

Oxidative stress, a new hallmark in the pathophysiology of Lafora progressive myoclonus epilepsy

Carlos Romá-Mateo^{1,2*}, Carmen Aguado^{3,4*}, José Luis García-Giménez^{1,2,3*}, Erwin Knecht^{3,4}, Pascual Sanz^{3,5}, Federico V. Pallardó^{1,2,3#}

¹ FIHCUV-INCLIVA. Valencia. Spain

² Dept. Physiology. School of Medicine and Dentistry. University of Valencia. Valencia. Spain

³ CIBERER. Centro de Investigación Biomédica en Red de Enfermedades Raras. Valencia. Spain.

⁴ Centro de Investigación Príncipe Felipe. Valencia. Spain.

⁵ IBV-CSIC. Instituto de Biomedicina de Valencia. Consejo Superior de Investigaciones Científicas. Valencia. Spain.

* These authors contributed equally to this work

Corresponding author:

Dr. Federico V. Pallardó

Dept. Physiology, School of Medicine and Dentistry, University of Valencia.

E46010-Valencia, Spain.

Fax. +34963864642

Federico.V.Pallardo@uv.es

ABSTRACT

Lafora Disease (LD, OMIM 254780, ORPHA501) is a devastating neurodegenerative disorder characterized by the presence of glycogen-like intracellular inclusions called Lafora bodies and caused, in most cases, by mutations in either *EPM2A* or *EPM2B* genes, encoding respectively laforin, a phosphatase with dual specificity that is involved in the dephosphorylation of glycogen, and malin, an E3-ubiquitin ligase involved in the polyubiquitination of proteins related with glycogen metabolism. Thus, it has been reported that laforin and malin form a functional complex that acts as a key regulator of glycogen metabolism and that also plays a crucial role in protein homeostasis (proteostasis). In relationship with this last function, it has been shown that cells are more sensitive to ER-stress and show defects in proteasome and autophagy activities in the absence of a functional laforin-malin complex. More recently, we have demonstrated that oxidative stress accompanies these proteostasis defects and that various LD models show an increase in reactive oxygen species and oxidative stress products together with a dysregulated antioxidant enzyme expression and activity. In this review we discuss possible connections between the multiple defects in protein homeostasis present in LD with oxidative stress.

1. Pathophysiology of Lafora disease

Lafora disease (LD, OMIM 254780, ORPHA501) is a rare, fatal, autosomal recessive form of progressive myoclonus epilepsy (PME) that occurs worldwide, but is relatively more frequent in Mediterranean countries. The term PME describes a group of disorders characterized by the occurrence of focal and generalized seizures, myoclonus, and progressive neurological deterioration, typically with cerebellar signs and dementia [1, 2]. The most common causes of PME are Unverricht-Lundborg Disease (ULD; due to mutations in the *CSTB* gene encoding cystatin B, a lysosomal cysteine protease inhibitor), myoclonic epilepsy with ragged red fibers (MERRF; mainly due to mutations in the mitochondrial gene *MTTK* encoding the tRNA^{Lys}), neuronal ceroid lipofuscinoses (NCL, a collection of inherited diseases due to mutations in more than ten different *CLN* genes), dentatorubropallidoluysian atrophy (DRPLA; due to an expansion of a CAG repeat in the *DRPLA* gene encoding atrophin-1), sialidosis (a lysosomal storage disease due mutations in the *NEU1* gene encoding the lysosomal enzyme α -N-acetyl-neuraminidase) and Lafora disease (LD; see below) [1, 2].

Lafora disease is unique among the PMEs due to the rapid neurological deterioration of the patients and the appearance of insoluble glycogen-like intracellular inclusions named Lafora bodies (LBs) in brain and other tissues [3, 4] (Table 1). LD initially manifests during adolescence with generalized tonic-clonic seizures, myoclonus, absences, drop attacks or visual hallucinations. As the disease proceeds, a rapidly progressive dementia with apraxia, aphasia, and visual loss ensues leading patients to a vegetative state and death, usually within the first decade from the onset of the first symptoms [5-7]. Mutations have been identified in two genes, *EPM2A* [8, 9] and *EPM2B* [10], although there is evidence for a third locus [11]. *EPM2A*, located on chromosome 6q24 [12], is mutated in approximately 60% of LD cases. *EPM2A* encodes laforin, a dual-specificity phosphatase of 331 amino acids with a functional carbohydrate binding domain at the N-terminus [13, 14]. A second gene, *EPM2B*, located on chromosome 6p22.3, was found to be mutated in 20-30% of LD patients [10, 15]. *EPM2B* encodes malin, an E3 ubiquitin ligase of 395 amino acids with a RING finger domain at the N-terminus and six NHL domains in the C-terminal region [16] [17]. More than 60 different mutations have been reported in *EPM2A* and *EPM2B* genes (see <http://projects.tcag.ca/lafora>, for a comprehensive relation of the reported

pathogenic mutations in both genes). They include missense point mutations, premature termination codons (PTCs, i.e. stop codons) truncating the majority of the corresponding protein, frameshift and deletion mutations. The final outcome of these mutations ranges from the formation of catalytically inactive proteins to the absence of the corresponding protein. To study the pathophysiology of LD, mouse models lacking functional *EPM2A* or *EPM2B* genes have been produced [either by deletion of exon 4 in the case of *EPM2A* (a four exon containing gene) [18] or by deletion of the whole gene in the case of *EPM2B* (a mono-exonic gene) [19]]. They both recapitulate the Lafora disease phenotype: they accumulate polyglucosan inclusions, show signs of neurodegeneration and have a dysregulation of protein homeostasis (proteostasis).

2. Laforin and malin, new players in the regulation of glycogen synthesis.

As indicated above, a hallmark of LD is the accumulation of Lafora bodies (LBs) [3, 4]. They are abnormal, poorly branched intracellular glucose polymers composed, like normal glycogen, of glucose residues joined by α -1,4-glycosidic linkages with α -1,6-glycosidic branches [20]. However, in LBs, the glycosidic branches in these glucose polymers occur less frequently than in glycogen and contain higher levels of phosphate. These two properties (less branching and higher phosphate content) make LBs largely insoluble [21-23]. Recent work has revealed that LBs appear as a consequence of dysregulation of glycogen synthesis due to the absence of either laforin or malin, pointing to a key role of these proteins in glycogen homeostasis [see [24], [25], for review]. Although all cells in LD patients can develop LBs, neurons are especially sensitive to energy perturbations and, for this reason, the accumulation of LBs may be more detrimental in these cells, triggering neuronal death [26-28]. For a long time, it was assumed that the role glycogen played in the brain was only as an emergency energy supply. However, recent data suggest that glycogen has a more dynamic role in brain metabolism [29-31]. In fact, it has been reported that glycogen breakdown in astrocytes is required for the reuptake of extracellular K^+ , a critical process for neuronal activity [29, 32]. In addition, it has been reported that glycogen plays a crucial role in learning and long-term memory formation [33]. It has also been assumed that astrocytes were the only neural cells that synthesize and degrade glycogen, but it has been recently demonstrated that neurons express glycogen synthase and glycogen phosphorylase and accumulate low levels of glycogen that are essential to

resist hypoxic conditions [34]. Thus, the general view of the importance of glycogen in neural physiology is changing.

Glycogen synthesis in neurons must be tightly controlled, since over-accumulation of this polysaccharide is detrimental for these cells [26-28]. Interestingly enough, during human aging neurons accumulate glycogen-like granules named “corpora amylacea” (CA), which both morphologically and in composition resemble LBs. Like LBs (see below), CA contain low branched polyglucosans and proteins (ubiquitinated proteins, advanced glycation-end products, chaperones). This raises the possibility that neuronal glycogen accumulation contributes to physiological aging as a key factor regulating age-related neurological decline in humans. If this neuronal glycogen accumulation is accelerated, as in LD, then a more pathological situation ensues [35].

The role that laforin and malin play in the regulation of glycogen synthesis is twofold. First, it has been described that laforin acts as a phosphatase of complex carbohydrates, being this function necessary for the maintenance of normal glycogen homeostasis [21, 36]. In the absence of laforin, glycogen becomes highly phosphorylated and less branched, decreasing the solubility of this polysaccharide [21-23] and leading to the formation of LBs (Figure 1). Second, it is known that laforin interacts physically with malin, forming a functional complex [16, 17, 37]. In this complex, laforin would recruit specific substrates to be ubiquitinated by malin (see below, section 3.2). Some of these substrates are involved in the regulation of glycogen biosynthesis, such as the muscle isoform of glycogen synthase (MGS) [26], the glycogen debranching enzyme (AGL) [38], and the R5/PTG and R6 glycogen targeting subunits of type 1 protein phosphatase (PP1) [26, 37, 39, 40] (Figure 1). Most importantly, the formation of the laforin-malin complex is a regulated process in which AMP-activated protein kinase (AMPK) plays a critical role. The involvement of AMPK in the regulation of the laforin-malin complex adds a metabolic component to our understanding of the pathogenesis of LD [37]. The laforin/malin complex thus appears to be part of a multiprotein complex that is involved in the regulation of glycogen synthesis. For this reason, the accumulation of LBs has led some authors to consider LD as a new member of the family of *glycogen storage diseases*, being the formation/accumulation of LBs the signal that triggers LD. Supporting this hypothesis is the fact that the elimination of the capacity of the cells to synthesize glycogen, either by depleting MGS or the protein targeting to glycogen (PTG) in animal models of LD,

not only results in the elimination of the accumulation of LBs, but also in the amelioration of the neuropathological symptoms [28, 41, 42]. These results have led those authors to suggest the use of MGS inhibitors as a putative strategy to treat LD.

3. Lafora disease and cellular proteostasis.

Protein homeostasis (proteostasis) is achieved by the coordinated action of an efficient folding and transport of newly synthesized proteins and an active quality control with degradative mechanisms reducing the load of unfolded/misfolded proteins, to prevent thereby abnormal protein aggregation [43]. When the folding capacity is saturated, unfolded/misfolded proteins are usually tagged with ubiquitin moieties to target them for proteasome and/or lysosomal degradation. If these protein clearance mechanisms are not efficient, then unfolded/misfolded proteins accumulate and tend to form oligomeric structures. Thus, the presence of protein aggregates is an indication of an altered proteostasis [43].

LBs contain, in addition to insoluble glycogen-like polysaccharides (polyglucosans), ubiquitinated proteins, advanced glycation-end products, chaperones, autophagy components and proteasome subunits [35, 44] (Table 1). Probably, polyglucosan fibers start to accumulate in the initial steps of LB formation due to a dysregulation in the synthesis of glycogen. These insoluble polyglucosans would act as a seed to attract components of the protein clearance machinery (ubiquitin proteasome system, autophagy, etc.), which will get trapped into the insoluble inclusions and will become unable to function properly. Therefore, LD can be considered as another example of proteostasis dysfunction, being then linked to the group of *neurodegenerative proteinopathies*. In fact, recent work has demonstrated that neuronal death in LD could be influenced not only by the accumulation of LBs, but also by an impairment in the proteostasis mechanisms (Figure 1), similarly to what it has been described in more frequent neurodegenerative diseases (*i.e.*, Parkinson's, Alzheimer's, etc.).

In this section we will review the relationships between LD, ER-stress, protein clearance (proteasome and autophagy) and oxidative stress.

3.1. Laforin, malin, and endoplasmic reticulum stress.

Endoplasmic reticulum (ER) is the sub-cellular compartment for the synthesis and folding of all ER, Golgi, lysosomal, secreted and plasma membrane proteins. ER function is highly sensitive to stresses that perturb cellular energy levels, red-ox status or Ca^{++} concentration. Such stresses reduce the folding capacity of the ER, which results in the accumulation and aggregation of unfolded proteins in the lumen. These events trigger a signal responsible for the activation of the unfolded protein response (UPR) that restores the ER function. In addition, unfolded proteins are retrotranslocated to the cytosol, polyubiquitinated and degraded by proteasomes in the ER-associated degradation pathway. The UPR pathway is characterized by the activation of two ER-resident kinases (PKR-like ER kinase, PERK; and the inositol-requiring protein 1, IRE1) and the translocation to the Golgi apparatus and subsequent cleavage of ATF6, a transmembrane ER-resident protein with a cytosolic domain with transcriptional activity. These components induce signaling cascades that lead to the overexpression of characteristic UPR-mediators, such as the heat shock protein BIP/Grp78, the protein disulphide isomerase (PDI), the transcriptional factor CHOP (a member of the C/EBP family of bZIP transcription factors that induces apoptosis) and the phosphorylated form of the eukaryotic initiation factor 2 alpha (p-EIF2 α), among others. However, if conditions of ER-stress persist, these mechanisms of adaptation are insufficient to handle the unfolded protein load and cells undergo apoptosis (see [45], [46], [47], [48], [43], for review). ER-stress conditions are also associated to the generation of intracellular reactive oxygen species (ROS) from ER. Electron transport during disulphide-bound formation is driven by protein disulphide isomerase (PDI) and ER-oxidoreductin 1 (ERO1), who uses a flavin-dependent reaction to transfer electrons from PDI to molecular oxygen. Under conditions of ER-stress, ERO1 is upregulated, leading to the accumulation of ROS [49]. ER-derived ROS production contributes to the establishment of a cellular oxidative stress status that is detrimental to the cell.

Several labs have reported that the laforin-malin complex plays a role in protecting cells from ER-stress conditions [44, 50-52]. For instance, in cell culture models depleted of malin or laforin, there is an increased ER-stress response that eventually leads to decreased proteasome function and increased apoptosis, which could be important factors in the development of LD [50, 51] (Table 1). This higher sensitivity of laforin/malin deficient cells to conditions of ER-stress may predispose them to the development of oxidative stress. In addition, since ER-stress conditions are

pro-inflammatory, as they activate the JNK and IKK pathways [53], it is quite possible that neuroinflammation could be a new trait in the physiopathology of LD.

3.2. Proteolytic impairment in Lafora disease

As indicated above, it was early described that besides glycogen-like polymers, LBs also contain about 6% protein [4, 54] and stain positive with anti-ubiquitin antibodies [18]. These observations suggested the presence of a certain amount of misfolded proteins in LBs and, thus, it was proposed that LD could be a disorder of protein clearance [55]. This proposal has been further supported by the presence in LBs of chaperones and proteasome subunits [35, 44], an observation that resembles similar situations in many proteinopathies in which the proteolytic mechanisms of the cells are impaired. Therefore, like in these pathologies, it is possible that in LD the protein folding capacity is exceeded, and that the intracellular protein degradation systems are also altered, resulting in the accumulation in LBs of chaperones, aggregate-prone proteins, free ubiquitin, ubiquitinated proteins, proteasome components and probably ATGs (autophagy-related gene products), among other proteins related with the proteostasis mechanisms. In fact, we have recently described a reduction in the total proteolytic capacity present in mouse embryonic fibroblasts from *Epm2a*^{-/-} and *Epm2b*^{-/-} mice, and also in fibroblasts from patients suffering from *EPM2A* and *EPM2B* mutations [19, 56, 57] (Table 1).

In mammalian cells the ubiquitin proteasome system and autophagy (Figure 2) are the major protein degradative systems [58] and an interconnection between both has been outlined [59]. In mammalian cells proteasomes and lysosomes are equally important in the degradation of short- and long-lived proteins, but their relative importance varies with the cell type, its metabolic situation, and the specific protein to be degraded [60, 61]. Our own results indicate that the reduction in the total proteolytic capacity present in LD cellular models described above, affects both proteasome and lysosome activities [19, 56, 57]. In the following sections we will describe the impact of the absence of laforin and malin on the different proteolytic systems.

3.2.1. Alterations of the ubiquitin-proteasome system (UPS) in Lafora disease

The UPS [62] acts in two parts, both consuming ATP (see Figure 2A): the polyubiquitination of protein substrates and their degradation by the downstream 26S or

30S proteasomes, composed by the 20S catalytic core and, respectively, one or two 19S regulatory particles. Canonical ubiquitination, which is a reversible process, involves three steps: i) activation of ubiquitin by a process that requires ATP and the E1 (ubiquitin-activating) enzyme, ii) transfer of the ubiquitin from the E1 to an E2 (ubiquitin-conjugating) enzyme, and iii) direct or indirect transfer of ubiquitin to an inner Lys of a specific protein substrate recognized by an E3 (ubiquitin-ligase) enzyme. Combinations of the large number of E2 enzymes and especially of E3 enzymes ensure the specificity of the whole process. The ubiquitination of a substrate protein can be sequentially repeated to generate a polyubiquitin chain, which is usually composed of four or more ubiquitin units attached to the substrate protein. In most proteasome substrates the ubiquitin moieties of the polyubiquitin chain are attached to each other by their Lys48, but there are also numerous exceptions. Since ubiquitin contains seven different Lys residues, a high variety of ubiquitin linkages and of polyubiquitin chains exists. This diversity may serve to regulate other cell functions different from proteasomal degradation, as it is also the case of monoubiquitination, but the important point here is that most proteins marked with Lys48-linked polyubiquitin chains (but also with some of the other linkages) are recognized, deubiquitinated to generate free ubiquitin and degraded in the 20S proteasome by processes that also consume ATP.

The fact that *EPM2B* encodes a protein, malin, which contained six NHL domains implicated in protein-protein interactions and an N-terminal RING-finger domain, immediately suggested a role of the protein as an E3-ubiquitin ligase [10], most probably involved in the degradation by the UPS of some proteins that produce the accumulation of LBs. Later, it was experimentally shown that malin interacts with laforin and catalyzes its polyubiquitination and degradation, at least under *in vitro* conditions [17, 63]. This brought into relation two apparently different proteins, an E3 ubiquitin ligase (malin) and a protein containing a dual specificity phosphatase domain (laforin), but whose defects produce the same disease. However, if laforin is a malin substrate and at the same time plays the main positive role to prevent the pathology, for example as a glucan phosphatase in the dephosphorylation of glycogen to avoid the formation of polyglucosans [21, 64], it could be expected that defects in malin would elevate the levels and, thus, the activity of laforin. This fact would be difficult to reconcile with data showing that defects in malin or in laforin produce a similar histological and neurological phenotype. The difficulty could be circumvented if the

elevated levels of laforin were not active in the absence of malin and/or if the excess of laforin becomes sequestered in specific compartments such as LBs [65]. However, even so, the role of malin in relationship with the glucan phosphatase activity of laforin would still require a different explanation from simply polyubiquitinating laforin for proteasomal degradation.

In this regard, it was crucial the discovery that laforin also interacts with proteins involved in glycogen biosynthesis [66], such as MGS and PTG, which brings protein phosphatase 1 to MGS. In fact, MGS [26], AGL [38] and PTG [26, 37, 39] were identified as substrates of the malin-laforin system. Therefore, it could be possible that it is the ubiquitin ligase malin who plays the main role in the molecular mechanisms that prevent LD, by facilitating the modification of enzymes involved in the synthesis of glycogen in neurons, thus avoiding an excessive accumulation of this polysaccharide that would be toxic in these cells. In this setting, laforin could act as an ancillary protein placing specific substrates involved in glycogen metabolism in the proximity of malin to be polyubiquitinated and possibly degraded by the proteasomes. However, later studies from different laboratories found that the levels of PTG, MGS or AGL were not altered in malin- or laforin-deficient mice [22, 67], making unlikely this possibility to explain the molecular basis of LD. Even the possibility that laforin is an *in vivo* malin substrate has been also recently questioned (see *e.g.* [68]). Therefore, it remains to clearly identify all substrates of malin under *in vivo* conditions and this would be probably very important for a better understanding of the molecular basis of LD and its associated accumulation of LBs.

Various reports indicate that the laforin-malin complex promotes the formation of Lys63-linked ubiquitin chains in different substrates [40, 69, 70] and although no information is known yet about the E2-conjugating enzyme that participates in this reaction *in vivo*, it has been described that malin can promote an *in vitro* efficient ubiquitination when associated with the E2-conjugating enzymes UbcH2, UbcH5 and UbcH6 [17, 37]. Also, it has been proposed that the U-box co-chaperone CHIP stabilizes malin [44]. Since CHIP may interact with the E2 UbcH13/Uev1a, a heterodimeric E2 enzyme that exclusively forms K63-linked ubiquitin chains [71, 72], it could be possible that laforin and malin could form a macrocomplex with CHIP and UbcH13/Uev1a, which would be responsible for the K63-linked ubiquitination of

specific substrates. Alternatively, laforin and malin could interact directly with UbcH13/Uev1a.

The accumulation of ubiquitinated and misfolded proteins in LBs could indicate a failure of the UPS to get rid of them in LD, as it occurs in many other neurodegenerative diseases. In fact, different studies with various LD models have illustrated the existence of defects in the ubiquitin-proteasome system [51, 57, 63, 73]. Although no UPS defects were observed in brain from laforin- or malin-deficient mice [19], these defects were observed by others using laforin- or malin-deficient mouse embryo fibroblasts under stress conditions [68]. Therefore, it is possible that in laforin- or malin-deficient cells, stress conditions overwhelm the activities of the chaperone machinery and the UPS, causing misfolding and ubiquitination of proteins that aggregate in LBs, and that this in turn reduces the activity of the UPS, causing further alterations that would be especially deleterious for sensitive cells, like neurons. These alterations could contribute to the pathology in LD, but because of the above mentioned proteasome-autophagy interconnection, they would be even worse if the other major intracellular protein degradation system, autophagy, is also altered. This possibility is discussed below.

3.2.2.- Alterations of lysosomes in LD

The lysosome is the final destination for endocytic, autophagic, and secretory molecules targeted for destruction by a variety of acid hydrolases, including numerous peptidases called cathepsins. Lysosomes degrade cellular proteins by different mechanisms being endocytosis, crinophagy and the various autophagies the best established of them [58]. Endocytosis (also called heterophagy) is the degradative route to lysosomes, *via* early and late endosomes, that is followed by extracellular material and also by certain plasma membrane proteins that, unlike the low density lipoprotein receptor and the transferrin receptor for example, do not recycle back to the plasma membrane for reuse. By crinophagy, secretory proteins are degraded by lysosomes when their demands outside the cell decrease and this occurs by fusion of the secretory granules with lysosomes or with late endosomes instead of with the plasma membrane. Finally, the main lysosomal mechanism for intracellular proteolysis is autophagy, a general term with at least three different forms of “self-eating”: macroautophagy, microautophagy, and chaperone-mediated autophagy (see Figure 2B). By

microautophagy specific cell areas enter into lysosomes by various modifications of the lysosomal membrane. In chaperone-mediated autophagy, specific proteins containing a KFERQ-like signal enter lysosomes through a receptor (LAMP2a) at the lysosomal membrane by a complicated process that requires ATP and cytosolic and lysosomal chaperones. Macroautophagy is by far the most important and best known lysosomal degradative route and, therefore, it is frequently and simply called autophagy, as we will do hereafter. It involves the sequestration by a segregating structure, called phagophore, of large areas of cytoplasm, typically including whole organelles, to make up an autophagosome. These pre-lysosomes fuse then with endosomes and lysosomes to form an amphilyosome or an autolysosome in which the sequestered cytoplasmic content is quickly degraded by the high concentration of acid hydrolases.

Based on the above mentioned dysfunction of the UPS in LD, it was possible that lysosomes in general and autophagy in particular, could be also affected in the disease. The main reasons to test this possibility were the following. First, autophagy complements the UPS for the degradation of polyubiquitinated proteins and can compensate defects on it. This is especially due to the fact that large autophagosomes can sequester material that cannot enter the narrow proteasome channel, such as aggregated proteins and altered mitochondria. Therefore, autophagy is more effective than proteasomes to eliminate aggregated or altered material of a certain size after delivery to lysosomes. Second, autophagy can also degrade polyubiquitinated proteins and it has been found that the laforin-malin complex promotes, at least in some of its substrates, the formation of K63-linked polyubiquitin chains [69]. These polyubiquitin linkages can target these substrates for degradation in lysosomes (see [58] for a review) and in relationship to LD, this was the case for the protein phosphatase 1 regulatory subunit R6 [40]. Finally and more important, lysosomes represent a well-established route in the degradation of glycogen, as exemplified by the accumulation of glycogen in lysosomes in Pompe's disease due to a deficiency in the lysosomal enzyme acid maltase [74]. Therefore it seems possible that the accumulation of polyglucosans in LD derives from a defect in autophagy.

In fact, impairment in autophagy in *Epm2a*^{-/-} and *Epm2b*^{-/-} mice models of LD was described, leading to decreased levels of LC3-II, a marker of autophagy, decreased degradation of long-lived proteins and increased levels of the autophagic component p62 [19, 56]. Later, different laboratories [28, 68, 75, 76] also noticed a similar

impairment in autophagy in various LD models (Table 1). This defect probably occurs through the mammalian target of rapamycin kinase (mTOR) pathway-dependent inhibition of autophagosome formation [68, 77], although other unidentified signalling pathways to autophagy may be also implicated in brain [78]. Since autophagy degrades practically all macromolecules and organelles in the cell, additional consequences of the autophagy defects in LD should occur, besides glycogen accumulation. In particular, the autophagy defect should produce, in LD, an accumulation of undegraded proteins and lipids and of defective mitochondria, and since these organelles are the primary source of reactive oxygen species, increased levels of these molecules should occur in LD. In fact, recent data obtained by the authors of this review have shown alterations in mitochondria and increased levels of reactive oxygen species in LD [79], which may contribute to neuronal stress and apoptosis. These possibilities will be further discussed in the following section.

An additional point relevant for the lysosomal alterations discussed here is that either malin or laforin could have independent functions of each other and in this regard, a specific malin function in the biogenesis and/or lysosomal glycogen disposal has been suggested [68]. However, the relevance of the specific loss of these functions, when one but not the other of the two proteins implicated in LD is defective, appears to be inconsistent with the observation that defects in malin or in laforin produce a quite similar phenotype in patients. Also, other alterations in the endocytic and lysosomal systems have been described in a laforin-deficient model of LD [80], which may or may not derive from the autophagic alterations.

In summary, defects in both UPS and autophagy in LD have been extensively documented. Now, a critical question is whether the defects in the UPS and in autophagy described above are a cause or a consequence of the LD pathology [81]. In the case of autophagy, more recent data indicate that inhibition of glycogen synthesis prevents LD in mice [41] and that glycogen accumulation produces the impaired autophagy and not the opposite [28, 68]. Also, unpublished data from various laboratories have indicated that enhancing the activity of the autophagic pathway is not an appropriate therapeutic option in LD. Therefore, although on the basis of the recent available results the observed alterations in the UPS and autophagy may appear to be secondary to the accumulation of polyglucosans, probably because LBs recruit critical factors involved in the regulation of the UPS and autophagy, further details are needed.

Also, the relevance of these alterations in the protein homeostasis machinery should not be minimized and needs to be further investigated to reach more definitive conclusions. For example, even if the observed defects are secondary, it is important to identify the signalling mechanisms by which LBs impair the main proteolytic pathways in the cells. Also and since it is obvious that the above mentioned alterations will considerably worsen the pathology, it will be important to know how they contribute to the clinical phenotype. All these and other aspects related with the impairment in the main pathways of intracellular protein degradation in LD will be certainly addressed in various laboratories in the near future.

4. Increased oxidative stress in Lafora disease

The relationships between defects in the cellular proteolytic pathways and the generation of reactive oxygen species (ROS) have been broadly documented. Impairment in protein clearance systems imperatively leads to ER-stress, as described above, and the accumulation of misfolded proteins and dysfunctional protein aggregates which act as targets of basal ROS levels, give rise to oxidized protein products [82]. In normal cell conditions, this accumulation is rapidly surpassed by the ubiquitination and targeting to the proteasome of oxidized protein products and misfolded aggregates, in a chaperone and co-chaperone mediated manner [83], as discussed in the previous section. It is interesting, however, that ubiquitination-dependent and independent processes are involved in oxidized protein degradation by proteasomes, keeping in mind that large aggregates can inhibit the proteasome and that the process differs with the length and degree of the oxidative stress insult [82]. The elimination of protein oxidation products and misfolded aggregates is hence tightly linked to prolonged oxidative stress, affecting proteasome and chaperone-mediated protein clearance at different levels, highlighting the importance of maintaining a refined balance between ROS production and protein homeostasis, as reflected in the numerous evidences of neurodegenerative diseases in which one or both of these processes are affected [84, 85].

A growing amount of evidence in recent years suggests that oxidative stress, via ROS and reactive nitrogen species (RNS), are key signal transducers sustaining autophagy [86]. Autophagy becomes the alternative solution to eliminate oxidized protein aggregates that overwhelm proteasomal activity. However, in a scenario of

proteolytic and/or autophagic impairment, as it is the case in LD (see previous section) [19, 51, 68, 81], these protein aggregates would accumulate. Antioxidant response elements provide a defensive shield against ROS by preventing the deleterious effect of protein oxidation and accumulation; hence, defective antioxidant systems worsen the previously described scenario, contributing to a positive, deleterious feedback. Autophagy also participates in the response against increased ROS levels: mitophagy, for example, is induced by oxidative stress, promoting the removal of dysfunctional mitochondria. In fact, in mitophagy-deficient cells, the excess of altered mitochondria leads to an overproduction of ROS [87]. In addition, ROS such as $O_2^{\cdot-}$ and H_2O_2 induce autophagy, although experimental evidence has shown that this induction depends on stress conditions and cell type. ROS also regulate different proteins relevant in autophagy, such as mTOR, AMPK, Beclin-1, the pro-autophagy protein HMGB1, Atg4, and Atg8 (LC3 in mammals), among others [88, 89]. Furthermore, it has been shown that chemically induced oxidation of glutathione (GSH) is able to promote autophagy even in the absence of any autophagic stimulus [90].

Thus, the proteolytic mechanisms represented by autophagy and proteasomal degradation are closely related to oxidative stress and cellular redox homeostasis, in both physiological and pathological conditions. This relationship is regulated by specific mediators, which play a pivotal role. Next, we will discuss the putative role of this particular kind of proteins in the development of LD, and the evidences of the involvement of antioxidant response impairment and/or an exacerbated ROS production in the ethiopathology of the disease.

4.1. Linking proteasome defects to antioxidant response in LD

One of the most important cellular sensors of oxidative stress are those proteins with enzymatic activity based in thiol reduction, including glutathione peroxidases (GPXs), thioredoxins (Trxs) and peroxiredoxins (Prdxs) [91, 92]. Proteins from these families act as regulators of disulfide bridge formation involved in reversible protein function activation, but they also participate in the elimination of abnormal disulfides produced by an unbalance in ROS production. In recent years, various evidences have demonstrated the linkage between some of these reducing agents and the proteolytic machinery of the cell: for instance, thioredoxin-1 (Trx1) and Txn1/TRP32 (thioredoxin-like protein1/thioredoxin-related protein of 32 kDa) interact both with misfolded proteins and proteasomal subunits [57, 93, 94]. In this line of evidence, we

described that Trx1 interacts with the 20S proteasome and translocates to the ER upon proteasome inhibition, producing an induction of the antioxidant response and an increase in ER-stress markers in human fibroblasts [57]. As discussed previously, the lack of laforin activity has been shown to correlate with impairment in proteasomal activity and ER-stress, both in siRNA-mediated gene silencing in culture cells and in specific tissues of laforin-deficient mice [51]. The fact that both ER-stress and proteasome activity is affected in LD cells and animal models, suggested that antioxidant defense systems are impaired. This was tested for the first time using fibroblasts from LD patients with mutations in the laforin gene, in which the impairment of proteasome activity paralleled a decrease in the levels of Trx1 [57]. Moreover, it was shown in this same study that LD fibroblasts exhibited a mislocalization of Trx1 and proteasomes, with diminished Trx1 and proteasome co-localization in the nucleus. Since Trx1 migration to the nucleus has been related with an active antioxidant response, the mislocalization observed in LD fibroblasts, together with the proteolytic defects, strongly pointed to an abnormal antioxidant response in these cells [57].

4.2. Defective antioxidant enzymes in LD

The data obtained in the study of Trx1 and its involvement in the proteasomal function of laforin-deficient cells [57] were further broadened by analyzing a series of antioxidant enzymes in both laforin- and malin-deficient cells from human patients and LD mice models [79]. Overall, this analysis showed a clear unbalance in the expression levels of concrete antioxidant enzymes, together with a significant decrease in their enzymatic activity. Particularly affected were cytosolic and mitochondrial superoxide dismutases (CuZnSOD and MnSOD, respectively), and catalase, whereas no apparent changes were observed for other antioxidant enzymes like glutathione peroxidases (GPXs) (Figure 3).

Using LD mice models [79], we also showed that malin KO mice displayed an increment in lipid peroxides in plasma, which indicates a systemic chronic oxidative stress as a consequence of excessive ROS production. This increase in oxidative stress could explain why patients suffering from LD show increased levels of oxidative stress markers such as 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 8-hydroxyguanosine (8-OHG) (markers of DNA and RNA oxidative damage) in cerebral cortex and urine [95].

The examination of oxidative stress parameters in LD depicts a landscape in which antioxidant enzymes appear severely affected, as expected in cells with an altered proteolytic system and a defective response to misfolded proteins [44, 63, 96, 97] together with an increase in ER-stress. Interestingly, our proteomic analysis performed on brain extracts from malin-deficient mice [79] showed some differences in its protein content profile as compared to control mice. The most relevant data provided by this analysis refer to a protein belonging to the family of Prdxs, peroxiredoxin-6 (Prdx6), and a mitochondrial membrane channel, VDAC2 (voltage-dependent anion channel 2, which will be discussed in the next section). Malin-deficient mice showed no differences in the total levels of Prdx6, but an increment in a post-translationally modified form that remains, to date, uncertain. Some authors have suggested that acidic forms of Prdx6 correspond to oxidized forms, although several works demonstrated that the modifications of Prdx6 are multiple and variable, including, among others, different degrees of cysteine oxidation, acetylation and phosphorylation [98, 99]. Prdx6 overexpression or post-translational modifications have been related to oxidative stress damage and proteolytic impairment in relation to several pathologies [100-105], including neurodegenerative disorders [106-108]. Prdx6 is particular among the rest of the peroxiredoxin family members, since it shows a bifunctional enzymatic activity both as glutathione peroxidase and as a phospholipase [109]. It has been proposed that the phospholipase activity of Prdx6 is related to autophagosome regulation, thus linking again oxidative stress responses with autophagy [110, 111]. Particularly interesting are recent results obtained by Yu and collaborators, in which Prdx6 hyperoxidation promotes its phospholipase activity, increasing cytotoxicity in astrocytes and contributing to neurodegeneration in a Parkinson disease mouse model [112].

Taking together all the data on antioxidant enzyme alterations in LD, it seems that the most affected antioxidant defense systems are those involving enzymes that decrease superoxide levels in the cell (see Figure 3). However, the unanswered questions concern the main source of ROS overproduction and the precise chain of events that leads to the proteolytic and oxidative stress defense alterations in LD and to neurodegeneration.

4.3. Mitochondrial dysfunction and ROS production: what came first?

Mitochondria are one of the main sources of ROS in the cell, and mitochondrial homeostasis is tightly linked to autophagy [113]. Thus, an increase in morphologically

and functionally altered mitochondria could reflect a failure in the autophagic process. Indeed, mitochondria from both laforin- and malin-deficient fibroblasts from human patients display an abnormal morphology, decreased membrane potential and ATP production, and an increment in ROS levels, particularly superoxide production [79]. Our findings in LD fibroblasts demonstrate that mitochondria from laforin- and malin-deficient cells have lost their normal morphology and appear aggregated, hence contributing to the loss of mitochondrial membrane potential and low efficiency of the mitochondrial transport chain [79]. These results seem to suggest that the origin of oxidative stress in LD could be found in the interplay between autophagy and mitochondrial function; however, the rest of defects on proteolytic systems and the related accumulation of aberrant glycogen granules and misfolded protein aggregates should also be taken into account, making difficult to assess which process constitutes the first failure that triggers a positive feedback that ultimately leads to cell malfunction (Figure 4).

Besides an altered morphology and physiological function of mitochondria from laforin- and malin-deficient cells, the aforementioned proteomic analysis performed on brain extracts from malin KO mice [79] highlighted an increase in the levels of VDAC2. VDAC1 and VDAC2 are voltage dependent channels located in the outer mitochondrial membrane and tightly related to mitophagy [114]. The relationship between mitophagy defects and ROS overproduction has been already documented [87]. Although no physiological explanation for the increase in VDAC2 levels has been found, it is interesting that using similar proteomic approaches other groups have found alterations in VDAC2 expression in other types of epilepsy [115, 116].

As a summary, the interplay between autophagy impairment, mitochondrial dysfunction and overproduction of ROS, together with the incapacity in the cell's response to oxidative stress (Figure 4 and Table 1), depicts a landscape that fits with the rest of disruptions reported in cell homeostasis in LD: ER-stress, proteasomal dysfunction and aberrant glycogen accumulation. The additive effect of these altered pathways ultimately leads neurons and other cell types in LD to dysfunction, and probably cell death.

5. Concluding remarks and perspectives

5.1. Laforin, malin and redox-regulated signaling pathways

As it has been explained above, laforin and malin are proteins with biochemically and functionally different features that form a functional complex, upon which several cellular processes of critical relevance for cell survival depend. In this review we introduce oxidative stress as a novel factor to be taken into consideration with the many other processes that we have discussed in the previous sections, which are regulated by, or directly linked to, the disruption of the laforin-malin complex. Noteworthy, little evidence pointed previously to a close linkage between redox regulation and laforin or malin. Some data, nonetheless, seem to favor this relationship. Degradation of misfolded proteins and ER stress are processes closely related to ROS overproduction, and hence the possibility that in the absence of laforin and/or malin the amount of oxidized proteins increases, is a plausible hypothesis.

Since laforin is a dual specificity phosphatase whose activity is dependent on a cysteine residue, redox conditions are critical to determine its enzymatic activity. The regulation of phosphatase activity by reversible oxidation of catalytic cysteine residues has been broadly documented [117, 118], but still little is known about the enzymes involved in regulating these oxidation-reduction processes. Recent findings describe the participation of Trx1 and TRP32 in the reactivation of tyrosine phosphatases closely related to laforin [119, 120], providing interesting clues on the precise regulated redox signaling that open novel paths to examine laforin's biochemical functions in relation to redox conditions in the cell. In addition, it has been found that laforin forms homodimers, being involved in this oligomerization a particular cysteine residue different from the one in the catalytic site. For this reason, laforin is prone to aggregate in the absence of a reducing environment [121, 122]. This indicates another layer of complexity in the regulation of laforin activity by oxidative conditions.

In all, this evidence fits with the relevance of a correct oxidative stress response and a careful regulation of redox environment for the proper physiological function of the laforin-malin complex, although further data are needed to completely assess their fine regulation, and the identity of other pivotal antioxidant enzymes.

5.2. Therapeutic potential of antioxidant treatments in LD

Oxidative stress has been linked to a myriad of human diseases. Excessive free radicals have been described in aging, and in almost all major human diseases including

cancer, atherosclerosis, stroke, emphysema, and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. Neganova and collaborators proposed that free radicals may also contribute to brain damage, neuronal death and persistent seizures in epilepsy [123]. Interestingly, epilepsy increases *NOSII* gene expression through NF- κ B induction, with concomitant NO, $O_2^{\cdot-}$, and ONOO dependent decrease of Complex I activity and increased Complex-III-dependent $O_2^{\cdot-}$ production in epileptic brain mitochondria [124, 125]. Since oxidative stress is a relevant factor in some epilepsies, antioxidant intervention has been described in some of these pathologies. For example, the use of acetyl-L-carnitine has shown beneficial effects in epilepsy [125], an antioxidant therapy has been proposed as an anticonvulsive and neuroprotective treatment strategy against epilepsy [126] and the use of tryptamine (a serotonin receptor agonist and serotonin releasing agent), which is a derivative of securinine [a gamma-aminobutyric acid (GABA) receptor antagonist], has been proposed as an antioxidant and anticonvulsant drug [123]. Consequently, and since oxidative stress dysfunction is present in LD, we suggest that the use of an antioxidant therapy as a therapeutic intervention in LD may be beneficial in the treatment of this terrible disease.

In summary, since enhancing the activity of the autophagic pathway appears not to be an appropriate therapeutic option in LD, we propose that the findings on mitochondrial malfunction, ROS overproduction and antioxidant defense alterations in LD become a novel piece in the intricate puzzle of LD neurodegenerative process, and might provide interesting and currently uninvestigated ways to understand and fight the development of this devastating disease.

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Figure legends

Figure 1. Molecular pathways altered in LD. Glycogen metabolism (A) and misfolded protein response (B) are two cellular processes in which severe alterations in LD have been described, producing an unbalance in glycogen homeostasis and protein clearance. Recently, mitochondrial function has been proposed as another critical process altered in LD, producing an increase in ROS levels and contributing to worsening the cellular homeostasis.

Figure 2. Implication of the major pathways of intracellular protein degradation in LD. The laforin-malin interaction facilitates the activity of malin as an E3-ubiquitin ligase that in coordination with still unknown E2-ubiquitin conjugating enzymes, promotes the ubiquitination of proteins involved in LD. A and B indicate alternative pathways in the degradation of these proteins, either by proteasomes (A) or by autophagy (B). Lysosomes degrade intracellular proteins by quite different mechanisms including microautophagy, chaperone mediated autophagy (CMA) and macroautophagy. The latter is by far the main lysosomal degradative route used under stress conditions, and includes elimination of dysfunctional mitochondria (mitophagy). Increased production of ROS affects the activity of proteasomes and also damages proteins and mitochondria that are in turn eliminated by the autophagic pathways (see text for details). (?) indicates possible degradative pathways implicated in LD but not yet tested.

Figure 3. Oxidative stress parameters and antioxidant enzymes affected in LD. In normal conditions, an excessive superoxide production is counteracted by the action of

cytosolic (CuZnSOD) and mitochondrial (MnSOD) dismutases, which produce hydrogen peroxide. The levels of hydrogen peroxide can then be decreased by three main pathways, catalyzed by catalase, peroxiredoxins (PRDXs) or glutathione peroxidases (GPXs) that use glutathione as the reducing agent. In LD, increased levels of superoxide seem to be the main source of excessive ROS in LD human fibroblasts (LD-Fs), and alterations (either at the protein level or in enzymatic activity) have been found in superoxide dismutases and catalase (colored in red), either in LD-Fs or LD mice models (LD mice). Peroxiredoxin-6 (Prdx6) and thioredoxin-1 (Trx1) (colored in pink) are also oxidative stress-related proteins altered in LD mice or human fibroblasts at a functional level (see text for details).

Figure 4. Interplay between oxidative stress and proteostasis in LD. Absence of laforin or malin, or defects in the function of the laforin-malin complex, have been independently related with defects in autophagy, proteasomal degradation, response to misfolded proteins and mitochondrial function. These processes interact with each other, contributing to the worsening of the loss of cellular homeostasis and probably also to the accumulation of LBs.

Table 1. Summary of cellular and molecular alterations found in the different LD models. (See text for references and details).







