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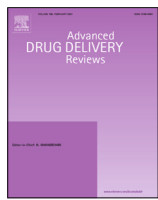
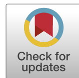


Models to study basic and applied aspects of lysosomal storage disorders



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A R T I C L E I N F O

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A B S T R A C T

The lack of available treatments and fatal outcome in most lysosomal storage disorders (LSDs) have spurred research on pathological mechanisms and novel therapies in recent years. In this effort, experimental methodology in cellular and animal models have been developed, with aims to address major challenges in many LSDs such as patient-to-patient variability and brain condition. These techniques and models have advanced knowledge not only of LSDs but also for other lysosomal disorders and have provided fundamental insights into the biological roles of lysosomes. They can also serve to assess the efficacy of classical therapies and modern drug delivery systems. Here, we summarize the techniques and models used in LSD research, which include both established and recently developed in vitro methods, with general utility or specifically addressing lysosomal features. We also review animal models of LSDs together with cutting-edge technology that may reduce the need for animals in the study of these devastating diseases.

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Abbreviations: AAV9, Adenoassociated Viral Vectors serotype 9; ASM, Acid Sphingomyelinase; ASMko, Acid Sphingomyelinase Knock-out; BBB, Blodd-Brain Barrier; BODIPY, Boron dipyrromethene; CNS, Central Nervous System; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; EM, Electron Microscopy; ER, Endoplasmic Reticulum; GAL4/UAS, GAL4/Upstream Activating Sequence; GFP, Green Fluorescent Protein; hiPSCs, human induced Pluripotent Stem Cells; iPSCs, induced Pluripotent Stem Cells; LAMP1/2, Lysosomal Associated Membrane Protein 1/2; LPC, Lysophosphatidylcholine; LPS, Lysophosphatidylserine; LSD, Lysosomal Storage Disorder; mTORC1, mammalian Target Of Rapamicine Complex 1; NBD, Nitrobenzoxadiazole; OMIM, Online Mendelian Inheritance in Man; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PS, Phosphatidylserine; PC-O, Plasmenylcholine; PE-O, Plasmenylethanolamine.

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1. Introduction

Due to their key role in the breakdown of cellular components [1], and for many years since their discovery in 1950 [2], lysosomes were considered merely "cellular trash cans". Nowadays, a plethora of functions beyond the degradation of intra- and extracellular molecules and organelles [3] have been assigned to lysosomes, including nutrient sensing [4], the quality control system involved in the ageing process [5] and participation in innate and adaptive immunity [6,7] (Fig. 1).

Aside from their involvement in multiple cellular processes, lysosomes make physical connections with several intracellular organelles such as mitochondria [8], the endoplasmic reticulum [9] or plasma membrane [10] by membrane tethering and fusion. These contact sites are crucial for the correct function of not only lysosomes, but also the subcellular organelles that fuse with them [11,12]. Proper lysosomal activity is vital for adequate cell, tissue and organ functions, and lysosomal impairment plays a relevant pathological role in many different diseases including cancer [13] and neurodegenerative [14–16] or autoimmune diseases [17].

Among the lysosome-related diseases are the lysosomal storage disorders (LSDs), whose aetiology is directly linked to lysosomal dysfunction. Most LSDs are caused by mutations in genes that encode lysosomal enzymes, resulting in more than 70 different rare diseases characterised by the lysosomal accumulation of molecules, in many cases the very substrates of the defective enzymes [18].

One way to classify LSDs is according to the type of molecule that accumulates in the lysosomes. This classification scheme (Table 1), which will be used to organize this review, includes three major groups: sphingolipidoses, mucopolysaccharidoses and glycoproteinoses/oligosaccharidoses [53] (Fig. 1). Sphingolipidoses are caused by defects in the lysosomal degradation of sphingolipids, and the accumulation of lipid in these diseases causes membrane defects that can affect cell survival, especially of neurons. As a result, neurodegeneration along with visceral complications are characteristic of many sphingolipidoses [54]. Mucopolysaccharidoses are the consequence of defects in the degradation of glycosaminoglycans (complex amino-sugar polymers). These diseases progress with both peripheral (i.e. upper airway obstruction or cardiac valve and muscle disease) and neuropathic (i.e. hyperactivity, impaired development and loss of cognitive function) features [55]. The third group, glycoproteinoses/oligosaccharidoses result from alterations in catabolism of oligosaccharide chains from glycolipids and glycoproteins, leading to the accumulation of these molecules. Their clinical manifestations are similar to those already mentioned for the mucopolysaccharidoses [56].

Besides the LSDs directly caused by the impaired function of lysosomal metabolic enzymes, some LSDs are the consequence of alterations in proteins that control trafficking of molecules through the endolysosomal compartment [57] or in lysosomal membrane proteins. This group of disorders are also characterized by storage inside lysosomes. Representative examples are Niemann-Pick type C (OMIM 257220; 607625), which results from mutations in the cholesterol transport proteins NPC1 or NPC2 [58,59] or Danon disease (OMIM 300257), which is caused by mutations in the gene encoding the integral lysosomal membrane protein LAMP2 [60].

Identification of the genetic cause in many LSDs has not been accompanied by a deep knowledge of the pathological mechanisms, much less by efficient treatments. Among the important caveats of current LSD therapies is the delivery of therapeutics for these diseases. Drug delivery systems may target and traffic to lysosomes after entering cells. However, accumulation of therapeutics in these organelles prior to degradation could cause deleterious effects. In addition, drug delivery systems that aim to disturb the endolysosomal compartment, so that the contained therapeutics can escape into the cytosol and reach other intracellular targets, may also affect lysosomal function. Particularly challenging is the delivery of therapeutics into the brain, which is necessary to treat the numerous neurological LSDs. Established and newly developed techniques and suitable cellular and animal models are required not only to understand pathological mechanisms in LSDs but also to assess lysosomal status and blood brain barrier penetrance and to evaluate therapeutic strategies and drug delivery systems in these diseases.

This review focuses on the current state of such techniques and models, considers advantages and drawbacks (Fig. 2) giving examples of their use in different types of LSDs in the past, and discusses in some instances the potential to advance therapeutic drug delivery and development in the future.

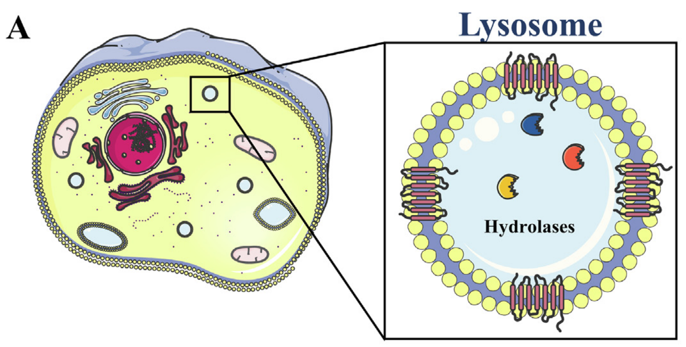
2. In vitro techniques in LSD research

Numerous in vitro approaches exist that can be applied to study lysosomal structure and function, as well as to diagnose and determine pathological alterations and therapeutic efficacy for LSDs. In this section we summarise the established approaches that are employed in the LSD context and techniques that have more recently been developed to specifically address lysosomes and their alterations.

2.1. "Omics"

"Omics" is a group of techniques used to collectively characterize and quantify pools of biological molecules, and they can be applied to a great variety of samples including cultured cells, animal models or patient-derived samples. Depending on the type of biological molecules that are characterized, "omics" can be divided in proteomics, lipidomics, genomics, metabolomics, etc. "Omics" tools are very useful in human clinical research because they allow a general characterization of disease-related alterations in different tissues and samples. In the case of genetic diseases like LSDs, genomic analysis can serve to detect key mutations. Additionally, proteomic and/or lipidomic analysis, which detect alterations in the levels of proteins or lipids, respectively, can give a comprehensive view of substrates that may accumulate in LSDs. Other important applications of "omics" in LSD research are those

A



Autophagy

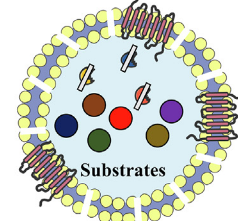
Quality control systems

Innate and adaptive immunity

Contacts with other organelles

B

LSDs



Sphingolipidoses

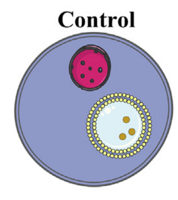
Mucopolysaccharidoses

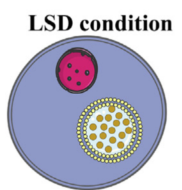
Glycoproteinoses

Non enzymatic LSDs

1. Accumulation of substrates

Control LSD condition



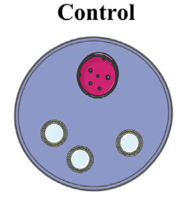


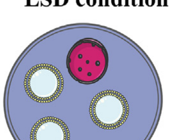
Hydrolase substrate detection

"Omics"; EM; Fluorescence-based methods

2. Increase in lysosomal area

Control LSD condition



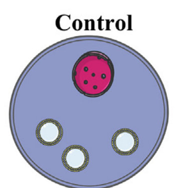


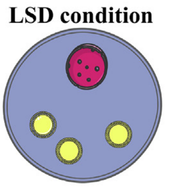
Immunostaining of lysosomal membrane proteins

Fluorescence-based methods

3. pH alteration

Control LSD condition



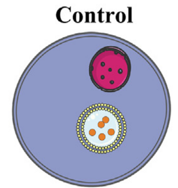


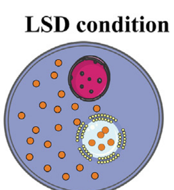
Use of fluorescent probes sensitive to pH alteration

Fluorescence-based methods; Optogenetics

4. Lysosomal membrane disruption

Control LSD condition



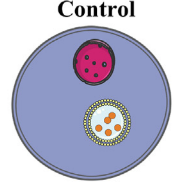


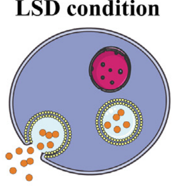
Cytoplasmic detection of lysosomal hydrolases

EM; Fluorescence-based methods; Optogenetics

5. Lysosomal exocytosis

Control LSD condition





Extracellular detection of lysosomal hydrolases

"Omics"; Fluorescence-based methods; Optogenetics

Fig. 1. A. Schematic of known lysosomal functions. **B.** LSD classification and different types of lysosomal anomalies found in the LSD context indicating the techniques that may help to analyse them. EM: Electron microscopy.

Table 1
List of representative LSDs, classified according to the nature of the storage molecule, indicating the gene affected, the encoded protein, the OMIM (Online Mendelian Inheritance in Man) identifier and the primary accumulating substrate in each of them. *Pompe disease is included in this group due to the accumulation of polysaccharides.

Disease	Gene affected	OMIM	Encoded protein	Primary accumulating molecule	Ref
	Sphingolipidoses				
GM1 gangliosidosis	GLB1	230,500	β-galactosidase	GM1 ganglioside	[19]
Sandhoff disease	HEXB	268,800	Hexosaminidase A/B	GM2 ganglioside	[20]
Tay-Sachs disease	HEXA	272,800	Hexosaminidase A	GM2 ganglioside	[21]
Fabry disease	GLA	301,500	α-galactosidase A	Globotriaosylceramide	[22]
Metachromatic leukodystrophy	ARSA; PSAP	250,100	Arylsulfatase A	Sulfatides	[23]
Krabbe disease	GALC	245,200	β-galactocerebrosidase	Psychosine	[24]
Niemann-Pick A/B disease	SMPD1	257200; 607,616	Acid sphingomyelinase	Sphingomyelin	[25]
Farber disease	ASAH1	228,000	Acid ceramidase	Ceramide	[26]
Gaucher disease	GBA	230,800	Glucocerebrosidase	Glucosylceramide	[27]
	Mucopolysaccharidoses				
Hunter syndrome	IDS	309,900	Iduronate 2-sulfatase	Heparan and dermatan sulfate	[28]
Hurler-Scheie syndrome	IDUA	607,015	α-l-iduronidase	Heparan and dermatan sulfate	[29]
Sanfilippo syndrome A	SGSH	252,900	Sulfamidase	Heparan sulfate	[30]
Sanfilippo syndrome C	HGSNAT	252,930	Acetyl CoA α-glucosaminide acetyltransferase	Heparan sulfate	[30]
Sanfilippo syndrome B	NAGLU	252,920	α-*N*-acetylglucosaminidase	Heparan sulfate	[30]
Sly disease	GUSB	253,220	β-glucuronidase	Chondroitin, heparan and dermatan sulfate	[31]
Sanfilippo syndrome D	GNS	252,940	*N*-acetylglucosamine-6-sulfatase	Heparan sulfate	[30]
Morquio A syndrome	GALNS	253,000	*N*-acetylglucosamine-6-sulfate sulfatase	Keratan and chondroitin-6-sulfate	[32]
Morquio B syndrome	GLB1	253,010	β-galactosidase	Keratan and chondroitin-6-sulfate	[33]
Maroteaux-Lamy syndrome	ARSB	253,200	Arylsulfatase B	Dermatan and chondroitin-4-sulfate	[34]
Natowicz syndrome	HYAL1	601,492	Hyaluronidase 1	Hyaluronan	[35]
	Glycoproteinoses/Oligosaccharidoses				
Sialidosis type I	NEU1	256,550	Neuraminidase	Sialyloligosaccharides	[36]
Sialidosis type II	NEU1	256,550	Neuraminidase	Sialyloligosaccharides	[37]
Galactosialidosis	CTSA	256,540	Cathepsin A	Sialyloligosaccharides	[38]
Schindler disease	NAGA	609,241	α-*N*-acetylgalactosaminidase	Galactose oligosaccharides, galactomannans and galactolipids	[39]
α-mannosidosis	MAN2B1	248,500	α-D-mannosidase	Mannose-containing oligosaccharides	[40]
β-mannosidosis	MANBA	248,510	β-mannosidase	Di- and trisaccharides with β-linked mannose at the non-reducing end	[41]
Fucosidosis	FUCA1	230,000	α-L-fucosidase	Fucosylated glycoproteins	[42]
Aspartylglycosaminuria	AGA	208,400	Glycosylasparaginase	Glycoasparagines	[43]
Pompe disease*	GAA	232,300	Glucosidase Alpha, Acid	Glycogen	[44]
	Non-enzymatic LSDs				
Danon disease	LAMP2	300,257	Lysosome Associated Membrane Protein 2	Glycogen	[45]
Niemann Pick type C	NPC1 NPC2	257220; 607,625	Niemann Pick type C1 Niemann Pick type C2	Cholesterol	[46]
Salla disease	SLC17A5	604,369	Sialin	Sialic acid	[47]
Cystinosis	CTNS	219,800	Cystinosin	Free cystine	[48]
Mucolipidosis IV	MCOLN1	252,650	Mucolipin-1	Phospholipids, gangliosides and mucopolysaccharides	[49]
Batten disease	CLN3	204,200	CLN3	Lipofuscin-like material	[50]
Ceroid lipofuscinosis type 7	MFSD8	610,951	Major facilitator superfamily domain-containing protein 8	Lipofuscin-like material	[51]
Ceroid lipofuscinosis type 12	ATP13A2	606,693	Polyamine-transporting ATPase 13A2	Lipofuscin-like material	[52]

aimed at finding biomarkers for diagnosis and assessment of treatment efficacy.

Given the versatility and insight that "omics" can provide, there are numerous studies using these techniques in the LSD context. In the sphingolipidoses, proteomic analysis of urine, blood and tissue samples from patients contributed to the finding of new biomarkers in Fabry disease (OMIM 301500) [61]. Proteomic profiling of lysosomes in Niemann-Pick type C (OMIM 257220; 607625), in which sphingolipids also accumulate, identified mTORC1 signalling as a targetable pathway for treatment [62]. Lipidomic analysis enabling the simultaneous quantification of lysosphingolipids allowed the screening of Fabry (OMIM 301500), Gaucher (OMIM 230800), infantile Krabbe (OMIM 245200) and Niemann-Pick diseases with high sensitivity and specificity [63]. This analysis permitted the discovery of *N*-acyl-O-phosphocholineserine, a class of lipid that had not been previously detected in biological samples, as a biomarker in plasma from Niemann-Pick type C (OMIM 257220; 607625) patients [64]. Lipidomic profiling of plasma and urine from Gaucher disease (OMIM 230800) patients was used to identify lipid species, which levels were significantly altered and could be modified by enzyme replacement therapy indicating that these species were directly or indirectly affected by the therapy [65]. Levels of plasma glucosylsphingosine are being used as reliable marker to follow therapeutic response in Gaucher (OMIM 230800) patients [66]. An example of lipidomic analysis unveiling differences in the brain phospholipid content in a mouse model for the acid sphingomyelinase deficiency is shown in Fig. 3.

In mucopolysaccharidoses, brain transcriptomic analysis assessed the effects of central nervous system (CNS) gene therapy in a mouse model for Hunter syndrome (OMIM 309900). Almost 80 % of the genes differentially expressed, of which many associated to inflammation and innate immunity, were corrected at a significant extent in their transcript levels by administration of adeno-associated virus vectors encoding iduronate-2-sulfatase

Cellular models

Primary cultures	Immortalised cell lines	hiPSCs	BBB models	Organoids
Closely resemble the host tissue	Numerous types from different tissues	Harbour the natural-disease causing mutations	Dynamic, uptake analysis	Close to physiologic environment
Can be obtained directly from human donors	Almost unlimited amount of material	Can be differentiated into neural cells	Numerous models using different cell types	May mimic different human organs
Limited life span and amount of material	Metabolic alterations due to genetic manipulation	Patient cell availability	Results are not totally extrapolable to physiological situation	Patient cell availability
Variability among cultures and cell heterogeneity	Genotypic and phenotypic variations due to passages	Difficult dedifferentiation/ differentiation protocols	Specialized equipment required	Specialized equipment required

Animal models

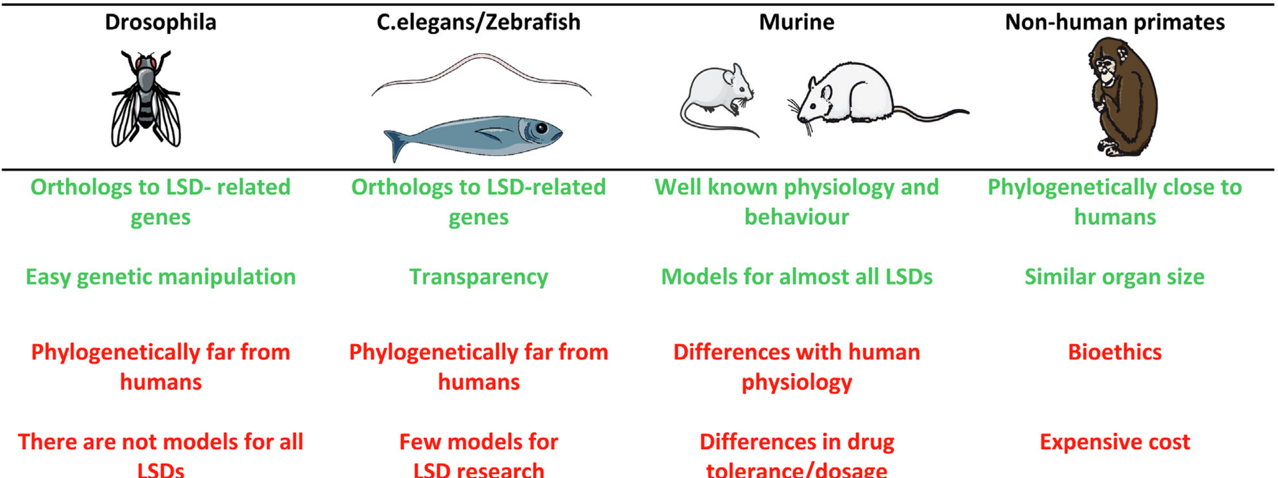


Fig. 2. Illustration of cellular and animal models used in LSD research indicating their main advantages (in green) and drawbacks (in red). Common advantage of the cellular models is the suitability to screen drugs and drug delivery systems and characterize pathological molecular mechanisms. Common disadvantages are the isolation from the physiological environment and, except for primary cultures, the genotypic/phenotypic variations that may arise during the culturing process. Common advantage of animal models is the possibility of studying pathology and therapy in the context of the whole organism. However, species-specific differences in physiology, metabolism and genetics must be taken into account when extrapolating results to the human condition. hiPSCs: Human-induced pluripotent stem cells; BBB: Blood Brain Barrier.

[67]. Whole exome sequencing screened missense changes in the SGSH gene in a group of adult onset Sanfilippo syndrome patients that presented very mild symptoms raising awareness of this unusual phenotype [68].

In glycoproteinoses, a based-electrospray mass spectrometry technique for the detection of carbohydrates was used to screen a complex mixture of O-glycosylated sialylated amino acids from urine of a Schindler disease (OMIM 609241) patient [69] although this method has not been widely adopted for diagnosis. Transcriptomic analysis of FUCA1 knock-down keratinocytes unveiled the altered expression of genes mainly related to differentiation and immune response in fucosidosis (OMIM 230000) [70].

2.2. Fluorescence-based techniques

Fluorescence-based techniques have greatly facilitated the detection of lysosomal damage and storage by microscopy or spectrophotometry. Lysosomes can be visualized by immunofluorescence techniques using antibodies against lysosomal membrane proteins such as LAMP1 (Fig. 4A). Cell-permeable fluorescent probes are available that target lysosomes. For example, the lysotracker red dye stains acidic compartments in live cells [71], and has been used to monitor lysosomal permeabilization in LSD cellular models [72,73] (Fig. 4B). SiR-lysosome is a fluorogenic and highly specific probe for lysosomes based on pepstatin A, a natural

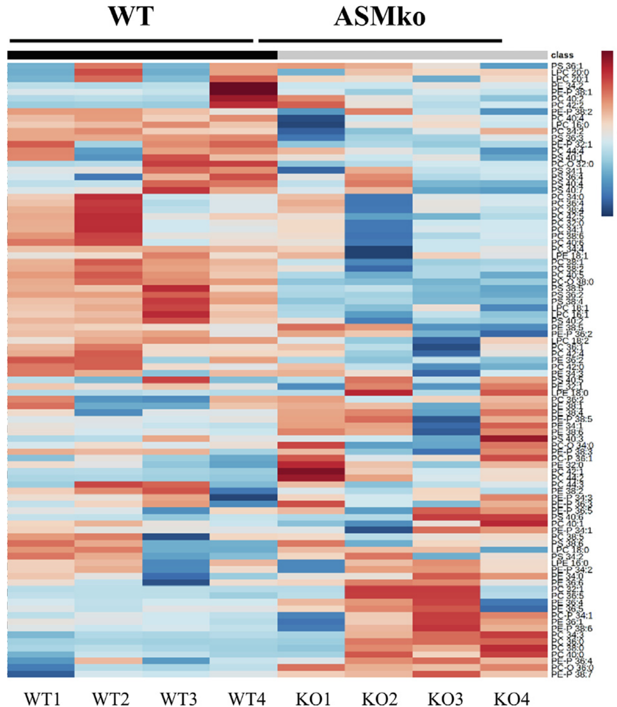


Fig. 3. Lipidomic analysis of the altered phospholipid composition in the cerebellum of a mouse model for Niemann Pick type A (ASMko). Phospholipid composition of the cerebral cortex was analyzed by mass spectrometry in different mice (4 wt and 4 ASMko) at 4 months of age. Differences in the levels of the indicated phospholipid species (PC; PE; PS; LPC; LPE; PC-O; PE-O) are represented for each individual mouse as a heatmap expressing the differences between the groups after normalization by using MetaboAnalyst 5.0 software.

cathepsin D-binding product [74]. It stains lysosomes in live cells without the need for genetic manipulation or overexpression, and has been used to monitor lysosome motility by single-particle tracking [75]. Fluorescence-based techniques are also available to monitor lysosomal function. Ratiometric assessment of lysosome pH using exogenous or genetically encoded probes can be used not only to determine lysosomal pathology in LSD models but also to assess the safety of drug delivery systems targeting the lysosome [76]. Newly developed exogenous probes to quantify lysosome pH, such as CQ-Lyso, utilize a single excitation wavelength minimizing background noise and autofluorescence interference. New genetically encoded pH lysosome biosensors allow measurement of lysosomal pH over extended periods of time although it is necessary to assess whether their overexpression has any effect on lysosomal size, positioning or activity [76].

There are different ways to associate fluorescence with the accumulation of specific lipids in the LSD context, including fluorescent analogues of native lipids (e.g. nitrobenzoxadiazole [NBD] or boron-dipyrromethene [BODIPY-lipids]) [77,78]; autofluorescent molecules such as the cholesterol-binding antibiotic filipin [79], or peptide sequences in toxins that mediate specific lipid binding. Examples of the latter are the earthworm toxin lysenin, which binds sphingomyelin [80] (Fig. 5A,B); the D4 domain of perfringolysin O, which binds cholesterol [81]; and the B subunit of cholera toxin, which binds the ganglioside GM1 [82]. Detection of these toxin-derived peptides by specific antibodies, transfection of their cDNAs fused with fluorescent proteins or by adding to them fluorescent tags, has helped characterize lysosomal storage in Niemann-Pick diseases [83,84]. They also allowed the discovery of lipid storage beyond lysosomes, which affects other cellular compartments with key relevance to pathology such as that of

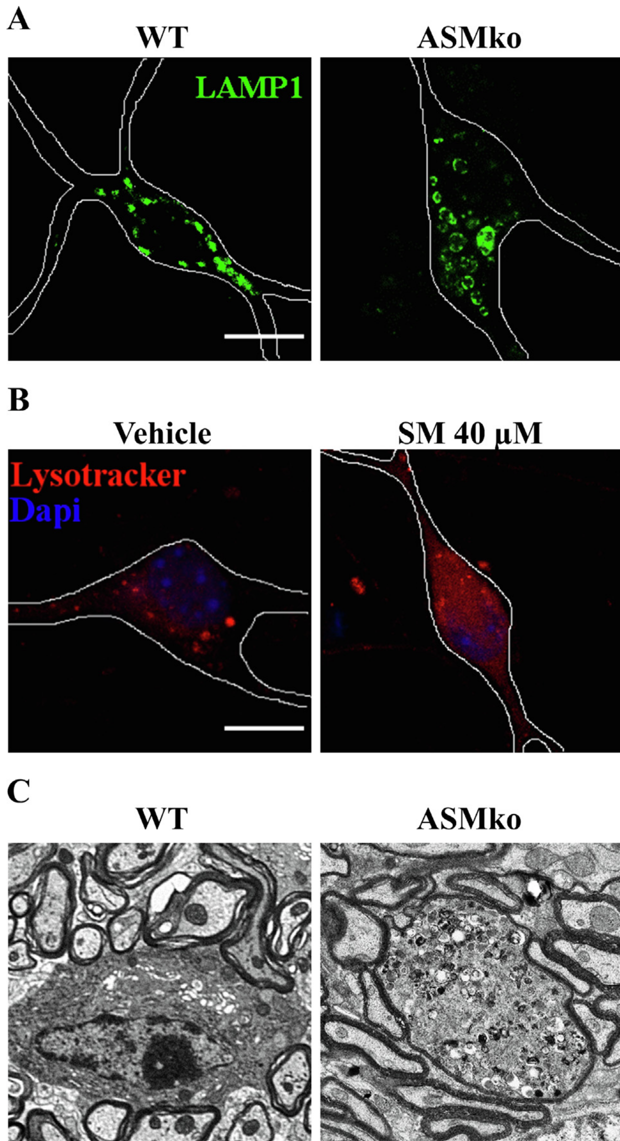


Fig. 4. Study of lysosomal alterations by microscopy. **A**: Immunofluorescence against the lysosomal membrane protein LAMP1 in cultured primary neurons from wt mice and from ASMko mice that mimic Niemann Pick disease type A. The fluorescence microscopy image illustrates the increase in lysosomal size due to lipid accumulation in the ASMko neurons compared to wt. White lines delineate the neurons. Scale bar = 10 μm. **B**: Fluorescence microscopy image of the lysotracker red dye in cultured primary neurons from wt mice treated with vehicle or with 40 μM sphingomyelin to increase the levels of this lipid as in the ASMko neurons. Lysotracker, which stains acidic compartments, shows a punctate pattern corresponding to intact lysosomes in the vehicle treated cells while having a diffuse cytosolic display in the SM treated cells indicating lysosomal permeabilization. White lines delineate the neurons. Dapi in blue indicates nuclei. Scale bar = 10 μm. **C:** Electron microscopy micrographs of microglia in WT and ASMko mice. The images allow visualization of expanded lysosomes and vacuoles in the cytosol of the ASMko microglia compared to the wt. Scale bar = 2 μm.

sphingomyelin at the plasma membrane in Niemann-Pick type A (OMIM 257200) neurons [80] (Fig. 5A).

The aforementioned probes have been used to determine the efficacy of potential therapeutic strategies. Reduction in filipin staining confirmed the usefulness of the cholesterol-extracting drug methyl-β-cyclodextrin [85] or the pharmacological activation of the cholesterol degrading enzyme CYP46 [84] in cellular and animal models for Niemann-Pick type C (OMIM 257220; 607625). Fluorescent-labelled cholera toxin subunit B unveiled

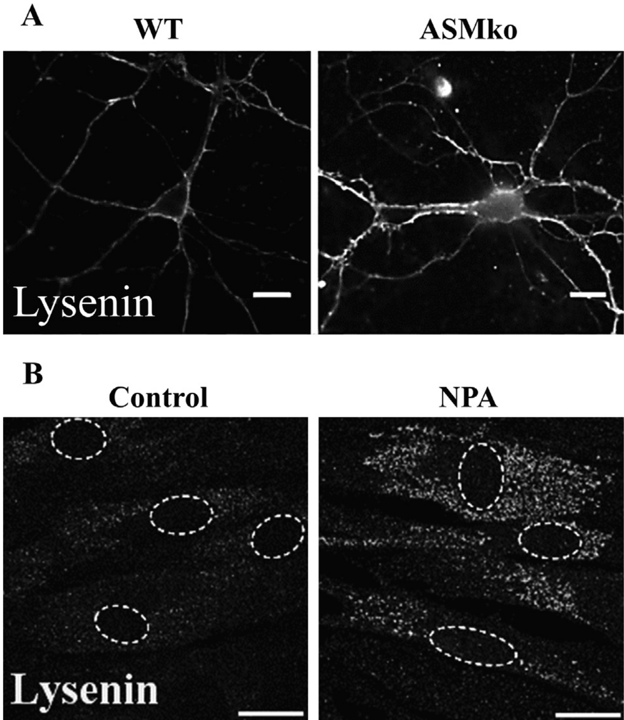


Fig. 5. Primary cell cultures to study lipid alterations in LSDs. **A**: Surface staining in non-permeabilized primary cultured neurons from WT mice and ASMko mice, which mimic Niemann Pick disease Type A, using an antibody against the toxin-derived peptide Lysenin that specifically binds sphingomyelin. This kind of experiment showed that in ASMko neurons sphingomyelin accumulation not only occurs in the lysosomes but also at the plasma membrane. Scale bar = 5 μm. **B**: Lysenin staining in permeabilized cultured fibroblast obtained from a control subject and a Niemann Pick disease type A patient evidencing the intracellular accumulation of sphingomyelin in the patient cell. White circles indicate the nuclei. Scale bar = 10 μm.

the effects of cyclodextrin on GM1-ganglioside levels in cellular models of gangliosidosis (OMIM 230500) [86]. BODIPY-labeled C12 sphingomyelin was used as a substrate to detect acid sphingomyelinase (ASM) activity in cerebrospinal fluid and plasma after adeno-associated viral vector-based gene therapy in the ASM knock-out mouse line that models Niemann-Pick type A (OMIM 257200) [87].

Fluorescence-based methods have been key in the clinical diagnosis of LSDs, which heavily relies on either detection of the accumulation of molecules or identification of defective activity of enzymes in patient cells and fluids. Staining of the autofluorescent antibiotic Filipin in patient fibroblasts has been traditionally used for the diagnosis of Niemann-Pick type C (OMIM 257220; 607625) [88]. The fluorescent-labelled ganglioside sulforhodamine-GM1 has been used to determine the activity of hexosaminidase A in skin fibroblasts and white blood cells from patients of GM2-gangliosidosis (OMIM 272800), allowing researchers to distinguish between the various subtypes of this LSD [89]. Keratin sulfate-dependent fluorescence has been used to diagnose Morquio A syndrome (OMIM 253000) from dried blood samples [90]. A simple method determined the enzymatic activity of α-L-fucosidase by measuring the fluorescence quenching of CdTe semiconductor quantum dots in serum samples from fucosidosis (OMIM 230000) patients [91].

2.3. Electron microscopy

Although less amenable than fluorescence-based microscopy methods, electron microscopy (EM) has also been widely used to analyse the ultrastructural changes related to the deposit of non-degraded molecules within the lysosomes and cytosol of cells from patients and LSD animal models (Fig. 4C). EM analysis of cells and tissues crucially contributed to the identification of autophagy alterations as a common pathological feature in many LSDs [92,93]. Single-particle cryo-EM has yielded protein structures with increasing levels of detail in recent years, and it can be applied to explore the dynamic conformational changes of lysosomal channels and proteins. Although EM does not have the scaling and output to be adopted for high throughput screening it has risen as a complementary method to traditional structural techniques and does show promise in structure-based drug discovery [94].

As EM has been used in the study of almost all LSDs, here we only wish to highlight a few relevant examples, mainly in human tissue. In sphingolipidoses, a recent comprehensive ultrastructural examination of the autopsy tissue from a Niemann-Pick type A (OMIM 257200) patient reported significant accumulation of sphingomyelin even in organs that did not show overt clinical manifestations [95]. Inclusions within the cytoplasm of podocytes were observed in a patient carrying unidentified gene variation associated with Fabry disease (OMIM 301500) [96]. EM carried out on skin biopsy samples from Farber disease (OMIM 228000) patients harbouring a new mutation revealed the presence of stacks of membranes in enlarged lysosomes also known as zebra bodies [97].

In mucopolysaccharidoses, membrane-bound vacuoles and deposits of sulfated acidic glycosaminoglycans were detected by EM in biopsied muscles from a Hunter syndrome (OMIM 309900) patient [98]. Keratocytes containing vacuoles and electron-dense lysosomes full of proteoglycans were described in the cornea from a patient of Maroteaux-Lamy syndrome (OMIM 253200) [99]. Single-particle cryo-EM helped to resolve the structural differences between distinct conformations of the TRPML1 channel, and may contribute to understand the potential mechanism of different mutations in mucolipidosis type IV (OMIM 252650) [100].

In glycoproteinoses, EM techniques allowed the description of an accumulation of terminal sialic acid at the non-reducing end of the sugar chain in the brain tissue of a sialidosis patient (OMIM 256550) [101]. Distended lysosomes within endothelial cells, fibroblasts and histiocytes were found in the skin of a fucosidosis patient (OMIM 230000) [102].

2.4. Optogenetics

Optogenetic methods allow precise manipulation of many cellular activities with light [103]. Through the use of illumination and the expression of photoproteins, protein–protein interactions and biochemical pathways can be controlled. Among the advantages of optogenetic methods are the cell type specificity, the high speed and accuracy and less damage to tissue. Among the main challenges for optogenetic implementation in humans are the expression of adequate amounts of photoproteins and the development of low-heat light sources. Different optogenetic strategies have been used in cellular and animal models to monitor and modulate relevant features of lysosome physiology including their pH or motility. As an example, light activation of lyso-pHoenix, a fusion protein made up of the light-activated proton pump Arch3, the pH-sensitive GFP variant pHluorin and the lysosomal-targeting CD63 protein, caused a rapid drop in lysosomal pH [104]. This probe enables the external control of lysosomal pH and can be applied to directly assess the acid-dependent activity of lysosomal proteases and the role of acidification in LSD pathology. Optogenetic strategies have also been used to control the transport and distribution of lysosomes. Upon light-induced recruitment of molecular motors such as kinesin or dynein, repositioning of these organelles in cells has been achieved [105]. Optogenetic modules

based on light-sensitive dimerizers have been used to translocate mTORC1 to lysosomes upon photoactivation [106]. Phototoxic proteins have been targeted to the cytoplasmic surface of lysosomes by fusion with the small GTPase Rab7. This strategy has been used to induce lysosomal membrane permeabilization [107] or oxidative stress [108] upon illumination.

2.5. Liposome-based techniques

Liposome-based techniques have contributed to both characterization and treatment of pathology in LSD models. Liposomes are spherical particles made up of different types of lipids that organise to form one or more lipid bilayers. They can be synthesized by different means [109] including the mechanical methods of thin film hydration [110] or solvent dispersion [111]. The broad application of liposomes mainly derives from their capacity to encapsulate different types of compounds or genetic vectors [112]. Liposomes can deliver their cargo to almost all organs and tissues. This includes the brain when strategies like coating or conjugation with antibodies are used to help liposomes cross the blood–brain barrier (BBB) [113]. Several key features must be determined in vitro before applying liposomes in clinical research settings, including solubility, stability, cargo uptake, ability to cross endothelial barriers and BBB structures, and effective delivery of cargo into the desired cell/organelle. We refer to the articles in this issue by Drs. Ventosa, Ceccini and Muro for excellent reviews on the use of liposomes and other nanoparticles in LSD therapy.

In addition to therapeutics, liposome-based techniques have been used as tools to study LSD pathological mechanisms and to create LSD models. In sphingolipidoses, a detergent-free liposomal assay uncovered the regulatory roles of distinct lipid classes on the hydrolysis of membrane-bound glucosylceramide by the lysosomal β-glucocerebrosidase in Gaucher disease (OMIM 230800) models [114]. Reconstitution of GM2 catabolism at liposomal surfaces was used as a model to study the effect of the accumulation of different membrane lipids on ganglioside metabolism and gangliosidosis [115].

In glycoproteinoses, liposomes loaded with glycolipids were used to demonstrate the deficient degradation of these lipids by skin fibroblasts from patients of Schindler disease (OMIM 609241) [116].

3. Cellular models in LSD research

Cellular models have been commonly used in LSD research. From traditional monocultured cells in isolation to cutting-edge technologies leading to the generation of multi-lineage cell platforms, these techniques have facilitated our understanding of pathological molecular mechanisms and driven the discovery of drug- and cell-based therapies.

3.1. Primary cell cultures

Primary cell culture is defined as the ex vivo culture of cells freshly obtained from a tissue. The main advantages of primary cells are their faithful transcriptomic and proteomic profile and closer proximity to physiological function and response. Some drawbacks are their requirements for specific substrates and nutrients and the acquisition of a senescent phenotype, which leads to an irreversible cell cycle arrest that limits the amount of material that can be obtained. This prevents the use of primary cultures for large-scale disease modelling and drug discovery projects.

Primary cultures of fibroblasts from patient-derived skin biopsies have been broadly used in LSDs, not only to understand disease mechanisms but also for diagnostic purposes (Fig. 4B). In mucopolysaccharidoses, transcriptomic studies on fibroblast cultures derived from patients suffering from all types of these diseases identified dysregulation of apoptosis-related genes [117]. In glycoproteinoses, the use of primary cultured fibroblasts from patients suffering from sialidosis (OMIM 256550) and galactosialidosis (OMIM 256540) demonstrated impaired elastogenesis that could be reverted by transduction with neuraminidase-1 cDNA or after treatment with bacterial sialidase [118]. Filipin staining to detect cholesterol accumulation in primary fibroblasts from Niemann-Pick type C (OMIM 257220; 607625) patients has been a key diagnostic tool for decades [119].

The use of primary neuronal cultures has been particularly informative for the neurological phenotypes in LSDs. Both monocultures and co-cultures of primary brain cells obtained from mouse models have been used. In sphingolipidoses, primary cultured neurons from ASM knock-out mice, which mimic Niemann-Pick type A (OMIM 257200) (Fig. 4A), unveiled alterations in autophagy [72], calcium homeostasis [80] and synaptic features [120,121]. In mucopolysaccharidoses, co-cultures of primary brain cells from mice lacking sulfamidase (which mimic Sanfilippo syndrome (OMIM 252900)) revealed the highest accumulation of heparan sulfate in astrocytes [122].

3.2. Immortalized cell lines

Immortalized cell lines are generated by naturally occurring or induced mutations that avoid senescence and allow cells to keep dividing. They can be used in research instead of primary cultures, providing pure populations of cells and more reproducible results. Among other advantages, they are cost effective, deliver an almost unlimited supply of material and bypass the ethical concerns associated with the use of animal and human tissue. However, due to the genetic manipulation that is required to generate them, immortalized cell lines may show altered phenotype when compared to primary cultures. Serial passage can also cause genotypic and phenotypic variations and contamination with mycoplasma is common [123]. Despite these drawbacks, immortalized cell lines have been instrumental in LSD research. They may express specific lysosomal enzymes to be used as therapeutic tools. Moreover, the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system, firstly discovered as a defence system in bacteria and archaea [124], facilitates genome editing and is a powerful tool [125] that is increasingly being used to generate LSD disease modelling cell lines. Of particular relevance in the LSD context are immortalized cells generated after transformation of patient-derived primary cells by, for example, hTERT or SV40 large T antigen technology [126]. Comparisons among single-patient derived cell lines are useful to understand the variable phenotype of LSD patients, which often depends on the specific mutations they carry.

In sphingolipidoses, a Chinese hamster ovary cell line overexpressing human GM2A was generated to purify the secreted enzyme and study its uptake by fibroblasts derived from Sandhoff disease (OMIM 268800) patients [127]. A human monocytic THP-1 cell line, in which the GBA1 gene was edited, was used to model Gaucher disease (OMIM 230800) [128]. GLA gene knock-out HEK-293T cells were generated by the CRISPR/Cas9 methodology to study the cellular pharmacokinetics of recombinant human α-galactosidase A [129]. The therapeutic potential of gene edited neural stem cells overexpressing galactosylceramidase was shown in Krabbe disease (OMIM 245200) [130]. An NPC1 knock-out HeLa cell line was generated by CRISPR/Cas9 methodology to study Niemann-Pick type C (OMIM 257220) [131].

In mucopolysaccharidoses, cathepsin B leakage from lysosomes to the cytoplasm was reported in a Hunter syndrome (OMIM 309900)-derived neuronal cell line [132]. NAGLU-deficient and

HGSNAT-mutated cell lines modelling Sanfilippo syndrome types B (OMIM 252920) and C (OMIM 252930), respectively, were generated by CRISPR/Cas9 technology [133,134].

In glycoproteinoses, RAG and LM/TK cell lines contributed to the chromosomal assignment of two genes associated with neuraminidase-deficiency disorders [135].

3.3. Induced pluripotent stem cells (iPSCs)

iPSCs are generated by reprogramming of somatic cells through the manipulation of specific transcription factors and exhibit the morphology, growth properties and marker genes of embryonic stem cells [136,137]. iPSCs can be obtained from different research animals, and even from human biopsies. Human iPSCs (hiPSCs) harbour natural-disease causing mutations and allow study of the role of genetic background in disease progression and severity. Importantly, they can be differentiated to produce cellular lineages that cannot be obtained directly from patients, such as neurons or neural stem cells. Therefore, they are instrumental to study the neurological phenotypes that characterize many LSDs (Table 2). Given their inherent patient specificity, hiPSC-derived cell types may be key to develop personalized drug treatments [163]. Efforts are currently underway to reduce the variability of directed hiPSC differentiation, which can lead to populations of multiple cell types that arise spontaneously [164].

Use of human iPSCs in LSD research has been extensively reviewed in [165]. Here, we provide only some examples that highlight their utility. In sphingolipidoses, oxidative stress and presynaptic dysfunction were described in neurons created from iPSCs obtained from skin-derived fibroblast of patients with Tay-Sachs disease (OMIM 272800) [142]. Patient-derived iPSCs and gene-editing technology were used to study the cardiac-related molecu-

Table 2
List of iPSCs and organoids generated for LSD research. hiPSCs: Human-induced pluripotent stem cells. iPSCs: Induced pluripotent stem cells. ND: Not described.

Disease	iPSCs	Organoids
Sphingolipidoses		
GM1 gangliosidosis	Neurons differentiated from patient-derived hiPSCs [138]	Cerebral organoids from iPSCs [139]
Sandhoff disease	iPSCs derived from mouse model neural stem cells [140]	Cerebral organoids from patient-derived hiPSCs [141]
Tay-Sachs disease	hiPSCs from patient-derived fibroblasts [142]	Cerebral organoids from patient-derived hiPSCs [141]
Fabry disease	hiPSCs from patient-derived fibroblasts [143]	
Metachromatic leukodystrophy	hiPSCs line from patient-derived fibroblasts [144]	Artificially iPSCs-derived whole-brain organoid [145]
Krabbe disease	hiPSCs line from patient-derived fibroblasts [146]	ND
Niemann-Pick A/B disease	hiPSCs line from type A patient-derived dermal fibroblasts [147] hiPSCs line from type B patient-derived dermal fibroblasts [148]	ND
Farber disease	hiPSCs line from patient-derived fibroblasts [149]	ND
Gaucher disease	hiPSCs line from patient-derived fibroblasts [150]	ND
Mucopolysaccharidoses		
Hunter syndrome	hiPSCs from patient-derived dermal fibroblasts [151]	ND
Hurler-Scheie syndrome	hiPSCs from patient-derived fibroblasts [152]	ND
Sanfilippo syndrome C	HGSNAT-mutated cell lines from healthy hIPSCs [134]	ND
Sanfilippo syndrome B	hiPSCs from patient-derived peripheral blood mononuclear cells [153]	ND
Sly disease	hiPSCs from patient-derived fibroblasts [154]	ND
Morquio A syndrome	hiPSCs from patient-derived dermal fibroblasts [155]	ND
Glycoproteinoses/Oligosaccharidoses		
Sialidosis type I	IPSCs-derived neural cells generated from patient-derived fibroblasts [156]	ND
Sialidosis type II	IPSCs-derived neural cells generated from patient-derived fibroblasts [156]	ND
Non-enzymatic LSDs		
Niemann Pick type C	Neurons differentiated from i^3Neuron iPSCs [157] hiPSCs from patient-derived fibroblasts [158]	Cerebral organoids from patient-derived hiPSCs [159]
Danon disease	hiPSCs from patient-derived peripheral blood mononuclear cells [160]	ND
Batten disease	Gene correction studies in hiPSCS from patient-derived dermal fibroblasts [161]	Cerebral organoids from patient-derived hiPSCs [162]

lar and functional consequences of mutations in GLA causing Fabry disease (OMIM 301500) that included increased excitability with altered electrophysiology and calcium handling [143].

In mucoplysaccharidoses, iPSCs from Hunter disease (OMIM 309900) patient-derived dermal fibroblasts showed classical embryonic stem cell morphology and pluripotency in vivo [151]. Genetically corrected iPSC-NSCs transplanted post-symptomatically into the striatum of adult Sly disease (OMIM 253220) mice were able to reverse neuropathology [154].

In glycoproteinoses, iPSCs from sialidosis (OMIM 256550)-patient skin fibroblast were differentiated into neural progenitor cells and neurons that mimicked the disease-like phenotypes including reduced neuraminidase activity, accumulation of sialyl-oligoconjugates and lysosomal expansion [156].

3.4. Multilineage cellular models: Organoids

Organoids are three-dimensional, self-organizing cellular aggregates that are derived from pluripotent stem cells or organ progenitors, and they are differentiated to form an organ-like tissue exhibiting multiple cell types [166]. While organoids derived from a variety of somatic tissue types have been generated, the brain organoid field has experienced great advances in recent years. Cerebral organoids contain not only neurons but also the different glial cells and can be used to study developmental processes and alterations [163]. Their similarity to real organs makes organoids a precious tool to characterize pathological phenotypes and mechanisms and to assess therapies. Moreover, their small size enables their use in high-throughput screening [167]. Such multi-lineage in vitro models may serve to reduce reliance on animals for research purposes and play a major role in the future of precision medicine [163].

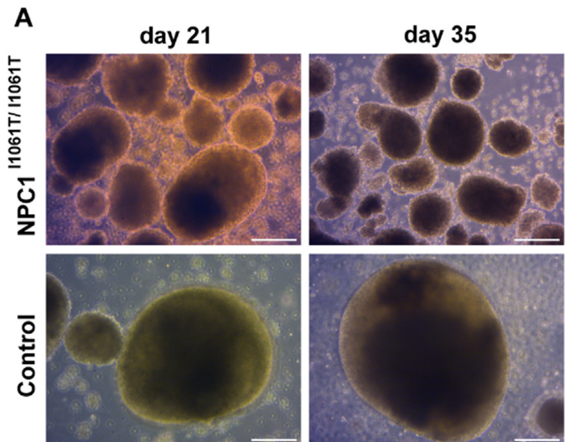
Although the use of organoids is quickly expanding in LSD research, only a few examples related to sphingolipidoses have been reported so far (Table 2). GLB1 knock-out cerebral organoids from knock-out iPSCs showed GM1 ganglioside accumulation, which could be prevented by AAV9-GLB1 vector injection that improved β-galactosidase activity in this organoid model for GM1 gangliosidosis (OMIM 230500) [139]. HEXB knock-out cerebral organoids derived from iPSCs accumulated GM2 ganglioside and showed impaired development [141]. Artificially induced whole-brain organoids, which use inexpensive computer simulations and may guide future wet lab research, were generated to evaluate factors associated with the pathogenesis and progression of metachromatic leukodystrophy and to assess new potential therapeutic options [145]. Niemann-Pick type C (OMIM 257220) brain and cerebellar organoids have been generated with induced neural stem cells from patient-derived fibroblasts and hiPSCs, respectively. The cerebellar organoids have smaller size compared to organoids that were differentiated from normal hiPSCs (Fig. 6). The brain organoids exhibit lower proliferative ability, cholesterol accumulation, impaired differentiation and lysosomal dysfunction [159].

A future task for the LSD field will be to use cutting-edge technologies for the artificial assembly of multiple organoids [168] or the generation of microfluidic organ-chips [169]. This technology allows culture of different cell types in separate, interconnected compartments, which enables replication of hydrodynamic forces and an environment closer to the in vivo situation. Organ-chips from hiPSCs derived from LSD patients would eventually allow personalised assessment of treatment efficacy.

3.5. BBB models

One of the most important challenges in LSDs is treating brain pathology, which is present in the vast majority of them. In order to access the brain, treatments (drug/small molecules/genes) have to cross the BBB that protects the CNS and maintains a homeostatic microenvironment [170]. This is a complex barrier formed by endothelial cells and other cell types such as astrocytes, pericytes and neurons [171]. In vitro BBB models have been generated to assess brain uptake, pharmacokinetics and pharmacodynamics of given compounds, and have also been used to identify pathologic BBB alterations. In vitro BBB models rely on cell-based primary and immortalized cell lines, co-cultures, stem cell-based methods or isolated microvessels [172]. More recently, a two-channel BBB chip that comprises human-derived endothelial cells, pericytes and astrocytes has been generated by microfluidic organ-on-chip technology [173]. These in vitro models offer multiple advantages compared to in vivo BBB models, e.g. the lower concentration of drug required and the easier identification of toxicity. A rationale for selection among the different BBB models has been extensively reviewed in [172].

In the LSD context, in vitro BBB models have been used to test the uptake of compounds and their targeting to lysosomes and activity therein. Dr. Pardridge's article in this issue offers an excellent review on BBB delivery for LSDs with IgG-lysosomal enzyme fusion proteins [174]. Here, we just highlight some examples. In



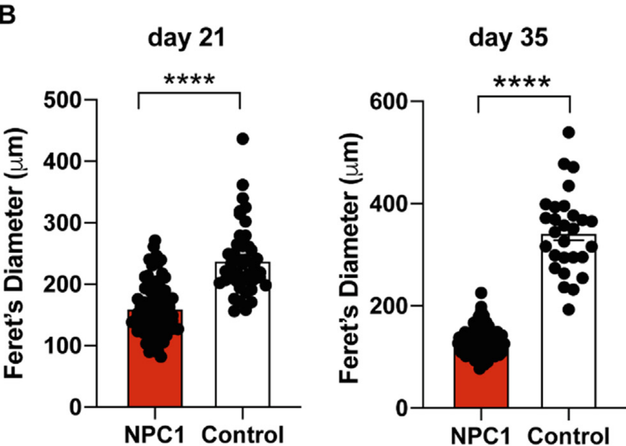


Fig. 6. Comparison of cerebellar organoids differentiated from NPC1$^{I1061T/I1061T}$ and healthy control-derived hiPSCs. **A)** Representative brightfield photomicrographs showing human cerebellar organoids differentiated from Niemann Pick disease type C (NPC) patient and healthy control-derived iPSCs at different timepoints. Scale bar, 200 μm. **B)** Feret's diameter of hiPSC-derived organoids along the cerebellar differentiation using NPC and healthy iPSC lines. Mann-Whitney test, ****p < 0.0001; Error bars represent SEM.

sphingolipidoses, bi-functional IgG-lysosomal enzyme fusion proteins, which were able to cross BBB models, were generated for hexosaminidase, ASM and β-galactosidase 1 based on a chimeric monoclonal antibody against the human insulin receptor. These fusion proteins acted as a "Trojan horse" allowing intracerebral delivery of the lysosomal enzymes [175]. Transendothelial transfer in a BBB cell culture model was assessed for five brain-targeting peptides to promote delivery of the lysosomal enzyme arylsulfatase A in metachromatic leukodystrophy (OMIM 250100) [176]. In vitro BBB models were used to assess polymer nanocarriers coated with antibodies against ICAM-1 that carried ASM. These nanocarriers effectively bound to ICAM-1 on cells and were internalized, trafficked to lysosomes, and restored degradation of lysosomal substrates [177]. A two chamber-based method confirmed the ability of recombinant NPC2, synthesized by genetically modified brain capillary endothelial cells, to cross an artificial BBB [178].

In mucopolysaccharidoses, a fusion protein consisting of the joining of a receptor-binding peptide from apolipoprotein E with the lysosomal enzyme α-L-iduronidase was able to bind to LDL receptors, subsequently endocytosed and the enzyme activity was detected in lysosomes in an in vitro BBB model [179]. A chemically induced β-glucuronidase that crossed the BBB corrected neuronal glycosaminoglycans deposit in murine mucopolysaccharidosis VII (OMIM 253220) [180]. More recently, in vitro BBB models were used for the study of the mannose-6-phosphate receptor-mediated endocytosis of the recombinant human sulfamidase. The results showing the ability of this recombinant protein to go through the BBB encouraged its in vivo administration, which reduced heparin sulfate storage and brain pathology in a mouse model of mucchopolysaccharidoses IIIA (OMIM 252900) [181].

4. Animal models in LSD research

Research on LSDs has always relied heavily (and still does) on the use of animal models to characterize the molecular and cellular pathological mechanisms and to assess safety and efficacy of therapies and drug delivery in vivo. Caution must be taken when extrapolating results obtained in animal models to the human condition due to species-specific differences in physiology, metabolism and genetics. Regardless, preclinical animal studies are required by regulatory agencies when considering approval of a new treatment in patients. Aside from naturally occurring animal models, the monogenic nature of many LSDs has enabled the generation of faithful animal models by genetic alteration (mutation/deletion), with the most common based on fruit flies, worms, zebrafish, and mice.

Generation of animal models by genetic alteration can be achieved by manipulation of the endogenous gene producing knock-out or knock-in models. While in Knock-out models expression of the gene is totally disrupted, knock-in models bear mutations that change the aminoacid sequence of the codified protein. This gene modification can be achieved by several techniques including homologous recombination [182] or CRISPR/Cas9 methodology [183]. Another way to manipulate endogenous gene expression is the use of antisense RNA, which decreases the target gene expression without supressing it completely, producing knock-down models [184]. Transgenic expression of exogenous genes including human ones is also possible and has been widely used to generate animal models in LSD research [185,186].

4.1. Naturally occurring animal models

One group of animal models in LSD research is composed of domestic or wild animals that carry naturally occurring mutations of proteins that cause LSDs in humans. This group includes both non-mammalian and mammalian vertebrates, which in many cases show phenotypes and symptoms quite similar to human patients. As examples, Sandhoff disease (OMIM 268800) has been characterized in sheep showing membranous cytoplasmic bodies within the nervous system and a decreased hexosaminidase A activity in the serum [187]. Cats presenting mutations in the NPC1 gene have been instrumental to demonstrate efficacy of methyl-β-cyclodextrin treatment and fostered clinical trials with this compound in Niemann-Pick type C (OMIM 257220) patients [188]. Deficient activity of iduronate-2-sulfatase and phenotypes characteristic of Hunter syndrome (OMIM 309900) were reported in dogs [189,190].

4.2. Drosophila melanogaster

Drosophila melanogaster, the fruit fly, is a highly manageable genetic model organism in which almost 75 % of human disease-causing genes have a functional homolog [191]. Genome manipulation in Drosophila can be achieved by several techniques including introduction of foreign genes using transposable elements [192] or by CRISPR/Cas9 editing [193]. Transgenic expression of exogenous genes is achieved using the GAL4/UAS system [194]. These techniques can be used to produce knock-out, knock-in, knock-down or conditional knock-out fly lines.

In sphingolipidoses, transgenic lines expressing variants of human α-galactosidase protein were used to demonstrate that mutations in the GLA gene triggered protein misfolding and ER retention, leading to the activation of the unfolded protein response and ER-associated degradation in Fabry disease (OMIM 301500) [195]. Fly lines that bear several mutated isoforms of arylsulfatase A led to propose a link between metachromatic leukodystrophy (OMIM 250100) and Parkinson's disease suggesting chaperone activity of arylsulfatase A towards α-synuclein protein [196]. Deletion of the GBA gene in a Gaucher disease (OMIM 230800) Drosophila model correlated with dopaminergic neuron loss and increased α-synuclein aggregation, pointing to a role of glucosylceramide accumulation in the metabolism of protein aggregates [197].

In mucopolysaccharidoses, fly lines knocked-out for the CG2135 gene (the Drosophila β-glucuronidase orthologue) recapitulated neuropathological abnormalities of mucopolysaccharidosis type VII (OMIM 253220), which were improved by using the polyphenol resveratrol [198]. A fly model for Hunter syndrome (OMIM 309900) was generated by knocking down the Ids/CG12014 gene through an RNA interference approach [199]. Neuronal specific knock-down of the SGSH/CG14291 gene was used to generate a fly model for Sanfilippo type A syndrome (OMIM 252900) in which lysosomal dysfunction and progressive defect in climbing ability, a hallmark of neurological dysfunction in Drosophila, were observed [200].

4.3. Caenorhabditis elegans

Several advantages make the nematode worm C. elegans a suitable model organism to study LSDs [201]: It is easy to maintain and breed having a very short life cycle; many of its genes have functional counterparts in humans; and its transparency makes it possible to observe the fate of individual cells in living animals. Together with its small and fully characterized CNS, these advantages make C. elegans especially appropriate to address questions of CNS pathology in LSDs.

Elimination of the two homologues of mammalian NPC1, whose mutation causes Niemann-Pick type C (OMIM 257220), caused impaired development of C. elegans that could be rescued by specific glycosphingolipids [202] and endocannabinoids [203]. Modula-

tion of the levels of proteins associated with the Endosomal Sorting Complex Required for Transport (ESCRT), which is a cellular mechanism that contributes to membrane remodelling and repair [204], almost fully supressed lysosomal defects and cell death in a C. elegans model for mucolipidosis type IV (OMIM 253000) [205].

4.4. Zebrafish

As in Drosophila and C. elegans, the use of zebrafish as an animal model for human diseases relies on the great genomic similarities (nearly 70 % of human genes have zebrafish orthologues [206]) and the development of genome editing techniques such as CRISPR/Cas9 [207]. A unique advantage of zebrafish is the clarity of the developing embryo [208], making this model especially suitable for analysis of developmental anomalies.

Zebrafish models have been used for research on the three principal types of LSDs. In sphingolipidoses, HexB knock-out zebrafish that model Sandhoff disease (OMIM 268800) were used to characterize lysosomal abnormalities in radial glia and microglia during development [209]. Down-regulation of the two zebrafish orthologs of GALC, the gene mutated in Krabbe disease (OMIM 245200), resulted in alterations of neuroD expression and apoptosis [210]. A chemically-induced Gaucher disease (OMIM 230800) zebrafish model was generated by treatment with cyclophellitol derivatives carrying a bulky hydrophobic substituent at C8, achieving GBA deficiency [211].

In mucopolysaccharidoses, knock-down of the iduronate sulfatase orthologue in zebrafish demonstrated the critical role of this enzyme during early vertebrate development, with its deficiency involved in misshapen trunk and abnormal craniofacial cartilage

Table 3
List of representative murine models generated for LSD research indicating their main phenotypic features. KO: Knock out. KI: Knock in.

Disease	Murine models	Main phenotypic features	Ref
Sphingolipidoses			
GM1 gangliosidosis	GLB1 KO mouse	Neurodegeneration in different brain areas	[215]
Sandhoff disease	HEXB KO mouse	No obvious behavioural or neurological deficits	[216]
Tay-Sachs disease	HEXA KO mouse	Neurodegenerative disease with spasticity, tremor and ataxia.	[216]
Fabry disease	α-GAL A KO mouse α-GAL A KO rat	Accumulation of ceramidetrihexoside and lamellar inclusions in liver and kidneys Pronounced mechanical pain behaviour; inclusions in dorsal root ganglia and sensory neurons	[217,218]
Metachromatic leukodystrophy	ASA KO mouse	Reduction of axonal cross-sectional area; astrogliosis; motor impairment	[219]
Krabbe disease	GALC point mutation KI mouse (Twitcher mouse)	Degeneration of myelin sheaths; multinucleated macrophages with PAS-positive cytoplasm	[220]
Niemann-Pick A/B disease	SMPD KO mouse SMPD point mutation KI mice	Cerebellar atrophy; Purkinje cell death and ataxia Different degree of neurological involvement	[182,221]
Gaucher disease	GBA Conditional KO mouse Chemically induced mouse	Progressive splenomegaly; modest storage in the liver Kupffer cells accumulating GL-1 in the liver	[222,223]
Mucopolysaccharidoses			
Hunter syndrome	IDS KO mouse	Progressive hearing loss leads to early deaf ness Progressive hearing loss leads to early deaf ness Early deafness; impaired learning and memory	[224]
Hurler-Scheie syndrome	IDUA KO mouse	Flattened facial profile and thickening of the facial bones and digits	[225]
Sanfilippo syndrome A	MGAT3 KO mouse	Distended bladder and hepatosplenomegaly; distended lysosomes in neurons	[226]
Sanfilippo syndrome B	NAGLU KO mouse	Abnormal hypoactive behaviour	[227]
Sly disease	GUS KI mouse	Peculiar facial dysmorphism and skeletal deformities	[228]
Morquio A syndrome	GALNS KO mouse	Normal appearance and life-span; Increased urinary glycosaminoglycan levels	[229]
Morquio B syndrome	GLB1 point mutation KI mouse	Lack of a evident phenotype	[230]
Maroteaux-Lamy syndrome	ASB KO mouse ARSB KO rat	Facial dysmorphia; bone abnormalities; poor fertility Excessive secretion of dermatan sulfate in urine	[231,232]
Glycoproteinoses/Oligosaccharidoses			
Sialidosis type I	NEU-1 point mutation KI mouse	Abnormal pattern of urinary oligosaccharides; Storage products in Purkinje cells	[233]
Galactosialidosis	PPCA KO mouse	Excessive excretion of sialyloligosaccharides in urine; severe organ dysfunction	[234]
α-mannosidosis	MAN2B1 KO mouse	Fertile; normal life-span and behaviour; membrane-limited cytoplasmic vacuoles in tissues	[235]
β-mannosidosis	MANBA KO mouse	Normal appearance and growth; cytoplasmic vacuolation in the CNS	[236]
Fucosidosis	FUCA1 KO mouse	Purkinje cell death; astrogliosis; psychomotor and memory deficits.	[237]
Aspartylglycosaminuria	AGA KO mouse	Motor impairment; Massively swollen bladders in older male mice	[238]
Non-enzymatic LSDs			
Niemann Pick type C	NPC1 loss of function mouse NPC1 point mutation KI mice	Purkinje cell loss; motor impairment; very short life span Abnormal cholesterol metabolism; altered glycolipid expression; motor impairment	[239–241]
Danon disease	LAMP2 KO mouse	Ultrastructural abnormalities in cardiac myocytes; reduced heart contractility	[242]
Batten disease	CLN6 point mutation KI mouse	Neurodegeneration; Impaired motor coordination, vision, memory and learning	[243]

[212]. CRISPR/Cas9 was used to mutate the sgsh gene in zebrafish, leading to progressive accumulation of heparan sulfate and CNS-specific features of Sanfilippo type A syndrome (OMIM 252900), including neuronal lysosomal overabundance, complex behavioural phenotypes, and neuroinflammation [213].

In glycoproteinoses, Neu1-knock-out zebrafish showed alterations quite similar to those observed in mouse models and human patients of sialidosis type I (OMIM 256550) such as changes in the expression pattern of genes involved in muscle differentiation and bone remodelling [214].

4.5. Murine models

A vast number of different murine models have been generated for the study of LSDs that include knock-out, knock-down and knock-in animals (Table 3). The knock-in models allow characterization of the effects of mutations that specifically occur in patients. Murine models, in which alterations in behaviour, anatomy and organ-specific biochemistry can be characterized, have greatly helped to identify pathological molecular mechanisms and enabled the preclinical assessment of therapies.

In sphingolipidoses, a mouse knock-out for the gene encoding ASM [182] faithfully mimics Niemann-Pick type A (OMIM 257200) and has been used to characterize the neurologic phenotypes that include altered autophagy [72], calcium homeostasis [80] and endocannabinoid system downregulation [244]. The endocannabinoid system consists of a family of modulatory lipid messengers together with their receptors and metabolic enzymes that regulate functions such as learning and memory, emotional processing, neurogenesis, sleep and pain control and inflammatory responses [245]. Pharmacological enhancement of the endocannabinoid system has been successfully tested in the ASMko mice to ameliorate cognitive and psychiatric alterations [244].

A knock-down mouse model deficient in α-galactosidase served to identify gene alterations in the prefrontal cortex of this animal model for Fabry disease (OMIM 301500) [246]. A knock-in mouse model for Niemann-Pick type C (OMIM 257220) was generated carrying a point mutation frequent in human patients [240], which allowed the characterization of the synaptic role of NPC1. It was determined that the reduction of NPC1 at the synapses leads to increased synaptic cholesterol and cognitive deficits in the knock-in mouse model, which were ameliorated by pharmacological activation of the cholesterol degrading enzyme CYP46A1 [84].

In mucopolysaccharidoses, iduronate-2-sulfatase knock-out mice were used to test the efficacy of enzyme replacement strategy with idursulfase in Hunter disease (OMIM 309900) [247]. A knock-in mouse model carrying the most common human missense mutation in the Glb1 gene showed reduced β-galactosidase as seen in Morquio B syndrome (OMIM 253010) [230].

In glycoproteinoses, a Neu1 gene knock-out mouse model presented clinical abnormalities reminiscent of early-onset sialidosis (OMIM 256550) in children, including severe nephropathy, splenomegaly and urinary excretion of sialylated oligosaccharides. This model also presented differences with the galactosialidosis mouse model including progressive deformity of the spine or lack of early degeneration of cerebellar Purkinje cells, which helped to distinguish these syndromes [248]. Knock-out mice lacking the α-L-fucosidase gene presented large amounts of the core-fucosylated glycoasparagine Fuc(α1,6)-GlcNAc(β1-N)-Asn and lysosomal storage as a model for fucosidosis (OMIM 230000) [237].

4.6. Non-human primates

Among animals, non-human primates show the highest phylogenetic proximity and genome homology with humans. This, together with the similar size of organs that is relevant to many therapies, makes non-human primates precious tools for the understanding and treatment of human disease. However, unlike the other mammalian systems, primates do not naturally develop LSDs. There is just one reported exception, in rhesus monkeys that developed Krabbe-like disease. The affected monkeys had very low GALC activity and presented multinucleated globoid cells, lack of myelin and psychosine accumulation in the white matter [249]. Bioethical concerns and methodological problems prevent the genetic editing of non-human primates to model LSDs. Still, these animals have been very useful in preclinical development to test safety, formulations and routes of administration of therapeutic candidates. For instance, injection of adenoviral particles encoding Hex protein in cynomolgus macaques provoked dyskinesias, ataxia, and loss of dexterity in a dose-dependent manner. Histopathological analysis showed severe white and gray matter necrosis. These results served to argue about variations in safety profiles of intracranial injection of adenovirus-associated vectors encoding Hex among different species [250]. In Fabry disease (OMIM 301500), marmoset monkeys were used to analyse serum levels of human α-galactosidase protein after its sustained delivery via nanoparticle-formulated mRNA [251]. Different methods of delivery of adeno-associated viral vector particles encoding human arylsulfatase, which is deficient in metachromatic leukodystrophy (OMIM 250100), were assessed in African green monkeys, concluding that the best delivery route was direct administration to the white matter [252]. Studies performed in cynomolgus macaques concluded that while intracranial injection of adeno-associated viral vectors encoding human ASM induced inflammation and motor defects, cisterna magna injection resulted in widespread transgene expression without toxicity, supporting the suitability of this strategy to treat Niemann-Pick type A (OMIM 257200) patients [87].

5. Conclusion

In this review we have summarised different techniques used to study lysosomal structure, function and the alterations observed in LSDs. The utility of these techniques apply to all LSDs, which we have classified by the accumulating substrate for the sake of simplicity. These techniques and models will serve to advance therapeutic drug delivery and development. Big challenges remain when addressing LSDs, whether using firmly established or newly developed techniques, or one of the numerous in vitro or in vivo models. Among the greatest challenges is the systemic nature of these diseases, which affect many different organs including the brain, and also variability among patients due to distinct genetic mutations and backgrounds. The need to confirm the ability of drug delivery systems to target lysosomes and assess the consequences or to cross the BBB requires the use of different specialized in vitro models. The generation of patient-specific cellular models, from cellular monocultures to organoids, is also essential for the precision medicine that may lead to personalized treatments. Developing new techniques that are powerful enough to greatly replace the use of live animals, which past LSD research has relied heavily on, is another important priority for the future.

Data availability

No data was used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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