



Review

Impaired autophagy: The collateral damage of lysosomal storage disorders

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ARTICLE INFO

Article History:

Received 28 October 2020

Revised 25 November 2020

Accepted 26 November 2020

Available online xxx

Keywords:

Lysosome

Autophagy

Gaucher disease

Batten disease

Danon disease

Pompe disease

Cystinosis

ABSTRACT

Lysosomal storage disorders (LSDs), which number over fifty, are monogenically inherited and caused by mutations in genes encoding proteins that are involved in lysosomal function. Lack of the functional protein results in storage of a distinctive material within the lysosomes, which for years was thought to determine the pathophysiology of the disorder. However, our current view posits that the primary storage material disrupts the normal role of the lysosome in the autophagic pathway resulting in the secondary storage of autophagic debris. It is this “collateral damage” which is common to the LSDs but nonetheless intricately nuanced in each. We have selected five LSDs resulting from defective proteins that govern widely different lysosomal functions including glycogen degradation (Pompe), lysosomal transport (Cystinosis), lysosomal trafficking (Danon), glycolipid degradation (Gaucher) and an unidentified function (Batten) and argue that despite the disparate functions, these proteins, when mutant, all impair the autophagic process uniquely.

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1. Autophagy: background

Autophagy (from the Greek for “self-eating”) is a set of pathways by which intracellular material is delivered into the lysosome for degradation and recycling. The fundamental role of autophagy is to salvage energy and critical nutrients, such as amino acids and fatty acids, in order for cells to survive under starvation [1,2]. Three distinct types of autophagy are recognized: Microautophagy, chaperon-mediated autophagy (CMA), and macroautophagy (Fig. 1). All three types terminate at the lysosome and therefore are called the autophagy-lysosomal pathways (ALPs).

Microautophagy, the least studied type, was originally described as the inward curvature of the lysosomal membrane to directly engulf a small portion of the cytoplasm. However, recent studies have expanded the definition to include the endosomal compartment and the endosomal sorting complexes [3]. There is no evidence as yet for impaired microautophagy in LSDs. In contrast, there is growing evidence for the involvement of CMA and macroautophagy in particular, in the pathophysiology and progression of many LSDs [4,5].

CMA is a selective process of lysosomal degradation of a subset of cytosolic proteins. The selectivity is conferred by the presence of at least one KFERQ-like pentapeptide targeting motif that is recognized by the heat shock-cognate chaperone 70 kDa protein (HSC70).

Approximately 30–40% of mammalian proteins contain this sequence motif, thus making them potential CMA targets. Upon binding to the HSC70 chaperone, the target protein is delivered to the lysosomal membrane where it interacts with the cytosolic tail of LAMP-2A, the CMA receptor [6,7]. Another form of HSC70, encoded by the same gene, is a luminal HSC70 which is required to complete the translocation of target proteins into the lysosomal lumen. The activity of CMA is upregulated in response to a variety of stress conditions, including nutrient deprivation, hypoxia, and oxidative stress [7].

Macroautophagy (often referred to as “autophagy”) is a major and most extensively studied autophagic pathway. Unlike microautophagy and CMA, macroautophagy is a multistep process that requires a complex vesicular trafficking network to connect the newly formed double-membrane autophagosomes with lysosomes. The formation of an autophagosome begins with the development of a double membrane (phagophore) that expands, curves, and finally fully encloses a portion of the cytoplasm. The outer autophagosomal membrane fuses with the lysosome where the sequestered cargo and the inner membrane are degraded [8]. The massive catabolic potential of macroautophagy relies on the ability of autophagosomes to sequester a large portion of the cytoplasm.

Autophagy is a non-selective “wholesale” degradation of cellular components in response to starvation – a universal stressor and a powerful inducer of autophagy [1]. However, autophagy also operates under physiological conditions when the nutrients are plenty.

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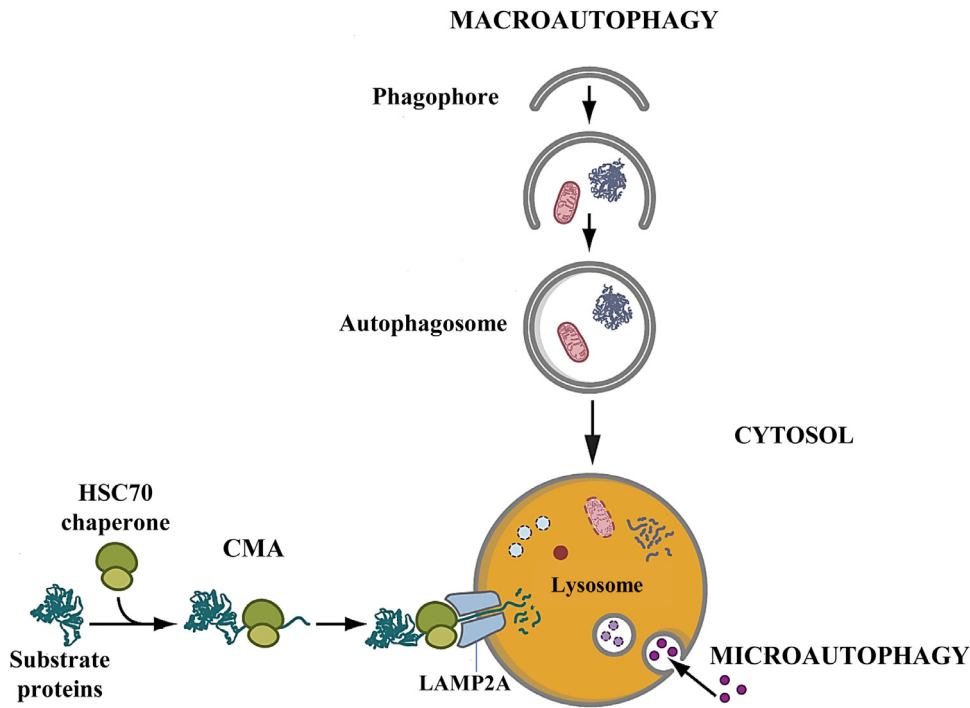


Fig. 1. Three types of autophagy.

All three types – macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) – converge on the lysosome. HSC70: heat shock-cognate chaperone 70 KDa protein; LAMP2A: lysosome-associated membrane protein 2 isoform A.

Under these conditions, autophagy is a highly selective process that rids the cells of worn-out organelles (including lysosomes themselves), toxic protein aggregates, intracellular pathogens, and other materials. The cargo is tethered to the growing phagophore by the action of specific receptors and adaptor proteins [8,9]. Autophagic flux refers to the overall rate of autophagic degradation activity: The flux is complete when the formation of autophagosomes is followed by their fusion with lysosomes and degradation of the autophagosomal content. If, however, autophagosomes fail to fuse with lysosomes, the flux is incomplete, a condition known as autophagic block [2].

The complete sequence of events comprising the macroautophagy process requires the recruitment and assembly of multiple autophagy-related protein complexes (referred to as “the autophagy machinery”). Here, we will name only those few that have been most commonly used to evaluate autophagy in LSDs.

Microtubule-associated protein light chain 3 (LC3) is a widely used marker of autophagosomes owing to its ability to form a stable association with the autophagosomal membrane. The protein exists in two forms: A cytosolic LC3-I and a lipidated membrane-bound LC3-II which has a faster mobility on SDS PAGE than LC3-I [10]. Elevated levels of LC3-II indicate either induction of autophagy or impaired fusion with lysosomes or both. Accurate measurement of autophagic flux still remains a challenge. Most commonly used techniques include inhibition of lysosomal function (i.e. by chloroquine) and evaluation of the level of autophagy substrates, such as ubiquitinated proteins and p62/SQSTM1 (sequestosome-1) [11]. A failure to increase LC3-II following chloroquine treatment and elevated levels of p62/SQSTM1 indicate an impairment of the flux.

A tandem-RFP-GFP tagged LC3 reporter [12] allows to distinguish autophagosomes from autolysosomes, a product of fusion between autophagosomes and lysosomes. Since GFP signal is quenched in the acidic pH of the lysosome, autophagosomes fluoresce in both red and green channels, whereas only red fluorescence remains following fusion with lysosomes. Autophagic flux can also be monitored by

using a CYTO-ID® Autophagy Detection Kit (Enzo) which utilizes a novel dye that selectively labels accumulated autophagosomes and autolysosomes; the assay is designed to monitor autophagy in live cells without the need for transfection.

The discovery of the transcription factor EB (TFEB) as a master regulator of lysosomal biogenesis [13] was of great importance in the field. The promoter region of many lysosomal genes was shown to contain one or more TFEB binding sites [10 base-pair sequence (GTCACGTGAC) named Coordinated Lysosomal Expression and Regulation (CLEAR)] motif [13,14]. Subsequent studies demonstrated that TFEB and TFE3 (another member of the same MiT/TFE family of transcription factors) upregulate the expression of genes involved not only in lysosomal but also in autophagosomal biogenesis, thereby establishing a coordinate upregulation of both lysosomes and autophagosomes [15,16]. These discoveries mark a seismic shift in the way we view the autophagy-lysosomal system. It makes perfect sense that a surge in the number autophagosomes should be matched with the increased degradative capacity of lysosomes. Moreover, these findings elevate the status of the lysosome to that of an active participant rather than a passive recipient in the autophagic process [17].

Nuclear translocation (activation) of TFEB/TFE3 is controlled by a number of kinases and phosphatases [18]. Among them, the most studied is the mammalian target of rapamycin complex 1 (mTORC1), a nutrient sensing protein kinase and a major regulator of cell growth and metabolism. When cells have sufficient amino acids, mTORC1 is recruited to the lysosome through Rag GTPases where it is activated by RHEB (Ras homolog enriched in brain); active mTORC1 phosphorylates TFEB (Ser211) and TFE3 (Ser321) and keeps them in the cytoplasm through binding to the 14-3-3 protein [16,19,20]. Under starvation, mTORC1 moves away from the lysosome, becomes inactive [21], and the less phosphorylated TFEB/TFE3 translocate to the nucleus where they trigger the expression of autophagy- and lysosome-related genes. Thus, in fed cells, active mTORC1 promotes cell growth and represses autophagy indirectly through TFEB/TFE3

phosphorylation. In addition, mTORC1 directly regulates autophagy by inhibitory phosphorylation of autophagy-initiating kinase Ulk1 (Ser 757) leading to a robust suppression of autophagosome formation [22].

Autophagy has been studied in a number of LSDs including mucopolysaccharidoses, sphingolipidoses, mucopolidosis, etc. [5,23]. Compromised autophagy emerges as a major common secondary abnormality in a complex pathogenic cascade culminating in tissue damage in many of LSDs, most of which are neurodegenerative disorders. Importantly, the survival of post-mitotic cells, such as neurons and muscle cells, relies heavily on autophagy to efficiently remove cellular waste and toxic materials. Here we review the findings of a defective CMA in a LSD, cystinosis, and discuss how the autophagy defects are uniquely expressed in several neurological and muscle LSDs (Fig. 2).

2. Cystinosis and CMA: the plot thickens

Cystinosis is a rare LSD caused by mutations in the gene that codes for the lysosomal membrane cystine transporter, cystinosin (CTNS). Faulty cystinosin function leads to the accumulation of the amino acid cystine in the lysosome [24,25]. Clinically, three major phenotypes are recognized: The most severe infantile nephropathic form leading to fatal renal failure in the first decade of life; the intermediate juvenile nephropathic form; the ocular non-nephropathic form without renal or systemic abnormalities. Both nephropathic forms account for ~95% of all cases.

Poorly soluble cystine forms crystals at the low pH of the lysosome leading to tissue deterioration throughout the body, including the kidney, liver, retina, muscle, endocrine glands, and the CNS. The renal tissue, in particular the kidney epithelial proximal tubule cells (PTCs), are most susceptible to the lysosomal cystine overload. Cystinosis is a major cause of renal Fanconi syndrome in children, a condition characterized by the failure of renal proximal tubular reabsorption leading to urinary loss of low-molecular weight proteins

and solutes. A typical narrowing of the proximal renal tubule (atrophy), known as swan-neck deformity, is a hallmark of the disease. If left untreated, glomerular dysfunction may lead to renal failure and the need for a kidney transplant [24,25].

Early studies reporting genotype-phenotype correlation identified patients with mild (rather than severe) illness despite near complete absence of CTNS activity [26], suggesting that additional mechanisms may be involved in the disease pathogenesis. Another clue came from data that showed the persistence of kidney disease (Fanconi syndrome) despite treatment with cystine-depleting drug, cysteamine, which has been used for years to alleviate the symptoms of cystinosis. Cysteamine interacts with cystine in the lysosome to form a cysteine-cysteamine mixed disulfide that can exit the lysosome using normal PQLC2, a lysosomal membrane transporter that differs from cystinosin [27].

Analysis of *Ctns*-deficient neonatal fibroblasts from *Ctns* knockout mice demonstrated that although the number of autophagosomes was increased both in vitro and in vivo (in the liver and kidney sections), their maturation and fusion with lysosomes was not affected, indicating that autophagic flux was fully functional [28]. These data suggested that the increase in the number of autophagosomes may have been a compensatory mechanism to counteract the deficiency of another form of autophagy – CMA. Indeed, markedly decreased levels of LAMP-2A (a lysosomal receptor of CMA) were found in *Ctns*-deficient fibroblasts; importantly, this decrease was observed in the context of overall increase in the total number of LAMP1 (another lysosomal membrane protein) positive lysosomes. Furthermore, LAMP-2A was misplaced in the diseased cells and co-localized with Rab11a-positive carrier vesicles (recycling endosomes) rather than with its natural partner LAMP1 at the microdomains of lysosomal membranes [28]. The impairment of CMA was further confirmed in vivo: Purified lysosomes from livers of starved *Ctns*^{-/-} mice were unable to efficiently degrade glyceraldehyde- 3-phosphate dehydrogenase (GAPDH), the substrate of CMA. The overexpression of WT cystinosin (or treatment with CMA enhancers) but not cysteamine

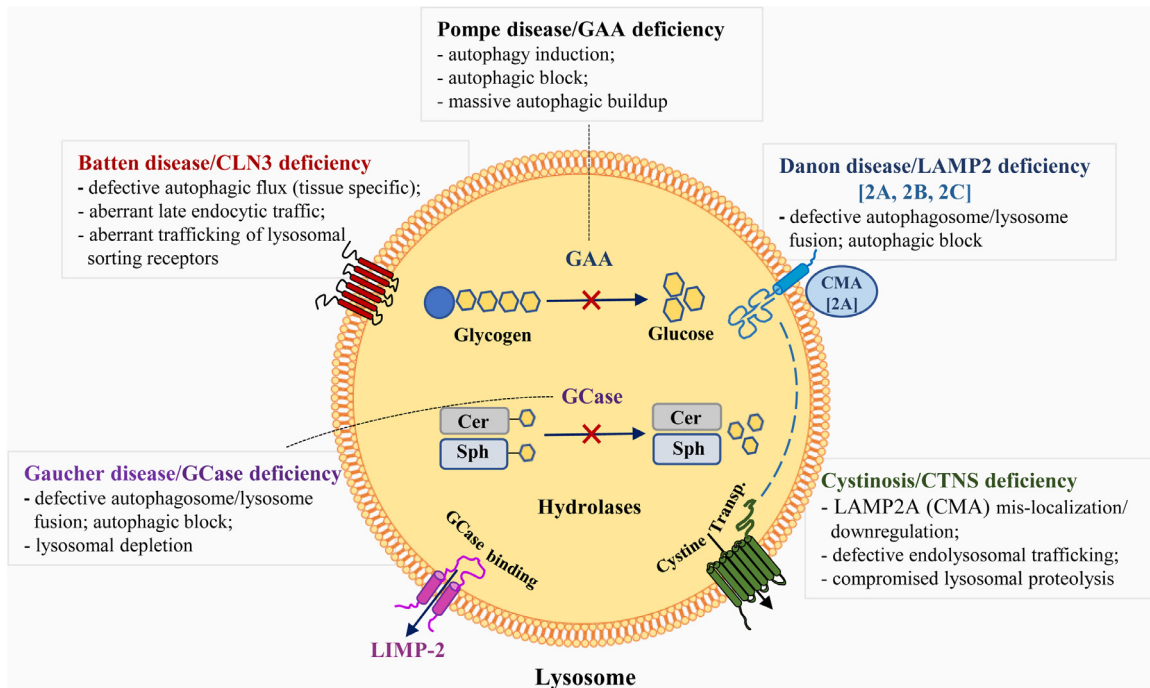


Fig. 2. Localization of affected proteins involved in five LSDs and their impact on autophagy.

GAA: acid alpha-glucosidase; CLN: ceroid lipofuscinosis neuronal; LAMP: lysosome-associated membrane protein; CMA: chaperone-mediated autophagy; GCase: glucocerebrosidase; Cer: ceramide; Sph: sphingosine. The dashed blue line indicates the link between cystinosin and CMA; LIMP-2 is a receptor for GCase.

treatment rescued aberrant CMA, indicating an additional function of CTNS that is independent of its cystine transporter activity [28].

Defective LAMP-2A trafficking in cystinotic cells was also linked to a markedly decreased expression of both Rab11 and the Rab7 effector RILP (Rab-interacting lysosomal protein). Upregulation of both proteins was sufficient to correct LAMP-2A mistargeting and improve oxidative stress-induced cell death in cystinotic cells [29].

The molecular mechanisms involved in the dual role of CTNS was further refined using a new in vitro model - human cystinosis-deficient PTC line (CTNS-KO PTCs) [30]. As in other cystinotic cellular systems, the new cell line exhibited apical PTC dedifferentiation, functional autophagic flux, and mis-localization and decreased LAMP-2A and Rab11 expression. In addition, a diminished expression and decreased apical plasma membrane localization of megalin were observed in the diseased cells. Megalin mediates the endocytosis of various plasma solutes for ultrafiltration and subsequent lysosomal degradation, and its apical distribution and recycling is a microtubule- and Rab11-dependent process [31]. CMA upregulation rescued the expression and localization of megalin in CTNS-KO PTCs [30].

Yet another molecule involved in endolysosomal trafficking - the dynein subunit, intermediate chain 2 (DYNC1LI2) - was shown to be downregulated in cystinotic cells [32]. The dynein family, a group of minus-end-directed microtubule motors, controls multiple cellular functions by transporting vesicles and organelles in a process called retrograde transport. DYNC1LI2 reconstitution in *Ctns*^{-/-} fibroblasts and PTCs re-established vesicular trafficking, rescued megalin expression, restored LAMP-2A lysosomal localization, and improved survival. This rescue required functional Rab7 and Rab11 GTPases, thus providing a functional link between DYNC1LI2 and these two GTPases [32].

Defective trafficking appears to be unique for LAMP-2A in cystinotic cells since other LAMPs reach the lysosome and function normally. Hence, cystinosis appears to function as a modulator of LAMP-2A trafficking to the lysosomal membrane in addition to its long-known role as cystine transporter.

Finally, although the impairment of CMA in cystinosis is not controversial, the involvement of macroautophagy is. An increased number of autophagosomes and their stalled clearance in the lysosome (abnormal autophagy flux) due to the defective cathepsin activation leading to a compromised lysosome proteolysis, were found in cystinosis-deficient PTCs [33]. Moreover, a recent study demonstrated an important role of cystinosis in the mTORC1-mediated nutrient sensing in kidney proximal tubular cells [34]. Thus, it appears that other functions may be added to the cystinosis repertoire.

3. Gaucher disease: the most common lysosomal storage disorder

Gaucher disease resides in a subcategory of the greater domain of lysosomal storage disorders called the sphingolipidoses. Their hallmark is storage of certain glycosphingolipids (GSLs), important building blocks of the outer leaflet of the cell membrane. The disease bears the name of the physician, Earnest Gaucher, who described the first case in 1882. Glucosylceramide (glucosylcerebroside) and glucosylsphingosine are the GSLs that accumulate mainly in the macrophages (referred to as "Gaucher cells") residing in the liver, spleen, lung and the CNS due to mutations in the gene (*GBA1*) coding for glucocerebrosidase [35]. Enlarged spleen, liver, anemia, thrombocytopenia, and bone disease are clinical features most frequently associated with Gaucher disease. The disorder is further classified into three types where Types 2 and 3 involve the CNS and the former is a clinically more severe form than the latter [35]. While the most common Type 1 displays no neuropathic involvement at onset during early childhood years, Gaucher patients as well as heterozygote carriers appear to harbor an increased risk for the development of Parkinson's disease later in life [36,37]. Glucocerebrosidase (GCCase) utilizes lysosomal integral membrane protein 2 (LIMP-2) encoded by the *SCARB2*

gene rather than mannose-6-phosphate receptor to gain entry into the lysosome [38]. The highly conserved amino acid sequence on GCCase that binds to LIMP-2 has been identified [39]. A mutation in *SCARB2* in addition to one in glucocerebrosidase could modify its expression and thereby affect the Gaucher phenotype [40].

Dysfunctional autophagy has been reported in several in vitro and in vivo models of Gaucher disease. Studies in a neuropathic mouse model displayed storage of glucosylsphingosine and glucosylceramide as well as the accumulation of membranous vesicles that resembled nonfunctional autophagosomes; accumulation of LAMP2 and p62/SQSTM1 in the brain suggested the impairment of autophagosome/lysosome fusion [41]. In addition, ultrastructural analysis in a neuron-specific glucocerebrosidase-deficient neuropathic mouse model displayed degenerating glucosylceramide-filled neurons containing autophagosomes, massive inclusions, and unusual ultrastructural features [42].

Autophagy was also examined at the cellular level by reprogramming iPSCs derived from Gaucher patients to differentiate into neurons. An increase in the number of autophagosomes, LC3-II and p62/SQSTM1 was observed in neuropathic but not in non-neuropathic cells; a further detailed analysis of neuropathic cells indicated defective autophagic flux and decreased autophagosomal clearance. In addition, there was a decrease in the levels of LAMP1 and TFEB, suggesting that lysosomal depletion and dysfunction may contribute to neurodegeneration in neuropathic cells. These features were ameliorated following treatment of the neurons with recombinant glucocerebrosidase, and the effect was enhanced when combined with TFEB overexpression [43]. In a recent follow up study, the researchers documented activation of mTORC1 in neuropathic iPSC neurons and suggested that the TFEB deregulation is linked to altered lipid sensing by mTORC1 [44].

In contrast, a decreased number of autophagic vacuoles and suppressed levels of LC3-II and p62/SQSTM1 were found in peripheral blood mononuclear cells (PCMB) isolated from the blood of Gaucher patients exhibiting both the neuropathic and non-neuropathic types, the results interpreted as inactivation of constitutive autophagy [45].

A neuropathic Gaucher disease model was also developed in *Drosophila melanogaster* which mimicked the human disorder [46]. Increase in Atg8-II (a homologue of LC3 in mammals) and accumulation of autophagy substrates, such as polyubiquitinated proteins and Ref(2)P (homologue of p62), suggested autophagic block in glucocerebrosidase deficient fly brain. The model displayed decreased mTOR activity and increased activity of the fly equivalency of TFEB (Mitf), the findings opposite to those in neuropathic iPSC neurons [43,44]. Further inhibition of mTOR with rapamycin corrected the disease pathology of the fly model [46]. Thus, the impairment of autophagy is documented in a variety of model system, but the underlying mechanisms remain controversial.

4. Batten disease: a mystery story

Batten disease, a disorder first described over a century ago, is currently an umbrella term which includes a group of thirteen diseases that are all fatal, inherited disorders of the nervous system, each of which is caused by a different mutant gene. Each of the thirteen disorders bears the common abbreviation CLN, ceroid-lipofuscinosis neuronal, followed by a distinguishing numeral. Neuronal accumulation of autofluorescent lipopigments resembling ceroid and lipofuscin as well as mitochondrial ATP synthase subunit c are found in CLN patients [47,48]. CLN3 is the most commonly occurring subtype. Clinically, CLN3 is a fatal autosomal recessive disorder of the nervous system, and typically appears in children between the ages of 5 and 10. Clinical manifestations include progressive loss of vision, seizures, loss of motor function, cognitive decline, and psychosis; most patients die in their twenties [48].

The most commonly found defect (a founder mutation) is a homozygous deletion of 966 base pairs inclusive of exons 7 and 8 ($\Delta\text{ex7/8}$) which results in a premature stop codon in exon 9 of the 15-exon gene [49]. The promoter of the *CLN3* gene contains CLEAR elements which makes it a part of the TFEB-regulated gene network [13,14], thus opening a possibility of TFEB modulation as a therapeutic approach to Batten disease [50]. While much detailed information has been documented concerning the normal *CLN3* gene as well as its mutant forms, the real function of the *CLN3* protein remains unknown. The problem is confounded by the lack of antibodies able to detect endogenous protein; therefore, most studies rely on the *CLN3* overexpression. We do however know that the structure of this mysterious protein clearly resembles that of a transmembrane protein [51], and that the protein predominantly localizes in the late endosomal-lysosomal compartments [49,52].

Dysfunctional autophagy was demonstrated in *Cln3 $\Delta\text{ex7/8}$* knock-in mice [53] and *Cln3 $\Delta\text{ex7/8}$* cerebellar cells, as indicated by the increase in LC3-II, downregulation of mTORC1, accumulation and defective maturation of autophagosomes, and altered turnover of ATP synthase subunit c [54].

Consistent with these data, defective autophagic flux was shown in *Cln3 $\Delta\text{ex7/8}$* -expressing HEK-293 cells, a conclusion based on the levels of LC3-II and p62/SQSTM1 and the outcome of overexpression of the RFP-GFP dual-labeled LC3 reporter [55]. Moreover, normalization of autophagic flux in these cells and in the *Cln3 $\Delta\text{ex7/8}$* model was achieved by using an antisense oligonucleotide that restored the open reading frame of the mutant gene [55]. Interestingly, loss of *CLN3* protein in the disease-relevant retinal pigment epithelium cells was associated with increased rather than decreased autophagic flux, suggesting a cell-specific effect of *CLN3* deficiency [56].

The absence of a defined function for the normal *CLN3* protein has made delineation of the mechanism of autophagosome-lysosome pathway disturbances by mutant *CLN3* quite difficult. *CLN3* has been implicated in the control of late endocytic traffic through interaction with Rab7A, a marker of late endosomes, and its downstream effector RILP (the Rab7-interacting lysosomal protein) [57]. Rab GTPases belong to a class of membrane trafficking regulators; they cycle between an active GTP-bound and inactive GDP-bound form and perform numerous cellular functions [58].

A recent study confirmed the interaction between *CLN3* and Rab7A in live cells and demonstrated that *CLN3* regulates Rab7A interaction with retromer, a protein complex required for efficient endosome-to-TGN trafficking of the lysosomal sorting receptors, such as cation independent mannose 6-phosphate receptor (CI-MPR) and sortilin, which were degraded in cells lacking *CLN3* or expressing mutant *CLN3* [59]. In addition, *CLN3* was shown to regulate Rab7A interaction with PLEKHM1 (pleckstrin homology domain containing protein family member 1), a protein involved in autophagosome-lysosome fusion [59]. This offers a possible pathogenic mechanism for Batten disease.

5. Pompe disease: first-in-class

Pompe disease was the first illness to be recognized as a LSD in 1963, with the discovery of lysosomal acid alpha-glucosidase (GAA) as the cause of massive deposition of glycogen in multiple tissues of infants [60]. The disease was medically described some thirty years prior by the Dutch pathologist J. C. Pompe. Fast forward to present day, and one will find that a myriad of discoveries germane to Pompe disease have been made [61]. Moreover, we now have years of experience with the first disease-specific treatment, enzyme replacement therapy (ERT), which was approved in 2006.

Pompe disease manifests as a continuum of clinical phenotypes. In the most severe classic infantile onset form (IOPD) with near complete lack of enzyme activity, the symptoms of muscle weakness, respiratory distress, and cardiomyopathy become apparent within a

few months after birth, and most babies, if untreated, die within the first year of life from cardiac failure [62]. In the less severe childhood and adult forms, cardiac muscle is usually spared, but slowly progressive muscle weakness, particularly the diaphragm, eventually leads to respiratory insufficiency and shortened lifespan [61].

Both pre-clinical studies in a mouse model and clinical data indicate that ERT with recombinant human GAA (rhGAA; alglucosidase alfa, Genzyme, a Sanofi Company) reverses the pathology of the heart and restores cardiac function. It is therefore no surprise that ERT provides the most benefit to IOPD patients who survive significantly longer than untreated patients and many are now in their teens. However, neurological manifestations of the disease are not amenable to ERT, and skeletal muscle, the target tissue affected in all forms of the disease, responds poorly to therapy [63–65].

A massive autophagic buildup in skeletal muscle is one of the culprits that contributes to the suboptimal response to therapy by negatively affecting the lysosomal delivery of the drug [66,67]. Both electron microscopy (EM) and immunostaining of single myofibres with lysosomal (LAMP1) and autophagosomal (LC3) markers point to the strikingly large autophagic areas that contain multivesicular bodies, glycogen, and multimembrane concentric structures in addition to classical double-membrane autophagosomes and glycogen-loaded lysosomes with broken borders [23]. The extent of this pathology in Pompe disease justifies its inclusion along with Danon disease and other disorders in a group of autophagic vacuolar myopathies [68].

The relevance of autophagy to the pathogenesis of muscle damage in Pompe disease is underscored by the evidence that glycogen, at least partially, enters the lysosome via autophagic pathway. In fact, glycogen particles are commonly seen inside double-membrane autophagosomes by EM, and muscle-specific inactivation of a critical autophagic gene, *Atg7*, alleviates lysosomal glycogen burden in a mouse model [69].

It appears that a combination of induction of autophagy and autophagic block is responsible for the autophagic defect in Pompe muscle. This conclusion is based on the increase in the levels of proteins involved in the initial steps of autophagy, such as VPS15, VPS34, and Beclin1, and on the time-lapse microscopy of live fibres, in which autophagosomes and lysosomes were stained with LC3 and LAMP1 respectively [70–72].

Induction of autophagy in Pompe skeletal muscle is supported by the findings of diminished mTORC1 activity and activation of AMP-activated protein kinase (AMPK) [72]. The nutrient-sensitive mTORC1 and the energy-sensing AMPK are two major kinases that control metabolic programs and have largely opposing effects. Like mTORC1, AMPK is also activated at the lysosome in response to low cellular energy levels (as is the case in Pompe muscle [72]) and promotes autophagy on two fronts - by TSC2 (the tuberous sclerosis complex)-mediated inhibition of RHEB (an activator of mTORC1) and by activating ULK1 at multiple sites (e.g Ser317 and Ser777) that are different from those of mTORC1 [22].

Indeed, AMPK-mediated phosphorylation of TSC2^{S1387} and ULK1^{S317} were markedly increased in Pompe muscle cells, whereas mTORC1 activity was decreased [72,73]. Therefore, it appears that at least some pieces of the puzzle come together to explain autophagy defects in Pompe disease. Defective autophagy leads to oxidative stress, accumulation of aberrant mitochondria and autophagic substrates, such as p62/SQSTM1 and potentially toxic ubiquitinated protein aggregates. As a proof-of-principle, a successful attempt to alleviate the burden of autophagy in the affected muscle was made by overexpression of TFEB in myofibres from *GAA^{-/-}* mice [70]. Finally, last but not least, the recently developed new rhGAA (AT-GAA, Amicus Therapeutics) [74] and gene therapy [75] address dysfunctional autophagy in Pompe disease much more efficiently than the current drug (Table 1). A significantly higher mannose 6-phosphate (M6P) content (both mono- and bis-phosphorylated forms) of AT-GAA compared to a very poorly phosphorylated alglucosidase alfa

Table 1
Approved therapies and clinical trials.

Cystinosis	Cystagon® Cysteamine Bitartrate Procysbi® (an extended release form of cysteamine) ERT: Cerezyme® (imiglucerase) VPRIV® (velaglucerase alfa) Elelyso® (taliglucerase alfa)
Gaucher disease	SRT: Cerdelga® (eliglustat) Zavesca® (miglustat)
Batten disease (CLN3)	Gene therapy: AT-GTX-502; AAV9-CLN3 Phase 1/2 NCT03770572 ERT: Myozyme/Lumizyme (alglucosidase alpha) Neo-GAA (avalglucosidase alfa) Phase 3/ NCT02782741 AT-GAA Phase 3/NCT03729362; Phase 3/ NCT03911505
Pompe disease	Gene Therapy: rAAV9-DES-hGAA Phase 1/2 NCT02240407 AAV2/8-LSPhGAA Phase 1/2 NCT03533673 SPK-3006 Phase 1/2 NCT04093349
Danon disease	Gene therapy: RP-A501; AAV9.LAMP2B Phase1 NCT03882437

ERT: Enzyme Replacement Therapy; SRT: Substrate Reduction Therapy; AAV: Adeno-associated virus. Clinical trials listed in this table are registered as of October 2020.

accounts for the superior effect of this new experimental drug. The presence of M6P moieties on the carbohydrate chains of lysosomal enzymes is a prerequisite for their efficient CI-MPR (cation-independent mannose-6-phosphate receptor)-mediated cellular uptake and lysosomal trafficking [76]. The advantage of gene therapy vs ERT is the continuous and sustained expression of the transgene as a source of GAA rather than a transient increase in enzyme activity following intermittent administrations of rhGAA at 2-week-intervals [77].

6. Danon disease: a curious twist

Almost forty years ago, the disease was defined as “Lysosomal glycogen storage disease with normal acid maltase” [78]. This definition was based on the examination of two boys, whose muscle biopsies were read as vacuolar autophagic myopathy with glycogen-laden lysosomes, similar to those seen in Pompe disease but with normal acid alpha-glucosidase (acid maltase) activity. As more cases were described, it became clear that this myopathy, much like chloroquine myopathy, does not involve changes in glycogen metabolism, and that the condition is a separate entity exhibiting X-linked inheritance [79]. Because glycogen is only one of the constituents that accumulate in the autophagic vacuoles, Danon disease is no longer included in the group of the glycogenoses.

Clinically the disease displays severe hypertrophic cardiomyopathy, heart failure, muscle weakness, retinopathy, and variable degrees of mental retardation in male patients. Female patients present with a milder phenotype mostly limited to cardiac abnormalities [80]. The hallmark of the disease in skeletal muscle is the presence of large LC3-positive membrane-bound structures, termed “autophagic vacuoles with unique sarcolemmal features (AVSF)” [81]. Increased vacuolization, lipofuscin accumulation, and myofibrillar disruption are also seen in cardiac muscle [71,80,81].

The disease is caused by mutations in the gene on chromosome Xq24 encoding lysosome-associated membrane protein 2 (LAMP-2) [82]. Extensive accumulation of autophagic vacuoles in multiple tissues was replicated in the LAMP-2 knockout mouse model [83]. This was the first example of cardiac and skeletal muscle myopathy caused by defects in lysosomal structural proteins rather than lysosomal enzymes.

The failure of autophagosomal-lysosomal fusion, excessive accumulation of autophagosomes, and partial mistargeting of a subset of lysosomal enzymes were documented in LAMP-2-deficient hepatocytes [84]. A block in autophagy that correlated with the disease severity was also found in patients' muscle biopsies, as indicated by the increase in LC3 and accumulation of 62/SQSTM1. In addition, altered localization of VPS15, a critical factor for autophagosome biogenesis/trafficking, and activation of TFEB were seen in the diseased muscle [71].

LAMP-2 protein is primarily localized in the limiting membranes of late endosomes/lysosomes; it contains a short cytoplasmic C-terminal tail with a lysosomal/endosomal targeting signal, a single transmembrane domain, and a large highly glycosylated intraluminal N-terminal domain. Three LAMP-2 isoforms – 2A, 2B, and 2C [81] – arise by alternative splicing of exon 9, the last exon of the gene, and they share identical luminal domains but have distinct transmembrane and cytoplasmic domains. All three isoforms, expressed in a tissue specific manner, are implicated in different types of autophagy: The cytoplasmic tail of LAMP-2A serves as a receptor in CMA [6,7]; LAMP-2B is involved in macroautophagy; LAMP-2C is thought to control the lysosomal uptake and selective degradation of RNA and DNA (RNautophagy and DNautophagy) mainly in the brain [85,86].

Most Danon patients carry mutations that result in deficiency of all three LAMP-2 isoforms. However, the disease pathogenesis and manifestations are largely attributed to the absence of LAMP-2B (abundant in the heart, skeletal muscle, and brain) since the defect of LAMP-2B alone is sufficient to cause the whole range of the disease manifestations [82].

Analysis of human cardiomyocytes demonstrated that, indeed, LAMP-2B is required for autophagosome–lysosome fusion through the interaction of its cytoplasmic tail with autophagosome-localized ATG14 (autophagy related 14) and the endosomal/lysosomal VAMP8 protein (vesicle-associated membrane protein 8) [87]. Cardiomyocytes derived from iPSCs (hiPSC-CMs) from Danon patients show decreased colocalization between ATG14 and VAMP8 and defective autophagosome–lysosome fusion. These findings elucidate the mechanism of cardiomyopathy in Danon patients. Thus, the LAMP-2B-mediated impairment of autophagosomal-lysosomal fusion appears to be the underlying pathogenic mechanism in Danon disease. However, the fusion between autophagosomes and lysosomes was not completely blocked in LAMP-2B knockout cells [87], indicating that other pathways may play a role.

7. Outstanding questions

The autophagic pathway has been examined in a significant number of LSDs. Those subject to such scrutiny have shown impairment of this pathway – the collateral damage of lysosomal storage. The selected five disorders showcase the idea that compromised autophagy is a universal feature of many LSDs. The question is whether correction of the lysosomal storage would take care of the autophagic abnormalities or an additional autophagy-targeting therapy upstream of lysosome may be needed. Given that the block of autophagic flux appears to be a shared feature in many LSDs [23], the attempts to overcome/reduce this blockage by stimulating autophagy seem reasonable. Autophagy induction by rapamycin – the mTORC1 allosteric inhibitor – has shown benefits in a small subset of LSDs, including a *Drosophila* model of Gaucher disease [5,46]. However, the caveat is that long-term rapamycin treatment leads to immunosuppression, thus precluding a broad use of the drug for the therapy of LSDs. An alternative, mTORC1-independent activation of autophagy, was achieved by the administration of trehalose, a disaccharide which activates TFEB and promotes autophagic flux; this approach was successfully used in a mouse model of Batten disease [50]. On the other hand, lysosomal glycogen clearance in Pompe skeletal muscle was observed when the standard of care (ERT) was combined

with suppression rather than activation of autophagy [69]. Thus, it appears that “one-size-fits-all” does not work. As demonstrated in this review, the devil is in the details – the exact breaking point in the autophagic process is likely to vary significantly in each of the myriad of LSDs.

In cystinosis, the cysteamine therapy greatly reduces lysosomal cystine clearance, but the early and severe dysfunction of the kidney epithelial proximal tubule cells (Fanconi syndrome) is not amenable to the therapy. The exact mechanism linking lysosomal defect and abnormal proliferation and dysfunction of these cells is the subject of controversy. Some researchers view the upregulation of macroautophagy in the context of fully functional autophagic flux as a protective mechanism to compensate for the deficiency of chaperone-mediated autophagy, whereas others present evidence of abnormal autophagic flux due to the inability of lysosomes to efficiently degrade the autophagosomal cargo. The reason for the discrepancy requires further studies.

In Gaucher disease, both inactivation of basal autophagy and an increase in the number of autophagosomes and their stalled clearance in the lysosome have been reported. Are there tissue and cell-specific effects of glucocerebrosidase deficiency and if so, what does it mean? Also, a decrease in the levels of TFEB in GBA1-deficient neuropathic cells does not agree with the findings in the *Drosophila* disease model, and raises the question of evolutionary conservation.

As to Batten disease, the outstanding question is straightforward: What is the function of the CLN3 protein?

The induction of autophagy in Pompe skeletal muscle is well-established, but it is still not clear what triggers this induction. It is also not clear whether recently developed experimental drugs for enzyme replacement therapy or gene therapy will be able to fully reverse the autophagic defect in skeletal muscle irrespective of when the therapy begins.

The molecular mechanism underlying defective autophagy in Danon disease is not controversial - LAMP-2 isoform B appears to be responsible for the autophagosomal-lysosomal fusion in cardiomyocytes. However, this fusion is not completely blocked in the absence of LAMP-2B which implies the involvement of other LAMP-2B-independent pathways in these cells. What are they?

7.1. Search strategy and selection criteria

Data for this Review were identified by searches of PubMed and Google Scholar, and references from relevant articles using the search terms “lysosome”, “autophagy”, and name of the disease. Only articles published in English between 1980 and 2020 were included except for the early references describing discoveries of LSDs. Considering constraints for the number of references, this manuscript includes many review articles in the bibliography.

Contributors

RM: collected and analyzed the literature, and shared in the writing; RP: provided intellectual input, analyzed the literature, and participated in the writing; NR: selected the subject material, collected and analyzed the literature, and wrote the paper. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors have nothing to disclose.

Acknowledgements

This work was supported by the Intramural Research Program of the NHLBI of the National Institutes of Health. The funder has no role in the writing of this review article.

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