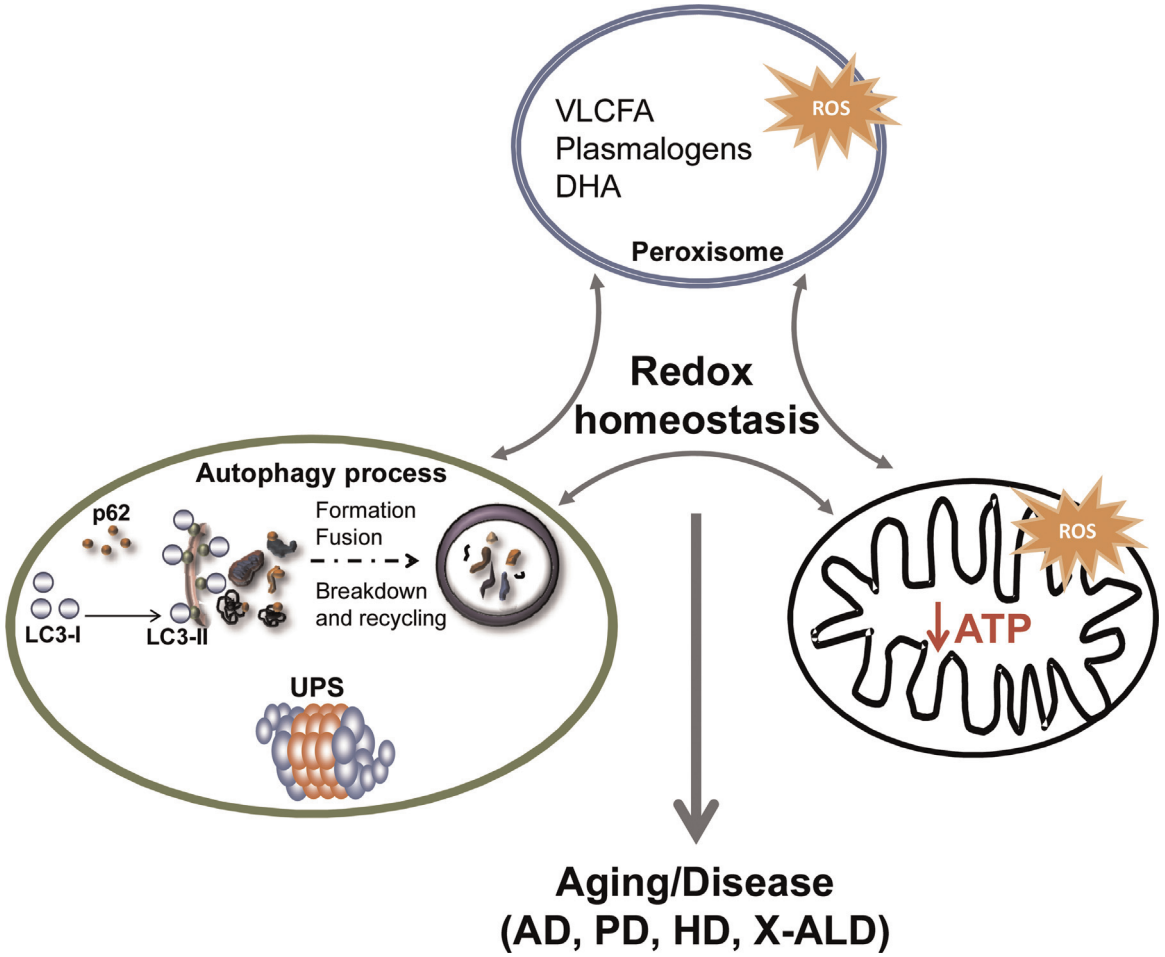

Fig. 2. Interplay of peroxisomes, mitochondria, UPS and autophagy in redox homeostasis. Peroxisomes, mitochondria, the UPS and autophagy machineries play a major role in cellular redox homeostasis. Peroxisome and mitochondria are directly responsible for ROS generation and ROS scavenging mechanisms, whereas UPS and autophagy degrade altered and oxydized proteins and organelles. Imbalance of redox status impacts on proteostasis (UPS and autophagy), and regulates functions of both organelles such as mitochondrial and peroxisomal biogenesis. Thereby alteration of one this component leads to a strong disturbance of redox homeostasis which is associated to aging and aged-related disorders such as AD, PD, HD, MS and X-ALD.

expression and activity [117]. Altogether these data suggest that not only UPS gets clogged with an excess of oxidized proteins during chronic oxidative stress pathologies, but also that OXPHOS dysfunction and/or mitochondrial ROS directly shut down UPS activity.

2.3.5. Oxidative stress regulates proteostasis in X-ALD: Autophagy malfunction

Along with degradation by the proteasome, autophagy constitutes the major route for eliminating misfolded or modified intracellular proteins [118], and is often malfunctioning in neurodegenerative diseases [119]. We therefore reasoned that induction of autophagy in X-ALD could be a plausible strategy to compensate for proteasome's deficient activity by increasing clearance of oxidized proteins. However, using the *Abcd1*$^{-}$ mouse model, we unexpectedly detected an impairment of autophagolysosomal formation and autophagic flux in spinal cords. We found lesser autophagolysosomes, raised p62 and decreased LC3 II levels. The mechanism appeared to be an aberrant overactivation of the mammalian target of rapamycin (mTOR) pathway, mediated by VLCFA excess (Fig. 1). We thus used a specific activator of autophagy through inhibition of mTOR, the rapamycin ester temsirolimus [120,121]. This in anticancer drug which has been used in rodent models of AD and PD with positive results [122,123]. In our hands, temsirolimus successfully blocked mTOR signaling, restored autophagic flux and arrested axonal degeneration [29], with little to no unwanted effects [33]. Interestingly, temsirolimus action preserved proteasome function along the way, together with maintaining redox and metabolic homeostasis, with conserved ATP and NADH production [33] (Fig. 1). These findings offer first evidence linking impaired autophagy to X-ALD, thus providing an additional therapeutic approach to assay in clinical trials.

In a similar manner, the autophagy process is often malfunctioning in neurodegenerative diseases [118], as well as in senescence and aging, possibly related to mitochondrial oxidative stress [124]. Indeed, ROS produced at the mitochondrial OXPHOS sites have been described as inhibitors of autophagy [125]. For instance, rotenone decreases autophagic flux, causing lysosomal vacuoles to accumulate in SH-SY5Y cells [126], and antimycin also acts as an specific autophagy blocker, lowering LC3-II levels [127]. Alternative origins of ROS, such as NADPH oxidase in *mdx* mice, the model of Duchenne's muscular dystrophy, may as well provokes mTOR activation and subsequent impaired autophagy [128].

On the other hand, an intriguing link between peroxisomes, oxidative stress and autophagy has been recently described. Peroxisomal ROS has been shown to suppress mTORC1 activity, in models of the tuberous sclerosis complex signaling node TSC1 and TSC2 proteins [129] (Fig. 2). These proteins are located in peroxisomes and bind to the import factors PEX19 and PEX5. This invites careful evaluation of the sites of ROS production in the different neural cell types in X-ALD.

Chronic oxidative stress is a hallmark of aging and may be a major contributing factor to the well-known decline of autophagy in aging, and thus can be a major culprit in cellular accumulation of waste and aggregated proteins in neurodegenerative disorders.

3. Role of peroxisomes in redox status and neurodegeneration

3.1. PeroxisomE-Mitochondrial CROSS TALK ON REDOX REGULATIOn AND beyond

We have previously discussed recent evidence indicating that oxidative damage and mitochondrial malfunction are major culprits in the pathogenesis of X-ALD, as an example of cross-talk between these organelles (Fig. 2) [58]. The concept is not novel. Besides morphological abnormalities in mitochondria found in patients suffering from the adult form of X-ALD, adrenomyeloneuropathy (AMN) [130,131], additional evidence of mitochondrial abnormalities or dysfunction in peroxisomal disease have been reported in Zellweger syndrome [132,133]; in patients with mutations in the HSD17B4 gene, which encodes for the peroxisomal D-bifunctional enzyme [134]; and in patients suffering from the mistargeting of peroxisomal EHHADH (peroxisomal L-bifunctional enzyme). The latter enzyme disrupts mitochondrial metabolism (decreasing OXPHOS activities) by interacting with the mitochondrial trifunctional proteins involved in mitochondrial fatty acid β-oxidation, HADHA and HADHB (hydroxyacyl-CoA dehydrogenase–3-ketoacyl-CoA thiolase–enoyl-CoA hydratase A and B) [135]. Similar morphological abnormalities have been reported in spinal motorneurons from *Abcd1*$^{-}$ and *Abcd2*$^{-/-}$ mice [41], in *Abcd1*$^{-}$ adrenocortical cells [136], in multiple *Pex5*$^{-/-}$ tissues [102,137,138], in the liver of *Pex2*$^{-/-}$ [139], in the cerebellum of *Pex13*$^{-/-}$ mice [140], and in the *Pex5* and *Pex7*-deficient strains of *Podospora Anserina*, suggesting a direct impact on mitochondria when peroxisomes are defective.

Beyond sharing metabolic functions in the β-oxidation of fatty acids, glyoxylate cycle and thermogenesis, these organelles exert common functions in redox homeostasis [141]. Catalase is the best known enzyme in peroxisomes, functioning as detoxifier of hydrogen peroxide [142]. Using specific probes able to discriminate between ROS produced in peroxisomes, cytoplasm and mitochondria, and also using a photosensitizer KillerRed protein generating ROS in these compartments, elegant experimental work from the group of Marc Fransen has shed light into the intricacies of redox regulation between these organelles [143].

Indeed, loss of peroxisomal catalase, and subsequent peroxisome-derived oxidative stress, induces redox imbalance in mitochondria, whereas no effect is observed in the cytoplasm. However, this relationship is non-reciprocal, as redox status is maintained in peroxisomes when ROS are generated inside mitochondria [97]. Further, an excess of peroxisomal ROS triggers mitochondrial fragmentation with subsequent cell death which is prevented by lipoic acid and L-histidine, a singlet oxygen quencher [98]. Similar protective effects were observed upon overexpression of antioxidant enzymes: catalase in the mitochondria, or GSTK1 (Glutathione S-transferase kappa 1) and SOD1 (Superoxide dismutase 1) in peroxisomes [98]. Finally, cells lacking functional peroxisomes (loss of Pex5, Pex19 or glycerone phosphate acyl transferase (GNPAT), which participates to plasmalogen synthesis), showed marked sensitization to cell death induced by oxidative stress originating from peroxisomes, mitochondria or the cytoplasm [98].

Besides mitochondrial fragmentation and subsequent cell death, there is unambiguous demonstration of direct detrimental effects of ROS excess on essential peroxisomal functions, such as pexophagy (the autophagic degradation of peroxisomes) and peroxisome biogenesis [144]. In particular, peroxisome biogenesis, peroxisomal content and division processes are controlled by oxidative stress *via* the redox-sensitive peroxins PEX5 [145], PEX11β [146] and PMP14 [147]. It is worth noting that the fundamental elements of mitochondrial fission/fusion machinery, the FIS1 and DRP1 proteins, are also regulating peroxisomal dynamics [148–150]. Remarkably, aberrant S-nitrosylation of DRP1 induces its GTPase activity, which is associated with mitochondria fission in AD and HD [151]. Non-surprisingly, the peroxisome proliferator-activated receptor PPARγ and PPARα agonists orchestrate both peroxisomal and mitochondrial fission-fusion dynamics [152–154].

3.2. The role of peroxisomes in neurodegeneration

Excess of VLCFA exerts toxicity on neural cell types, as reported by several laboratories (Lizard G, Reiser G and Singh I labs).

Lizard's and Reiser's labs have observed cell death in neurons, oligodendrocytes and astrocytes, with doses of VLCFA ranging from 20 to 40 μM depending on the cell types [66–68,95]. However, no cell death was shown by these teams when *ABCD1* was silenced in murine oligodendrocyte cell line [67] or primary astrocyte culture generated from *Abcd1*-null mice [95]. In contrast, Singh's lab described cell death after *ABCD1* loss, in rat B12 oligodendrocytic cells, but not in human U87 astrocytes, suggesting that oligodendrocytes are more sensitive to *ABCD1* knockdown than astrocytes [155]. This higher sensitivity of oligodendrocytes to VLCFA but not the other neural cell types is consistent to the axonal loss and demyelination found out when functional peroxisomes are absent in oligodendrocytes [156] but not in astrocytes nor neurons [157]. However, neither oligodendrocyte loss nor demyelination has been observed in X-ALD mouse models [18,40] indicating that *ABCD1* deletion, alone, is not sufficient to kill oligodendrocytes *in vivo* in the mouse. Therefore, we posit that unknown factors which protect oligodendrocytes *in vivo* ought to exist, which may be differentially activated in oligodendrocytes lacking of ABCD1 and ABCD2, compared with loss of functional peroxisomes in the same cells.

There is growing evidence, gained mostly from conditional transgenesis experiments in the mouse, of the pivotal role played by peroxisomes in nervous system development and maintenance, in particular in myelin sheaths. Selective loss of functional peroxisomes in oligodendrocytes leads to demyelination and axonal loss in *Cnp-Pex5* mice, after having exhibited a normal myelination process during the first months of life [156]. Absence of peroxisomes in astrocytes provokes defects in the myelin with increase of VLCFA content but no axonal loss nor demyelination. This implies that an excess of VLCFA levels in the myelin is not sufficient to exert a major direct impact on neurological functioning [157]. Similarly, mice with specific deletion of Pex5 in neurons (*Nex-Pex5*) exhibit no metabolic disturbance neither axonal damage [157]. Finally, *Nestin-Pex5* mice, which harbor a deletion of Pex5 in the central and peripheral nervous system (of expression in oligodendrocytes, neurons, astrocytes and microglial cells), show an earlier onset phenotype than *Cnp-Pex5* mice, which exhibit demyelination and axonal abnormalities associated with locomotor and cognitive deficits [158]. This suggests that peroxisomes of neurons, astrocytes and microglia contribute to some degree to myelin maintenance when peroxisomal functions are abrogated in oligodendrocytes devoid of the organelles.

Aging is characterized by a progressive loss of physiological integrity, leading to impaired cellular function, increased vulnerability to insults and decreased regenerative capacities. Redox status, mitochondrial fitness and proteolysis capacities are affected during and may contribute to aging [159] (Fig. 2). As noted elsewhere, peroxisomes are involved in these different processes, suggesting an as-yet-poorly explored role of the organelle in aging (Fig. 2). A few reports support this notion; it has been observed that peroxisome morphology is altered with age in rodent hepatocytes [160] and peroxisomal ROS are elevated which impacts mitochondria in a vicious cycle [97]. As a consequence of excess of ROS with aging, peroxisome biogenesis is decreased, as some peroxins (PEX5, PEX14, PEX11β) involved in this process are redox-sensitive [43], thus leading to a general decrease of peroxisomal functions during aging, which may contribute to the aging process itself [160].

This notion is underlined by the observed peroxisomal alterations in Alzheimer's (AD) and Multiple Sclerosis (MS) patients, as well as in Parkinson's disease (PD), which may contribute to these pathologies. For instance, in AD patients with Braak V-VI stage, VLCFA are elevated and plasmalogens lowered (Fig. 2). Both are good indicators of peroxisomal malfunction, and are directly correlated to the amount of neurofibrillary tangles but not to amyloid plaques [161]. Peroxisomes but not mitochondria are absent in neuronal processes with phosphorylated TAU [161], which may indicate a selective toxicity of phosphoTAU to peroxisomes. Moreover, a direct link has been demonstrated between DHA — the major polyunsaturated fatty acid in the brain, which is synthesized by peroxisomes — and AD. Indeed, dietary supplementation with DHA in the 3xTg AD mouse model reduced both types of β-amyloid fibrils, the soluble form and the Aβ-plaques [162,163]. Moreover, an alteration in peroxisomal protein contents (induction of ABCD3, Acyl-coA oxidase and Pex5, concomitant with a reduction of thiolase and catalase in the hippocampus) was readily observed at early stages of pathology in the Tg2576 AD mouse model [164]. In MS patients, VLCFA are increased in grey matter, consistent with a decrease of peroxisomes in grey matter neurons [165]. Peroxisomes have also been linked to PD [132]. Firsthly, aggregation of α-synuclein in the brain of *Pex2*$^{-/-}$, *Pex5*$^{-/-}$ and *Pex7*$^{-/-}$ mice was evidenced, and Lewy-like inclusions were formed when α-synuclein thereby was overexpressed in *Pex5*$^{-/-}$ fibroblasts [166]. Further, reduced catalase activity was reported in the brains of A53T α-synuclein mice, which was then correlated to lower expression of other peroxisomal genes such as *ABCD3*, *AcylCoA oxidase* and *PEX14*, and to raised carbonyls levels. However, no accumulation of phytanic acid, pristanic acid or VLCFA was observed in these mice, indicating that neither peroxisomal α- nor β-oxidation were altered in the PD mouse model at this age [167]. Altogether, these data suggest that peroxisomes may contribute directly to neurodegeneration, or indirectly *via* cross-talk with mitochondria in redox homeostasis [94,95] (Fig. 2).

4. Concluding remarks

Compelling evidence indicates that X-ALD recapitulates the pathological hallmarks of the main neurodegenerative disorders, with intertwined peroxisomal, mitochondrial, proteasomal and autophagy dysfunction. Neutralization of ROS prevents these alterations along with axonal degeneration, which indicates that oxidative stress is a major culprit in this disease. Thus, therapeutic strategies that hold therapeutic potential may include: i) Antioxidants targeted to mitochondria and/or protectors of mitochondrial membranes; ii) Mitochondrial biogenesis boosters for generation of new, intact mitochondria; iii) Activators of intrinsic antioxidant pathways like autophagy and the proteasome, which will remove damaged or dysfunctional proteins and organelles, and can be considered as endogenous antioxidant systems; and iv) A combination of the above (Fig. 1). Thus, the identification and validation of biomarkers to monitor optimal dosing and biological efficacy of the antioxidant treatments is of prime interest. We have formerly used MALDI-TOF to identify a set of quantitative oxidative lesion markers to proteins (GSA, AASA, CEL, CML and MDAL) in murine nervous tissue of the *Abcd1*$^{-}$ mouse [49], in peripheral mononuclear cells of AMN patients [46], and are currently in the process of validating them in a larger setting, a phase II clinical trial with a combination of antioxidants for AMN patients (NCT1495260).

X-ALD, a rare monogenic peroxisomal disorder, may constitute a suitable paradigm to dissect molecular mechanisms and altered pathways in neurodegeneration, and thus a model to uncover new disease-modifying treatments that may also help in other, multifactorial or inherited neurodegenerative diseases in which axonal degeneration is at the bulk of clinical symptoms.

We posit that neurodegenerative disorders may be considered as secondary peroxisomal and mitochondrial disorders to some extext, in which redox homeostasis and proteostasis plays a major role in the neurodegenerative cascade and thus, constitute prime therapeutic targets. The scenario is however complex, and precludes straightforward extrapolation of conclusions from one model to another from

arising, while inviting to a cautious examination of the specific pathomechanisms operating in every particular disease model prior to designing the most suitable therapies (Fig. 2).

Funding

This study was supported by grants from the European Leukodystrophy Association [ELA2012-033C1], the Spanish Institute for Health Carlos III [FIS PI11/01043, FIS PI14/00410, FIS ICI14/00076], the Oliver's army, the Hesperia Fundation, the Autonomous Government of Catalonia [2009SGR85, 2014SGR1430] to A.P., and the Spanish Institute for Health Carlos III [Miguel Servet program CP11/00080] to S.F. The CIBER on Rare Diseases (CIBERER) and the CIBER on Neurodegenerative Diseases (CIBERNED) are initiatives of the ISCIII.

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