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SYSTEMATIC REVIEW

Systematic Review of Genetic Substrate Reduction Therapy in Lysosomal Storage Diseases: Opportunities, Challenges and Delivery Systems

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Abstract

Background Genetic substrate reduction therapy (gSRT), which involves the use of nucleic acids to downregulate the genes involved in the biosynthesis of storage substances, has been investigated in the treatment of lysosomal storage diseases (LSDs).

Objective To analyze the application of gSRT to the treatment of LSDs, identifying the silencing tools and delivery systems used, and the main challenges for its development and clinical translation, highlighting the contribution of nanotechnology to overcome them.

Methods A systematic review following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) reporting guidelines was performed. PubMed, Scopus, and Web of Science databases were used for searching terms related to LSDs and gene-silencing strategies and tools.

Results Fabry, Gaucher, and Pompe diseases and mucopolysaccharidoses I and III are the only LSDs for which gSRT has been studied, siRNA and lipid nanoparticles being the silencing strategy and the delivery system most frequently employed, respectively. Only in one recently published study was CRISPR/Cas9 applied to treat Fabry disease. Specific tissue targeting, availability of relevant cell and animal LSD models, and the rare disease condition are the main challenges with gSRT for the treatment of these diseases. Out of the 11 studies identified, only two gSRT studies were evaluated in animal models.

Conclusions Nucleic acid therapies are expanding the clinical tools and therapies currently available for LSDs. Recent advances in CRISPR/Cas9 technology and the growing impact of nanotechnology are expected to boost the clinical translation of gSRT in the near future, and not only for LSDs.

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Graphical Abstract

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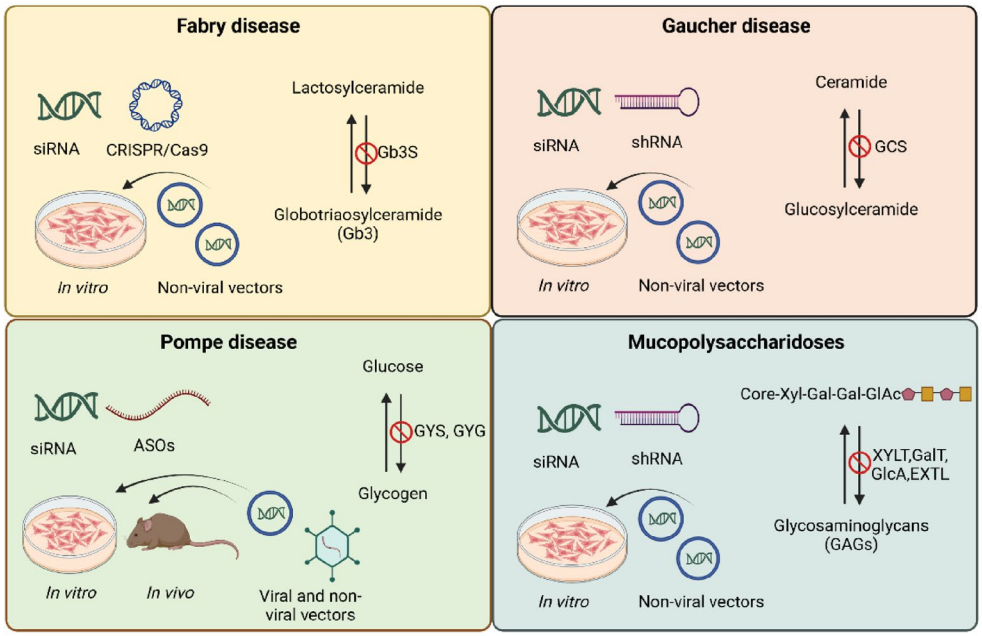

Genetic substrate reduction therapy (gSRT) is considered an alternative therapeutic option for the treatment of lysosomal storage disorders (LSDs). This systematic review describes and critically discusses strategies, silencing tools and delivery systems used for the implementation of gSRT in different LSDs. The main challenges and opportunities for its development and clinical translation have been identified, highlighting the contribution of the nanotechnology to the progress and implementation of gSRT in LSDs.

siRNA small interfering RNA, *CRISPR/Cas9* clustered regularly interspaced short palindromic repeats, *Gb3S* Gb3 synthase, *shRNA* short hairpin RNA, *GCS* glucosylceramide synthase, *ASOs* antisense oligonucleotides, *GYS* glycogen synthase, *GYG* glycogenin, *Xyl* xylose, *Gal* galactose, *GlucAc* glucuronic acid, *XYLT* O-xylosyltransferase, *GalT* β-galactosyltransferase, *EXTL* EXT-like protein, *GAGs* glycosaminoglycans

Key Points

Genetic substrate reduction therapy (gSRT) is a promising strategy for treatment of lysosomal storage diseases (LSDs) but it has been applied only at the preclinical level; the main challenges for its clinical use are rare disease conditions, organ-targeted delivery, limited understanding of the physiopathology of LSDs, and a lack of suitable animal models.

siRNA has been the most widely used silencing tool, but CRISPR/Cas9 technology has emerged more recently, with gene-editing technology expected to have a growing impact on the progress of gSRT.

Nanotechnology is playing a key role in advancing gSRT by driving the development of personalized treatments based on organ-targeted, LSD-specific nucleic acid nanomedicines. The lack of treatments that address CNS involvement is one of the most important obstacles to overcome for new therapeutic strategies.

1 Introduction

Lysosomal storage diseases (LSDs) are a group of more than 50 inherited metabolic disorders characterized by a defective lysosomal metabolism and its subsequent substrate accumulation [1]. LSDs typically arise due to mutations in genes responsible for lysosomal enzymes, transport proteins, activator proteins, or other essential gene products crucial for normal lysosomal activity. Upon alteration of those genes, different types of substrates form intracellular deposits, leading to lysosomal enlargement, cellular impairment, and systemic clinical symptoms [2, 3]. Traditionally, the classification of LSDs is based on biochemical data obtained from diagnosis techniques used for early detection of the diseases, in terms of the nature of the primary storage material: sphingolipidoses (sphingolipids) [4], mucopolysaccharidoses (MPSs) (glycosaminoglycans) [5], glycoproteinoses (glycoproteins)[6] and glycogenoses (glycogen) [7].

The multisystemic substrate deposits result in a range of clinical manifestations, including visceral, ocular, hematological, skeletal, and neurological symptoms, which may partially overlap across different disorders [1, 8]. LSDs are genetically and clinically heterogeneous disorders but frequently they are presented as pediatric neurodegenerative diseases usually associated with visceromegaly [1]. However, it is also common to detect skeletal dysmorphia caused by bone pathology, developmental delay, or other defects that affect the central nervous system (CNS).

Individual LSDs are considered as rare diseases, with an estimated incidence ranging from 1 in 50,000 to 1 in 250,000 live births. Nevertheless, as a group, LSDs present an estimated incidence of 1 in 5000 to 1 in 5500. Although the prevalence depends on the geographical area [9], LSDs represent a great socio-economic burden. In addition, LSDs remain underdiagnosed, although there has been a growing implementation of newborn screening (NBS) for LSDs in the last few years, and prevalence data are improving [10]. The incorporation of LSDs in NBS programs also raises issues regarding the identification of individuals with attenuated or late-onset phenotypes, as well as the detection of individuals with genetic variants, resulting in decreased enzyme activity but with unknown clinical significance [9]. The prevalence data registered in the Orphanet Data Base are shown in Table S1 (Online Supplemental Material (OSM)) [11]. The most common LSDs are Fabry disease (FD), Gaucher disease (GD), metachromatic leukodystrophy (MLD), and Pompe disease (PD). Considering the nature of the storage material, the most prevalent LSDs are the sphingolipidoses, followed by the MPSs.

Restoring defective enzymes through enzyme replacement therapy (ERT) is an essential objective of the current treatment protocols for specific LSDs, including FD, GD, PD, acid sphingomyelinase deficiency (ASMD, historically known as Niemann-Pick disease), some MPSs, and MLD, as it is shown in Table S1 (OSM). The treatment, which relies on the regular intravenous infusion of a functional recombinant human enzyme, is effective in slowing down the advancement of the disease and to enhance the quality of life of the patients [12, 13]. Nevertheless, it presents some disadvantages: short half-life of the therapeutic enzymes [14], which requires frequent administrations, variable bioavailability [15], inability to cross the blood brain barrier (BBB) [16], and the possibility of developing IgG antibodies against the recombinant enzyme [17, 18].

Pharmacological chaperones are an alternative to treat LSDs. Although they are only available for FD, they have also demonstrated efficacy in preclinical research in PD and GD [19, 20]. Chaperones are small molecules that are useful only in patients with amenable mutations affecting the protein folding [21]. They bind to the affected enzyme, stabilize its structure and, consequently, avoid degradation, and the storage substances can be metabolized [22]. The use of oral chaperones could represent an alternative therapy in patients with low response to ERT.

Substrate reduction therapy (SRT), which consists of the inhibition of enzymes that synthesize the storage substances, may also help to restore the substrate balance (Fig. 1). The SRT concept was suggested in 1996 by Radin et al. [23], and is currently available for GD and Niemann-Pick disease

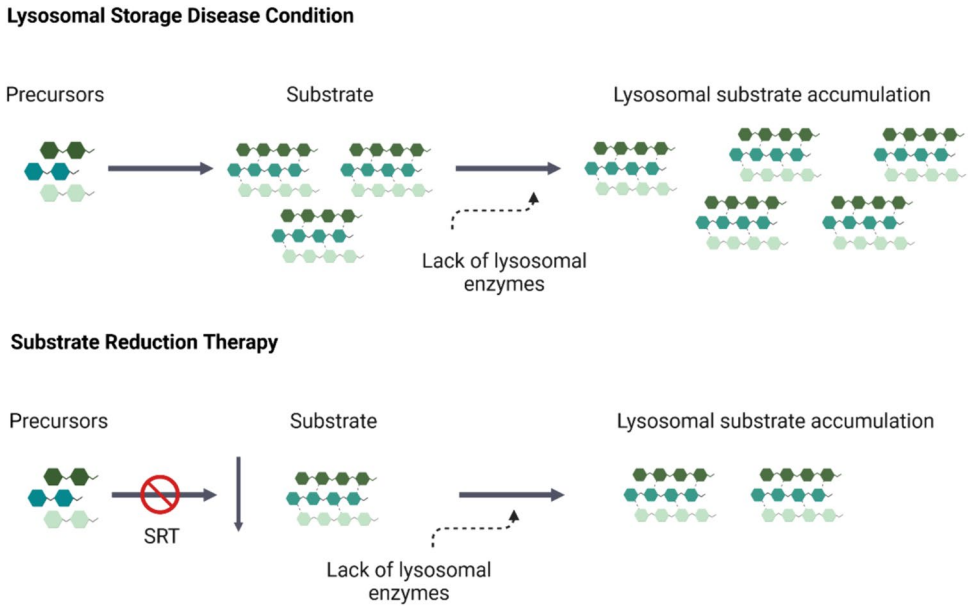

Fig. 1 Substrate reduction therapy concept. *SRT* substrate reduction therapy. Created with BioRender.com

(ASMD). Unfortunately, SRT options also present some problems including metabolic interactions and undesired gastric effects. Therefore, current therapeutic options for treating LSDs are not efficient enough and new strategies are needed to address the different unmet medical needs of each of these rare disorders [14]. Among the new options, genetic SRT (gSRT) has been proposed as an approach to selectively downregulate genes responsible of the biosynthesis of storage substances. gSRT involves the use of RNA-silencing technologies (interference RNA (iRNA) or single-stranded antisense oligonucleotides (ASOs)) and gene-editing tools.

Silencing strategies have emerged in the last decades, with a special focus on ocular disorders, cancer, cardiovascular issues, and viral infections [24]. iRNA consists of an innate post-transcriptional gene-silencing process, which is carried out by diverse molecules. Short interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) are the most studied iRNA tools. On the one hand, siRNAs are short double-stranded RNA segments with 21–23 nucleotides complementary to the target mRNA sequence, and are responsible for its degradation after transcription, preventing translation of the protein [25]. On the other hand, shRNA is a synthetic molecule made up of two sets of complementary RNA sequences, each containing 19–22 nucleotides, connected by a short loop of 4–11 nucleotides. It is based on plasmid-coded RNA, which must undergo transcription within the cellular nucleus. Upon reaching the cytoplasm, the Dicer complex is responsible for processing the hairpin loop to generate a double-stranded siRNA. [26]. As a result, the targeted mRNA is degraded or its translation process into proteins is blocked [27]. iRNA represents the most studied silencing strategy at present [28], and this technology reached its greatest achievement in 2018, when the US Food and Drug Administration (FDA) approved the first siRNA therapeutic for the treatment of the transthyretin-mediated amyloidosis, ONPATTRO^{TM} (patisiran) [29]. Since then, five more siRNA-based products have been authorised [30–34]. Therapeutic applications of antisense technology relies on diverse mechanisms, including translation blocking, RNase H-dependent degradation, and splicing modulation [35, 36]. Currently, nine ASOs have obtained FDA approval [37]. Apart from iRNA and ASOs, genome editing represents a set of technologies based on engineered nucleases that lead to gene silencing. Zinc-finger (ZFN), transcriptor-activator-like effector nuclease (TALEN), meganucleases, and clustered regularly interspaced short palindromic repeats (CRISPR) technology are the main gene-editing strategies [38, 39], the latter being the most employed technique.

The clinical implementation of gene therapy medicinal products faces the major challenge of developing delivery systems tailored to the specific nucleic acid as well as to the therapeutic purpose. Nucleic acid-based medicinal products must have the capability of protecting the genetic material, promoting the association to target cells, and ensuring appropriate intracellular disposition. As can be seen in Table S2 (OSM), viral vectors are at the forefront of gene therapy clinical trials for LSDs; however, their clinical translation is limited by safety concerns, including among others immunogenic and oncogenic potential, and large-scale production [40]. Progress in the science of material and nanotechnology has resulted in a major boost to the development of non-viral vectors, with a better safety profile and advantages for industrial manufacturing, although they do not have the efficacy of viral vectors [41].

The goal of this work was to analyze, through a systematic review, the application of gSRT as a therapeutic strategy

for the treatment of LSDs. The silencing tools and delivery systems used for the implementation of gSRT in this field have been described. This review includes a critical discussion about the main challenges and opportunities for the development and clinical translation of this therapy, with a special focus on the contribution of nanotechnology to the progress and implementation of gSRT in LSDs.

2 Methods

In order to fulfill the objective of this review and to guide data collection and analysis, we formulated four research questions: (i) in which LSD has gSRT been applied?, (ii) which are the silencing strategies studied?, (iii) which are the most employed delivery systems?, and (iv) which are the main challenges for clinical translation? Table 1 shows the objectives of the four research questions formulated to focus the review.

2.1 Adherence to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Guidelines

This systematic review was carried out following the guidelines outlined in the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) Statement [42].

2.2 Data Selection

The data collected in this study were obtained from three databases: PubMed, Scopus, and Web of Science (WoS). To make the search as expansive as possible, the systematic search was conducted up to and including 8 February 2024. The search terms employed are shown in Table 2. The search was additionally limited to English-language articles. Searches were modified to include truncation symbols (*) to ensure all search terms were covered. Full search strategies are presented in Table S3 (OSM).

2.3 Eligibility Criteria

Full-text records/articles that reported information on gSRT in LSDs were included. Articles were excluded if they referred exclusively to the development of cellular or animal models or if they were reviews, preprints, letters to the editor, book chapters, or conference abstracts.

2.4 Data Extraction

Study characteristics were extracted by two authors and included the following: LSD, target (gene/enzyme), silencing strategy, kind of research (in vitro/in vivo), and type of vector (viral/no viral).

2.5 Quality Assessment of the Studies Included

Assessment of methodological quality for the studies involving the use of animal models followed the Animal Research: Reporting of In Vivo Experiments 2.0 (ARRIVE 2.0) guidelines [43]. Studies were scored using the recommended set of 21 items (Table S4 (OSM)). If information corresponding to the subitems was included, it was indicated with "Reported" (= 2 points), while if no reporting was done, it was indicated as "Not reported" (= 0 points). In the case of not all subitems being compliant, it was noted as "Unclear" (= 1 point). A predefined quality coefficient (< 0.5: poor; 0.5–0.8: average; 0.8–1: excellent) was applied for each study. Assessments were independently carried out by three authors, and disagreements were resolved among the authors. Studies were not excluded based on those assessments, but the results were considered in the overall discussion.

3 Results

Using the PRISMA criteria (Fig. 2), 1048 records were identified, and after removing duplicates, 851 records were screened. At the eligibility stage, a title review was first

Table 1 Research questions

	Question	Purpose
Q1	In which LSDs has gSRT been applied as therapeutic strategy?	To study which features are present in those LSDs that are suitable for gSRT
Q2	Which are the silencing strategies employed for gSRT in LSDs?	To identify the possible silencing strategies for each LSD and therapeutic approach
Q3	Which are the most employed delivery systems for gSRT in LSDs?	To evaluate the most suitable delivery system considering the application and the kind of nucleic acid, and to discuss the contribution of nanotechnology to the advancement of the gSRT
Q4	Which are the main challenges for clinical translation of gSRT?	To detect the main issues that gSRT has to overcome to become a medicinal product and achieve clinical translation

Q question, *LSDs* lysosomal storage disease, *gSRT* genetic substrate reduction therapy

Table 2 Search strategy

LSD search terms	Silencing strategy search terms	Search strategy
Lysosomal storage OR Fabry OR Pompe OR Gaucher OR Mucopolysaccharidoses OR Hurler OR Hunter OR Sanfilippo OR Morquio OR Maroteaux OR Metachromatic OR Niemann OR Wolman OR Jansky	Gene silencing OR interference RNA OR iRNA OR siRNA OR shRNA OR microRNA OR Gene editing OR CRISPR OR ZFN OR TALEN OR ASO* OR Gapmer OR Antisense OR oligonucleotide	*Title/Abstract* (LSD Keywords) AND *Title/Abstract* (Silencing strategy Keywords) AND *Language* (English)

LSD lysosomal storage disease, *iRNA* interference RNA, *shRNA* short hairpin RNA, *CRISPR* clustered regularly interspaced short palindromic repeats, *ZFN* zinc finger, *TALEN* transcriptor-activator-like effector nuclease, *ASO* antisense oligonucleotide

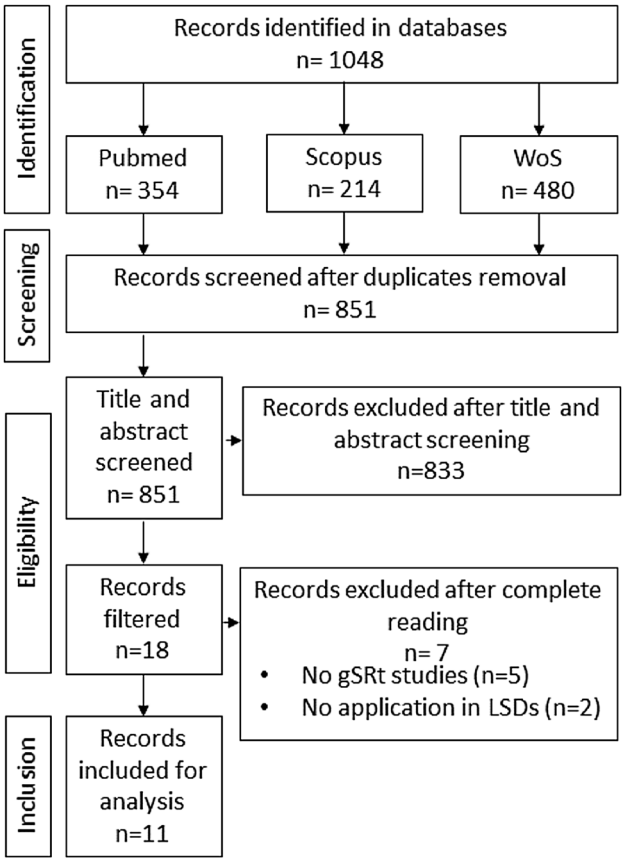

Fig. 2 PRISMA flow diagram of the selection process of the records from the three databases. *WoS* web of science, *LSD* lysosomal storage disease, *gSRT* genetic substrate reduction therapy

conducted. In a second stage, the abstract-screening process was focused mainly on being in accordance with eligibility criteria. After both processes, 833 records were excluded and 18 were considered for complete reading. Finally, 11 articles were included in the final analysis.

The reasons for excluding the records were: the objective was the development of new cellular and animal models but not the evaluation of new gSRT treatments, and gSRT was not applied to LSDs.

3.1 The Studies

The publications included in the systematic review are summarized in Table 3. A description of the main achievements of these studies is presented below.

3.1.1 Fabry Disease Studies

Three reports about the application of gSRT in FD were identified, all of them published in 2023, as shown in Table 3. Kim et al. [44] developed a delivery vector bearing siRNA against globotriaosylceramide (Gb3) synthase

Table 3 Summarized information from the eligible studies

References	Authors	Year	LSD	Target (enzyme/gene)	Silencing strategy	Kind of research (in vitro/in vivo)	Type of vector (viral/non-viral)
[44]	Kim et al.	2023	Fabry disease	Gb3 synthase/*A4GALT*	siRNA	In vitro A549 cells	Polyhistidine-incorporated lipid nanoparticles
[45]	Beraza-Millor et al.	2023	Fabry disease	Gb3 synthase/*A4GALT*	siRNA	In vitro IMFE-1 cells	Solid lipid nanoparticles with cationic lipids
[46]	Cui et al.	2023	Fabry disease	Gb3 synthase/*A4GALT*	CRISPR/cas9	Kidney organoids	Electroporation
[47]	Diaz-Font et al.	2006	Gaucher disease	Glucosylceramide synthase/*GCS*	siRNA shRNA	In vitro HeLa cells RAW264.7 (mouse macrophage cells)	Lipofectamine 2000 ™
[48]	Douillard-Guilloux et al.	2008	Pompe disease	Glycogen synthase/GYS Glycogenin/*GYG*	shRNA	In vitro Primary myoblasts and C2C12	Lentiviral
						In vivo Gaa mice (i.m., gastrocnemius)	AAV2/1
[49]	Clayton et al.	2014	Pompe disease	Glycogen synthase/ *GYS1*	Antisense oligonucleotide (phosphorodiamidate morpholino oligonucleotide) for exon skipping	In vivo Gaa and C57BL/6 mice (i.v., tail vein injection) Once every 2 weeks during 12 weeks	Cell-penetrating peptide
[50]	Kaidonis et al.	2010	MPSs I (Hurler) and IIIA (Sanfilippo A)	Heparan sulphate sythesising enzymes EXTL2 and EXTL3 /*EXTL2, EXTL3*	shRNA	In vitro 293T cells and human fibroblasts	Lipofectamine™ Lentiviral
[51]	Dziedzic et al.	2010	MPS III A (Sanfilippo A)	Heparan sulphate sythesising enzymes XYLT1, XYLT2 GALTI, GALTII /*XYLT1, XYLT2* *GALTI, GALTII*	siRNA	In vitro MPS fibroblasts	HiPerFect® Transfection reagent (blend of cationic and neutral lipids)
[52]	Dziedzic et al.	2012	MPSs	Heparan sulphate sythesising enzymes EXTL2 and EXTL3/*EXTL2, EXTL3*	siRNA	In vitro MPS fibroblasts	HiPerFect® Transfection reagent (blend of cationic and neutral lipids)
[53]	Chmielarz et al.	2012	MPS I (Hurler)	Heparan sulphate sythesising enzymes XYLT1, XYLT2 GALTI, GALTII/*XYLT1, XYLT2* *GALTI, GALTII*	siRNA	In vitro MPS fibroblasts	HiPerFect® Transfection reagent (blend of cationic and neutral lipids)
[54]	Canals et al.	2015	MPS III C (Sanfilippo C)	Heparan sulphate sythesising enzymes EXTL2 and EXTL3/*EXTL2, EXTL3*	siRNA	In vitro MPS fibroblasts	Lipofectamine 2000™

AAV adeno-associated viral vectors, *A549 cells* adenocarcinomic human alveolar basal epithelial cells, *Gb3* globotriaosylceramide, *IMFE-1* immortalized Fabry endothelial cell line-1, *siRNA* small interfering RNA, *shRNA* short hairpin RNA, *CRISPR/Cas9* clustered regularly interspaced short palindromic repeats, *MPSs* mucopolysaccharidoses, *i.m.* intramuscular administration, *i.v.* intravenous administration

(Gb3S) siRNA, the enzyme implicated in the production of Gb3, which is the substrate that forms deposists in FD-affected cells. They first selected the optimal siRNA sequence that was formulated in a novel polyhistidine-incorporated lipid nanoparticle (LNP) (pHis/LNP) by a microfluidic method to ensure siRNA protection from degradation. In A549 cells (adenocarcinomic human alveolar basal epithelial cells), *Gb3S* gene (*A4GALT)* silencing by siRNA pHis/LNP was greatly improved (6.0-fold) compared to that by siRNA/LNP.

Recently, novel nanomedicines, which consist of various siRNA molecules targeted to *Gb3S*, encapsulated in solid lipid nanoparticles (SLNs), have been developed. These SLNs-vectors were further modified with different ligands, including gold nanoparticles, protamine, and polysaccharides [45]. The siRNA-golden SLNs showed efficient association with FD model cells (IMFE-1), leading to silencing rates of up to 90% at the GB3S-mRNA level. The effectiveness of silencing depended on the specific composition and preparation technique of the system. The silencing of Gb3S-mRNA resulted in a reduction of the enzyme expression, and the combination of different siRNA sequences induced a synergic effect, allowing a reduction of the total siRNA-dose.

Cui and co-workers [46] investigated whether the suppression of the gene-encoding Gb3S (*A4GALT*) by CRISPR-Cas9 technology could reverse the phenotype of FD nephropathy (FDN) in a kidney organoid system derived from human-induced pluripotent stem cells (hiPSCs). They generated a FDN patient-derived hiPSCs (CMC-Fb-002) and a FD-specific hiPSCs (GLAKO) by knock-out (KO) of *GLA* (the gen that encodes α-galactosidase A, the enzyme deficient in FD patients) in wild-type (WT) hiPSCs. Then, they performed CRISPR/Cas9-mediated A4GALT KO in both cell models and generated kidney organoids. The authors found a rescue of FDN phenotype, and hence, they proposed this silencing strategy as a therapeutic approach to treat FDN.

3.1.2 Gaucher Disease Study

gSRT with siRNA for GD was first studied in 2006 by Diaz-Font et al. [47] (Table 3). They designed four different siRNAs for the human glucosylceramide synthase (*GCS*) gene, which catalyzes the first step in glycosylceramide (GSL) synthesis. Two of the siRNAs resulted in significantly reduced *GCS* mRNA levels (up to 70%), and GCS enzyme activity in HeLa cells, using Lipofectamine™ as the delivery system. With the two siRNAs, the efficacy depended on the siRNA concentration. A significant decrease in glucosylceramide formation with one of the siRNAs was also observed. Based on those results, the authors constructed two different shRNA expression vectors (targeting the same sequences), which were also able to inhibit *GCS* expression (49 and 65%) and GCS activity (58 and 50%).

3.1.3 Pompe Disease Studies

The use of iRNA with the aim of reducing the lysosomal accumulation of glycogen in PD was first tested by Douillard-Guilloux et al. [48] in 2008, as it is shown in Table 3. They used shRNAs targeted to glycogenin and glycogen synthase genes (*GYG* and *GYS*), respectively, the two major enzymes involved in glycogen synthesis. shRNAs were formulated in lentiviral vectors and tested in myoblast C2C12 cells and in primary myoblasts from PD mice. Silencing of *GYG* and *GYS* resulted in a decrease of glycogen deposits in transduced cells, as well as reduction of lysosomal size. Additionally, intramuscular injection of recombinant AAV-1/2 (adeno-associated virus-1/2) vectors expressing shRNA targeted to GYS mRNA into newborn Gaa PD mice demonstrated the efficacy of this strategy to reduce glycogen accumulation in vivo.

As an alternative, Clayton and co-workers [49] proposed the suppression of muscle-specific glycogen synthase 1 (Gys1) with ASOs. They formulated a phosphorodiamidate morpholino oligonucleotide (PMO) with a cell-penetrating peptide (GS-PPMO) for facilitating muscular delivery. The PMO induced exon skipping and premature stop codon in the *GYS1* gene, and after systemic administration of GS-PPMO to Pompe mice, a 90% reduction of Gys mRNA in the quadriceps and in the diaphragm was detected. The reduction of mRNA in heart was only achieved with the highest dose administered. The reduction of transcript levels was accompanied by decreased levels of muscular GYS and enzymatic activity, as well as lower lysosomal glycogen deposits in those tissues.

3.1.4 Mucopolysaccharidoses Studies

In 2010, Kaidonis et al. [50] used iRNA technology to target *EXTL2* and *EXTL3*, two genes involved in the synthesis of heparan sulfate (HS), which is the glycosaminoglycan (GAG) that accumulates in MPS III (Sanfilippo syndrome) patients (Table 3). They designed several shRNA constructs specific to *EXTL2* or *EXTL3*. Among them, three *EXTL2- and* one *EXTL3*-specific shRNAs, delivered by Lipofectamine™ 2000, were able to knock down endogenous target gene expression in kidney epithelial 293T cells, and decreased GAG synthesis. Lysosomal GAG levels were also reduced in MPS IIIA and MPS I fibroblasts. Following this, the administration of shRNAs into a lentiviral vector decreased gene expression, and one of the shRNAs targeted to *EXTL2* was effective in reducing GAG levels. These results demonstrate the potential of shRNA therapy to treat HS-storing MPSs.

In other study, Dziedzic et al. [51] employed siRNA to control the expression of genes involved in the synthesis of GAGs and in the first steps of HS. They were able to downregulate the mRNA levels of *XYLT1*, *XYLT2*, *GALTI*, and *GALTII* genes in MPS IIIA fibroblasts (20–40% with respect to control samples). A reduction of the levels of transcripts led to a decrease in levels of the corresponding proteins. Additionally, reduction of GAG synthesis in the fibroblasts was achieved (up to 40%). Taking into account their results, the same research group later studied if the combination of two different and specific siRNA sequences would be able to silence the expression of the two genes involved in particular steps of GAG synthesis in a more effective manner than the use of single siRNAs [52]. However, they found no statistical differences in the GAG synthesis between the use of a pair of siRNAs and the use of single siRNAs.

Chmierlarz et al. [53] evaluated in three MPS I cell lines the effectiveness of ERT (laronidase, Aldurazyme®) in combination with siRNAs targeted to *XYLT1, XYLT2, GALTI*, and *GALTII* genes. Depending on the cell line, the decrease in GAG storage after treatment with the combination of ERT and siRNA was less pronounced, similar, or stronger than that obtained with the two strategies used alone.

In order to reduce lysosomal GAG accumulation inside the lysosomes, Canals et al. [54] employed different siRNAs targeting *EXTL2* and *EXTL3* genes, and tested them in fibroblasts from two different patients suffering from MPS III C (Sanfilippo C) by using Lipofectamine™ 2000 as a transfection agent. This achieved a silencing of EXTL mRNAs of about 90%, 30–60% decrease in GAG production after 3 days, and up to 24% decrease in GAG storage after 2 weeks. A clear reversion of the phenotype after treatment was also detected.

3.2 Quality Assessment

Quality assessment was applied to the two studies involving animal models (Douillard-Guilloux et al. [48] and Clayton et al. [49]). The most frequently reported items in the in vivo experiments were study design, outcome measures, statistical methods, experimental animals, results, abstract, background, and objectives. Conversely, items least frequently reported included randomization, blinding, housing and husbandry, protocol registration, and data access. The quality coefficients were 0.40 and 0.54, respectively.

4 Discussion

4.1 Q1. In Which LSDs has gSRT Been Applied as a Therapeutic Strategy?

The systematic review revealed that gSRT has been applied to LSDs only at a preclinical level. The identified studies in which gSRT has been applied include sphingolipidoses (specifically in FD [44–46] and GD [47]), glycogen storage PD [48, 49], and MPSs I and III [50–54]. Figure 3 summarizes where in the metabolic pathways gSRT has been applied to treat these LSDs. No studies on lipid storage disorders or neuronal ceroid lipofuscinosis were found in the systematic search.

FD and GD are sphingolipidoses for which SRT is available or is under clinical research. Lucerastat (NCT03425539, NCT03737214) and venglustat (NCT02228460, NCT02489344) are two oral iminosugars that inhibit GCS, limiting the amount of glucosylceramide availability, and thus preventing the accumulation of Gb3 in FD patients, whose kidney, heart, brain, and peripheral nervous system are the main affected organs [55–59]. However, it is noteworthy that depending on the step or enzyme of the metabolic route inhibited, crucial pathways for the production of a whole range of glycosphingolipid intermediates with important biological functions could also be blocked, resulting in potential hazards. In this sense, glucosylceramide is a common precursor in the synthesis of many other glycosphingolipids, and not only of Gb3; therefore, its inhibition would also affect other products, such as lactosylceramide, whose reduction might induce neurological side effects. As an alternative to avoid those side effects, SRT can be directed to the inhibition of Gb3S, which plays a part in the final step of the Gb3 synthesis. In this regard, gSRT for FD has been successfully tested in vitro in three recent studies to selectively downregulate Gb3S. The reduction of Gb3S-mRNA was achieved by siRNA technology in A549 cells (a pulmonary epithelial cell line derived from a human alveolar cell carcinoma) [1], and in IMFE-1 cells [2], a cellular model of FD. As another strategy to treat FD nephropathy, Cui et al. [3] applied CRISPR/Cas9-mediated A4GALT KO in kidney organoids generated from patient-derived hiPSCs.

With regard to GD, current therapies include three ERT products, imiglucerase (Cerezyme®), velaglucerase alfa (Vpriv®) and taliglucerase alfa (Elelyso®), with efficacy and safety demonstrated by long-term observations [60–62], and two SRT orally administered products. Miglustat (*N*-butyldeox-ynojirimycin, Zavesca®) and eliglustat tartrate (Cerdelga®), which reduce the biosynthesis of GCS [61], although eliglustat is unable to cross the BBB and miglustat is not active in neuropathic symptoms [63]. However, despite the success of the five approved therapies, there are still many unresolved issues that highlight the need for the improvement and development of new therapies. Among others, development of antibodies in ERT or drawbacks resulting from inhibitory effects on undesired metabolic pathways for SRT, as with mentioned above for FD [64]. In addition, approved therapies have a limited effect on neuropathic signs, although the negative impact is limited

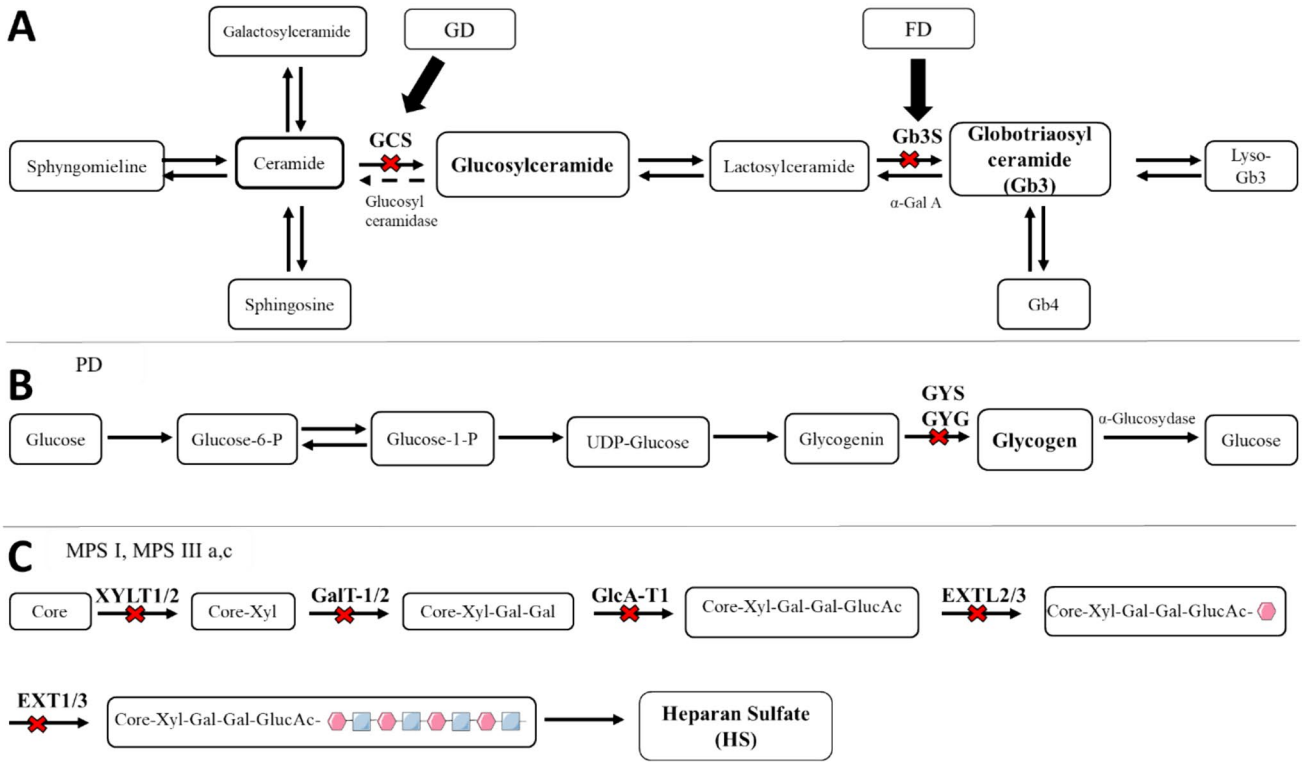

Fig. 3 Target enzymes of LSDs metabolic pathways addressed by gene substrate reduction therapy (gSRT). **A** Application in Gaucher disease (GD) and Fabry disease (FD). **B** Application in Pompe disease (PD). **C** Application in MPSs I and III. *GCS* glucosylceramide synthase, *Gb3S* Gb3 synthase, *α-Gal A* α-Galactosidase A, *GYS* glycogen synthase, *GYG*, glycogenin, *MPS* mucopolysaccharidosis, *Xyl* xylose, *Gal* galactose, *GlucAc* glucuronic acid, *XYLT* O-Xylosyltransferase, *GalT-1/2* β-galactosiltransferase 1/2, *GlcA-T1* glucosyltransferase 1, *EXTL2/3* EXT-like protein 2 and 3, *EXT1/3* glycosiltransferase EXT1 and EXT3

because of the majority of GD patients are type 1, without neuropathic features. Apart from that, the success of ERT coupled with the availability of SRT treatments may be the reason for the lack of recent studies about the application of gSRT in this disease. There is only one study, published in 2006, in which the GCS enzyme was inhibited in vitro through siRNA, resulting in reduction of glucosylceramide accumulation in HeLa cells [47].

Lysosomal accumulation of glycogen in PD triggers cell dysfunction, especially in cardiac, smooth, and skeletal muscle cells and motor neurons [65]. ERT is the only approved therapy for PD. It is based on recombinant GAA (rhGAA), alglucosidase alfa (Myozyme®, Lumizyme®), or the recently authorized avalglucosidase alfa (Nexviadyme®). Lifelong intravenous administration of ERT is able to clear glycogen effectively in the heart and resolve cardiomyopathy; however, its effectiveness is limited in skeletal muscle and does not address respiratory or neurological deterioration [66, 67]. In addition, the effectiveness of ERT is influenced by antibody development, which is a relevant issue in FD and PD (mostly in subgroups with no residual GAA enzyme activity) as well as in MPSs. To ameliorate the PD condition, gSRT has been evaluated in vitro and in vivo as a specific tool to inhibit the expression of GYG and GYS (Fig. 3B). Two silencing strategies (shRNA or ASO) have been evaluated in a mouse model of PD: on the one hand, intramuscular administration shRNA reduced glycogen in the muscle [48], on the other hand, intravenous injection of the ASO resulted in substrate reduction in the quadriceps, diaphragm, and heart 1 month after administration.

MPSs are a group of LSDs caused by the deficiency of enzymes directly involved in glycosaminoglycan (GAG) catabolism [68, 69]. MPS III (Sanfilippo syndrome), which is subclassified into four subtypes (IIIA-IIID), is the most frequent MPS, and patients suffer from severe neurological dysfunctions [70, 71]. MPS I (Hurler syndrome) is caused by a deficiency of the α-L-iduronidase (IDUA) enzyme, which catalyzes the degradation of both HS and dermatan sulfate (DS) GAGs. ERT is available for MPS I, MPS II, MPS VI, MPS IVA, and MPS VII. Weekly intravenous infusion has shown efficacy in some MPSs, but limited effects in the CNS have been observed [72, 73]. Apart from ERT, hematopoietic stem cell transplantation (HSCT) represents the gold standard for MPSI patients, especially for neuropathic forms [74, 75]. Although the natural isoflavone genistein is an SRT option [76], no attenuation of the CNS symptoms

has been observed in clinical trials [77]. gSRT has been evaluated in vitro as a therapeutic option for MPS I [53] and III [50–52, 54]; the objective was to inhibit different enzymes involved in the synthesis of HS (Fig. 3C). The different strategies evaluated were targeted to two of the three steps involved in the synthesis of GAGs (tetrasaccharide linkage, chain elongation and maturation [78]). Downregulation of *XYLT1*, *XYLT2*, *GALTI*, and *GALTII* genes involved in the tetrasaccharide linkage step by siRNA-mediated gSRT reduced the levels of xylosyltrasnferase 1 and 2 and β-galactosiltransferase 1 and 2, as well as GAG synthesis in MPS IIIA and MPS I cell lines [8, 10]. gSRT targeted the *EXTL2* and *EXTL3* genes, encoding enzymes, which act on the elongation of the HS chain, also reduced the GAG synthesis [7, 11]. shRNA delivered by a lentiviral vector downregulated the EXTL2 enzyme and reduced the GAG synthesis in kidney epithelial 293T cells [50]. Canals et al. [54] by using siRNA targeted to the same gene observed a decrease in GAG synthesis and storage, as well as a clear reversion of the phenotype of MPS III C fibroblasts.

4.2 Q2. Which are the Silencing Strategies Employed for gSRT in LSDs?

Several molecular strategies are available to achieve the silencing required for gSRT, including interference RNA (iRNA), antisense oligonucleotides (ASOs) [49], and CRISPR/Cas9 gene-editing technology [46]. Among the iRNA technologies, only siRNA [44, 45, 47, 51–54] and shRNA [47, 48, 50] have been employed for gSRT in LSDs. Figure 4 describes the mechanisms of action of silencing molecules and the LSDs where they have been applied for gSRT.

siRNA are short double-stranded RNA segments able to cleavage the target mRNA sequence once incorporated into the RNA-induced silencing complex (RISC) [25, 79]. siRNA technology has been used to test gSRT in vitro for Gb3S-mRNA downregulation in FD [46], and several siRNAs have been evaluated in different cell lines for MPS IIIA [8], MPS III C [54], and MPS I [10]. A single siRNA molecule can bind to and regulate multiple mRNA copies; consequently, low siRNA concentrations are able to induce efficient gene knockdown [80, 81]. However, cellular, plasma, and tissue degradation by nucleases often results in short-term silencing activity [82]. Therefore, due to the gradual reduction of siRNA concentration in the cytoplasm, siRNA-based treatments would require multiple dosing, especially in dividing cells, limiting the implementation in clinical use [79, 83].

shRNA is an alternative for long-term gSRT based on iRNA technology. shRNA is a plasmid-coded RNA processed into a pre-shRNA molecule in the nucleus that, once in the cytoplasm, forms a double-stranded siRNA [26, 83]. Therefore, transfected cells can synthesize siRNA molecules continuously, and, consequently, the effect of shRNA is longer lasting compared to siRNA [83–85]. However, the need for shRNA to enter the cell nucleus limits its potential and considerably complicates the development of delivery systems, making it difficult to introduce into clinical use. shRNA molecules have been designed and evaluated as gSRT tools in vitro for GD [47], MPS IIIA and MPS I [50], and in mice for PD [48]. Nevertheless, despite the potential of shRNA, currently the iRNA-based therapeutics marketed include only siRNA molecules. It is important to note that thanks to strategies such as *N*-acetylgalactosamine (GalNAc) conjugation for tissue targeting, and chemical modification of siRNAs for stability against nucleases, iRNA therapeutics can be administered at lower doses and with longer dosing intervals; two examples are vutrisiran and inclisiran, which are administered every 3–6 months. Strategies to improve the siRNA stability and effectiveness involve the modification of the 2′-hydroxyl group, the use of modified locked nucleic acids, conjugation with cholesterol and polyethylene glycol (PEG), or sugar and backbone modifications [79, 86, 87]. Apart from conjugation with GalNAc, siRNA design generally involves 2′ modifications across the entire siRNA and stabilization of the ends. Most RNAi therapeutics approved or in clinical development feature a 19- to 21-nucleotides duplex structure with a single 2-nt overhang at the 3′ end of the antisense strand [88].

ASOs are short synthetic oligonucleotides (15–25 nucleic acid length) that bind to complementary RNA sequences (targeted mRNA) through Watson–Crick base pairing for gene downregulation [89]. As represented in Fig. 4, they can regulate the expression of specific genes through a variety of mechanisms, including inhibition of 5′ cap formation, steric blocking of protein translation, RNA splicing modulation, and activation of RNAse H to degrade the target mRNA [90], or modification of pre-mRNA splicing at the cellular nucleus [91]. Various chemical modifications of ASOs have been implemented in order to reduce the possibility of degradation by nucleases. In fact, unmodified ASOs are five to ten times more vulnerable to degradation than modified ones [67]. Chemical modifications include changes in phosphodiester bonds [68] or in 2′ nucleotide sugar, like 2′-O-methyl (2′O-Me) and 2′-O-methoxyethyl (2′-O-MOE) [69]. The design of PMO and peptide nucleic acids (PNAs) has enhanced stability and nuclease resistance of ASOs, maintaining their high mRNA target affinity [70]. In order to develop a gSRT for PD, Clayton et al. [49] employed a PMO to induce skipping of exon 6 and premature stop codons present in the Gys1 transcript to produce an unstable mRNA. Advances in chemical development and improvement of the backbone structure have provided an important impetus for clinical translation of ASOs. To date, nine ASOs have been approved by the FDA, and most of them achieved marketing authorization in the last 5 years [92].

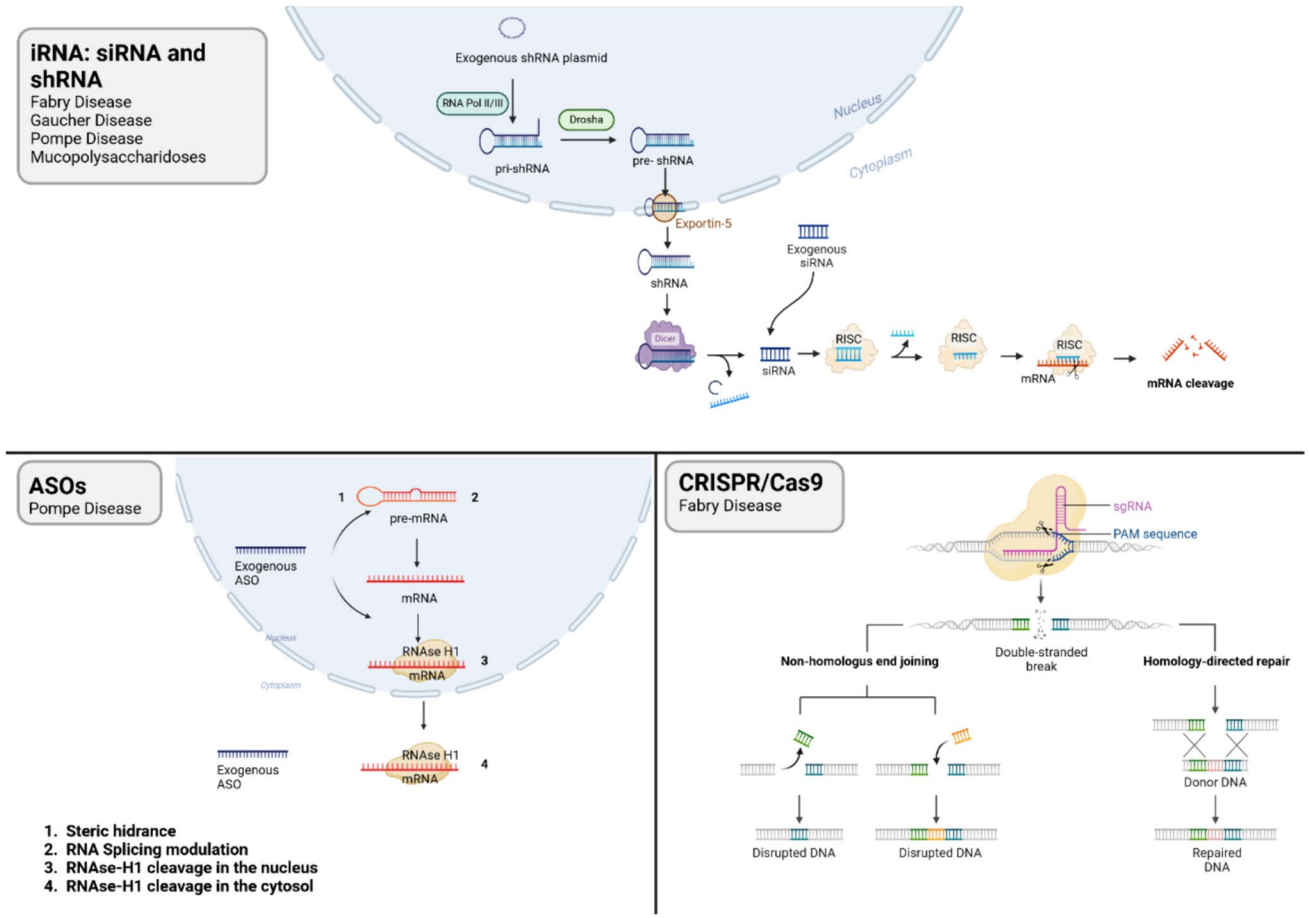

Fig. 4 Silencing and gene-editing strategies used for gSRT in LSDs. *siRNA* short interfering RNA, *shRNA* short hairpin RNA, *RNA Pol II/III* RNA polymerase II/III, *RISC* RNA-induced silencing complex, *mRNA* messenger RNA, *ASO* antisense oligonucleotide, *CRISPR/Cas9* clustered regularly interspaced short palindromic repeats, *sgRNA* single guide RNA, *PAM sequence* protospacer adjacent motif. Created with BioRender.com

Gene-editing technology has recently emerged as a precise DNA modification technique that leads to a permanent effect. Editing platforms cleave the double-stranded DNA at a specific location by programmable nucleases, so cellular repair mechanisms become active and induce sequence changes at the cleaved site [93]. The reparation of the double-strand break can be done by homology-dependent repair (HDR) or non-homologous end joining (NHEJ). A NHEJ mechanism results in an insertion-deletion mutation in target sites [94]. However, in the presence of an appropriate DNA template, HDR leads to a precise replacement of a gene nucleotide [95]. Among different editing tools, CRISPR/Cas9 technology is the only one employed to date for gSRT [46]. Cui et al. [3] performed ex vivo CRISPR/Cas9-mediated *A4GALT* KO in kidney organoids generated from patient-derived hiPSCs as a strategy to treat FD nephropathy. Therapeutic genome-editing processes have been greatly accelerated in recent years because of improvements in sequence-specific nuclease technology, despite the possibility that CRISPR/Cas9 technology may also present limitations related to immunological effects [96, 97]. Recently, an ex vivo CRISPR/Cas9 therapy for sickle cell disease and beta-thalasemia (exagamglogene autotemcel, Casgevy®) has obtained approval from the FDA and the European Medicines Agency (EMA) [98]. This therapy has shown high levels of allelic editing in bone marrow and blood in treated patients more than a year later [99]. Gene-editing technology is able to provide a very high suppression of the targeted sequence permanently. However, for LSDs a minimal expression of the target enzyme is necessary to maintain cellular homeostasis and functions, and complete suppression is not desired [59, 100]. In addition, the non-permanent activity of other silencing strategies, such as siRNA or ASO, could be beneficial because their effect can potentially be reversed.

A major concern for silencing strategies is off-target effects due to unintended genes affected in treated cells. In this regard, the off-target effects of CRISPR/Cas9 occur

when Cas9 acts on untargeted genomic sites. Some studies have shown that off-targets with shRNA are less frequent than with siRNA [84]. To minimize off-targets, different strategies are emerging, including in silico prediction [101, 102].

4.3 Q3. Which are the Most Employed Delivery Systems for gSRT in LSDs?

gSRT strategies for LSDs require the development of innovative delivery systems that preserve nucleic acids from degradation, afford specificity to target the desired cell, and an appropriate intracellular distribution, preventing off-targets and the activation of the immune response. Overall, delivery systems are categorized as viral and non-viral vectors, and the latter into chemical and physical systems. Viral vectors are the most widely used delivery systems in the clinic, but nanomedicines, and in particular LNP-based therapeutics, have recently revolutionized the field of nucleic acid therapies.

The most suitable delivery system would depend not only on the type of LSD, but also on the therapeutic nucleic acid. As it can be observed in Fig. 5, once a delivery system reaches the target cell, it undergoes several steps that impact its effectiveness. The interaction between vectors and cell surface by electrostatic interactions are favored for cationic nanosystems, but the inclusion in the vectors of ligands with the ability to interact with specific cell surface receptors can also enhance cell binding [103]. The entry of the delivery system into the cell is mainly driven by endocytosis, which involves many complex processes determining the intracellular disposition of nucleic acids [104]. Upon entering the cell, vectors follow the endosome-lysosome pathway, moving gradually from early to late endosomes, with a pH reduction until they reach the lysosomes. The acidic pH and digestive enzymes inside the lysosome are factors inducing nucleic acid degradation [105], and a suitable delivery system should ensure their protection and endosomal escape. Moreover, specific nuclear delivery strategies should be implemented for shRNA, CRISPR/Cas9, and for ASOs acting in the nucleus.

Viral vectors allow high transfection efficiencies in vitro, ex vivo and in vivo. The choice of the optimal viral system is determined by a variety of factors such as the target cell type, the ability to integrate the genetic material, and the size of the nucleic acid [106]. All except one of the gene therapy clinical trials for LSDs (Table S2, OSM) use lentiviral and adeno-associated virus (AAV) delivery systems, but none of those clinical trials involved the gSRT approach. In the case of gSRT, lentiviruses have been employed by Douillard-Guilloux et al. [48] and Kaidonis et al. [50] for the delivery of shRNA in vitro. Lentivirus can incorporate constructs up to 9–10 kB in size [107], providing long-term and stable gene expression [108], and are also good candidates for ex vivo transfection [109]. Integration-defective lentiviruses that deliver nucleic acids without permanently integrating into the host genome are a suitable option to avoid the oncogenicity risk associated with integration processes [110]. AAVs can provide long-term expressions [111] without presenting integrating features [112]. A large number of AAV serotypes allow for tissue-targeted gene delivery. High muscle tropism recombinant AAV2/1 vectors co-expressing EGFP (enhanced green fluorescent protein) and short hairpin GYS2 (AAV.shGYS2) were developed by Douillard-Guilloux to address PD [48]. AAVs have been widely used for gene therapy research because of their relatively low immunogenicity and high efficiency. The AAV packaging capacity of 4.7 kb limits carrying large genes, such as CRISPR/Cas9 machinery [113]. In order to overcome this obstacle, dual AAV systems, with an expanded capacity of approximately 9 kb, have been developed and shown to be effective in vivo for CRISPR-mediated gene corrections [114, 115]. However, electroporation was applied in the first and only study using CRISPR/Cas9 technology for gSRT [46]. Non-viral physical transfection methods include electroporation, sonoporation, gene gun, and microinjection [116]. Electroporation can mediate extremely efficient delivery, creating pores in the cell membrane by using electric pulses, and although cell toxicity has limited its clinical application, it is an interesting alternative for ex vivo therapies [104].

Apart from transgene expression and integration process, immunogenicity is another factor that influences the suitability of a vector for specific therapeutic application [117]. Pre-existing antibodies against viruses, especially against adenovirus, are prevalent within the population, and result in decreased expression of the transgene and exacerbated virulence of the vector [118]. Non-viral vectors are not exempt from immunogenicity, although it is considered to be much lower than that of viral vectors [119]. Vector immunogenicity is an additional disadvantage when repeated doses are required, as in the case of iRNA and ASO-based nanomedicines.

Non-viral nanodelivery systems involve an extensive variety of structures including lipids, polymeric and polypeptidic systems, dendrimers, inorganic nanoparticles, and hybrid systems. They show advantages such as safety, cost, ease of manufacture, reduced immune responses, multidose capability, large payloads, and flexibility of design. In addition, although clinical translation is hindered by the transfection effectiveness, important progress has been made through different approaches [120, 121].

Lipid-based vectors are the most widely used, and in this systematic review, eight out of the 11 identified studies applying gSRT to LSDs employed them for siRNA and ASO delivery [44, 45, 47, 49, 51–54]. Cationic lipids, which consist of hydrophobic alkyl chains linked by a

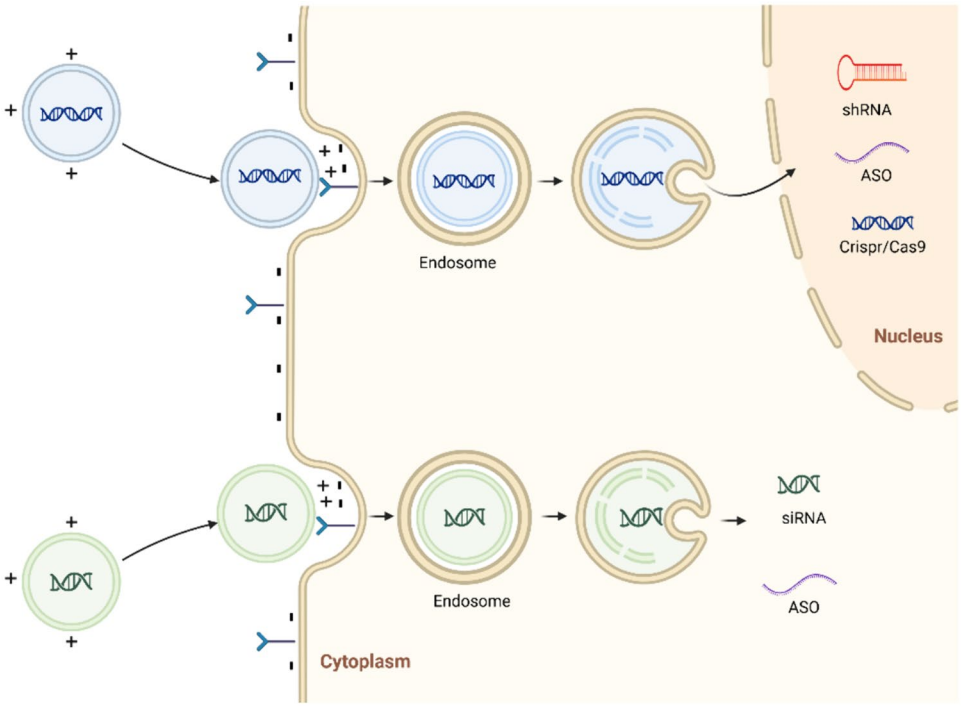

Fig. 5 Intracellular barriers for nucleic acid delivery systems. *shRNA* short hairpin RNA, *ASO* antisense oligonucleotide, *CRISPR/Cas9* clustered regularly interspaced short palindromic repeats/Cas9, *siRNA* small interfering RNA. Created with BioRender.com

binding structure to a polar group, are common components of lipid systems. These lipids interact with the anionic charges of the cell membrane, condense, protect, and achieve efficient nucleic acid loading with high delivery effectiveness. One of the most employed cationic lipids is 1,2-di-O-octadecenyl-3-propane (DOTAP), which has been used for siRNA-mediated gSRT in FD [45]. The combination of *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) with 1,2-dioleoyl-sn-gycero-3-phosphoethanolamine (DOPE) was marketed as Lipofectamine™, one of the most employed transfection reagents [122]. Lipofectamine™ and HiPerFect®, another commonly used commercial transfection reagent based on a blend of cationic and neutral lipids, provide high transfection effectiveness and have been widely employed in in vitro studies of gSRT for LSDs [47, 50, 52–54]. However, clinical applications of cationic lipids are limited by the toxicity related to their positive charges, which lead to non-specific binding to anionic cellular and extracellular elements, a short circulation half-life, activation of the immune response, and the low efficient endosomal escape [123–125].

As an alternative to permanent cationic lipids, ionizable lipids have been recently developed. They show reduced toxicity while maintaining their transfection capacity. Ionizable cationic lipids present a tertiary amine that can protonate in an acidic pH environment and, through the proton sponge effect, promote endosomal escape, a bottleneck for successful cell transfection [126, 127]. Ionizable lipids were first developed for DNA delivery, but lately they have been employed for siRNA delivery. The incorporation of the ionizable lipid (6Z, 9Z, 28Z, 31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino) butanoate (DLin-MC3-DMA; MC3) resulted in the first siRNA commercialized product, Onpattro™ [128]. In addition, the leading mRNA COVID-19 vaccines, from Pzifer/BioNTech and Moderna, are based on LNP technology containing the ionizable lipid ALC-0315 and the SM-102, respectively [129]. Currently, nanocarriers that include ionizable cationic lipids are considered as one of the main options in the field of delivery systems, showing potential for siRNA delivery applications. The effect of the incorporation of the ionizable lipid 1,2-dioleoyl-3-dimethylammonium-propane (DODAP) in SLNs-based vectors for gSRT was studied in FD [45]. For most vectors, the incorporation of DODAP did not increase the silencing efficiency; instead, factors like the technique used for SLNs preparation or the inclusion of various ligands had a higher influence on the reduction of Gb3S expression in an FD cellular model (IMFE-1 cells). This strategy was also evaluated by Kim et al. [44] using polyhistidine, a polypeptide biodegradable by peptidases in the cytoplasm that promotes endosomal escape via the proton sponge effect because of the histidine residues (pKa 6.0–6.4) [130, 131]. The incorporation of polyhistidine into lipid nanoparticles, which also contained the ionizable lipid 2-hexyl-decanoic acid and 1, 1′-[[(4-hydroxybutyl)- imino]di-6,1-hexanediyl] ester, resulted in a greatly improved Gb3S gene silencing (6.0-fold).

The modification of nanoparticles surfaces with polymers can increase the nanomedicines in the organism by reducing

their interaction with biological components, improving their effectiveness. PEG is widely used for this purpose but the generation of PEG-specific antibodies after the administration of PEG-coated LNPs has been described [132, 133]. Other components such as polysaccharides can also hamper interactions with biological components and could be beneficial for in vivo therapies [134]. For example, Delgado et al. [135] incorporated dextran on the surface of SLNs and observed a prolongedretention of SLNs in the bloodstream. In this regard, different polysaccharides have been included in SLNs containing siRNA targeted to silenceGb3s for FD [45].

Cell-penetrating peptides (CPPs) have also found application in gSRT as a peptidic non-viral system for nucleic acid delivery. The precise mechanisms for overcoming cell membranes and enhancing cellular internalization are not completely understood. It is hypothesized that CPPs could induce the clustering of negatively charged glycosaminoglycans present on the cell surface, thereby initiating micropinocytosis and lateral diffusion, or directly causing disruption of the lipid bilayer [127]. Clayton et al. [49] employed an arginine-rich CPP to improve the biodistribution of a PMO to muscle after systemic administration. In addition, the CPP presented non-α-amino acids, such as 6-aminohexanoic acid and β-alanine, which previously had resulted in increased serum and intracellular stability, reduced endosomal trapping, and enhanced nuclear uptake [136]. Chemical modification and conjugation of PMO to CPP allowed a significant dose reduction in Pompe mice and led to a dose-dependent decrease in glycogen synthase transcripts.

4.4 Q4. Which are the Main Challenges for Clinical Translation of gSRT?

The lack of clinical trials for the treatment of LSDs using gSRT, despite the current paucity of treatment options, underscores the many hurdles that must be overcome for successful clinical translation. Nanotechnology offers a wide variety of smart therapeutic tools that are easily adaptable to different activities and applications, but important issues such as tissue-targeted delivery beyond the liver, immunogenicity, and low effectiveness still need to be addressed before its use can be expanded. Apart from the aforementioned challenges related to gene-silencing and -editing mechanisms and delivery strategies, other limitations have been identified, such as the need for relevant in vitro and in vivo disease models and the consideration of LSDs as rare diseases.

4.4.1 Organ Delivery

LSDs affect different organs and tissues given their multisystemic nature, but depending on the specific LSD, some organs are more affected, such as the heart and kidney in FD or skeletal muscle in PD. Particularly, the delivery of active molecules to the CNS is one of the most important milestones for new therapeutic strategies for LSDs. The BBB along with other systems such as the P-glycoprotein hinder the access of bioactives in the brain after systemic administration [137–139]. Current research efforts aim to overcome this hurdle, employing strategies such as direct injection, intrathecal administration [140] and BBB disruption [141]. siRNA has been successfully delivered by intraventricular administration [142, 143], but with many associated complications, demonstrating the need for new delivery strategies.

Active organ-targeting can be achieved by incorporating the carrier ligands on the surface that specifically bind to receptors exclusively present in the target tissue or cell [144]. Active targeting has been mostly studied for liver [145–147] followed by brain delivery [148, 149]. CNS delivery may be achieved by colloidal systems, including micelles, liposomes, and nanoparticles, and active targeting could represent an interesting strategy [150, 151]. Surface-modified nanosystems with the transferrin receptor [152], insulin receptor [153], or glucose transporter-1(GLUT-1) [154] are suitable candidates for targeting the brain. Several CPPs have been tested along with transferrin in liposomes, including poly-L-arginine, penetratin, vascular endothelial-cadherin-derived peptide, pentapeptide QLPVM, HIV-1 trans-activating protein, and melittin [155–160]. In an animal model of MPS VII, a therapeutic pDNA for ERT was administered intravenously in neutral PEGylated immunoliposomes conjugated with the OX26 monoclonal antibody targeting the rat transferrin receptor [161]. In the case of siRNA, Wei et al. [162] designed a siRNA delivery system based on core-shell nanoparticles decorated with transferrin, resulting in a more specific accumulation in brain tumor tissues compared to the non-targeted nanoparticles. CNS targeting has not been evaluated with gSRT for LSDs; however, these strategies could be applied to develop nanomedicines for these lysosomal disorders.

An alternative non-invasive delivery strategy to the CNS is the nasal-to-brain route [163]. Nanotechnology has been also used to develop nose-to-brain strategies. Rodríguez et al. [164] successfully delivered siRNA to the brain of mice by intranasal administration of polyethylenimine nanoplexes, showing the potential of this route for gene-silencing-mediated therapy in the CNS. Few studies utilize active targeted systems for delivery to the kidney, smooth muscle cells, or vascular endothelial cells, which represents a significant challenge in the treatment not only of LSDs but also for other pathological conditions. Some studies have functionalized liposomes with anti-Thy 1 antibody OX-7 to target the kidney [165] or with antibodies against vascular cell adhesion protein 1 (VCAM-1) for vascular endothelium targeting for siRNA delivery [166]. Successful gSRT

in muscle is especially challenging in PD with a general muscular affectation [167]. Skeletal muscle composes more than 40% of human body weight and, consequently, systemic drug delivery is preferred because of the larger distribution covered [168]. However, poor distribution in muscle and the need for relatively large amounts of the drug are some of the disadvantages associated with systemic administration [169]. Indeed, the dose of the recombinant human enzyme needed in PD patients is significantly higher than those used in other LSDs, i.e., in GD, glucocerebrosidase is employed at a dosage of 1 mg/kg, while for PD rhGAA 20 mg/kg is administered [48]. In addition, liver and spleen clearance and biodistribution are key issues for successful muscle targeting by intravenous administration of nanomedicines [169–171]. In this regard, proteins on the surface of skeletal muscle cells can be used as targeting signals for delivering active substances to muscle tissue [172]. Currently, several examples of muscle-targeting peptides have reported increased muscular affinity, such as the peptide sequence ASSLNIA [173] and the conjugation with PMO [174]. A CPP-conjugated PMO [49] packed in a high muscle tropism recombinant AAV2/1 vector as a gSRT tool has been successfully evaluated in Pompe mice after intramuscular administration [48] and systemic injection.

A new methodology termed selective organ targeting (SORT) enables delivery of nucleic acids to specific tissues by the use of SORT molecules, such as charge-based lipids. SORT has been developed mainly to target liver, lung, and spleen, although other organs can also be targeted [175]. However, a deeper understanding of the endogenous targeting mechanisms by modifying lipid composition to modulate the in vivo protein corona is still required [176]. Guimares et al. [177] developed a collection of lipid nanoparticles containing personalized tailor-made mRNAs; they found that a formulation composed of C12-200/DOPE/cholesterol/DMG-PEG with a ratio of 35/16/46.5/2.35 effectively delivered the mRNAs to the muscles. In another study, it was suggested that the ionizable cationic lipid C12-200 has the potential to improve the specificity of LNPs to the muscle [178]. The influence of DOTAP for muscle delivery was studied by Wei et al. [179], delivering a Cas9/sgRA for Duchenne's muscular dystrophy.

4.4.2 Availability of LSD-Relevant Models

In most situations, efforts to demonstrate the therapeutic potential of any drug relies on studies on model organisms, both in vitro and in vivo. Primary cells are the most representative cell models for studying the molecular hallmarks of diseases, as they are directly isolated from the patient's tissues [180]. Despite having been used as LSDs cell models to test gSRT strategies, since they present several common features such as increased lipid accumulation, their use is limited. To overcome limitations of primary cells, different strategies have been proposed, such as the immortalization of primary cells by reducing their replicative senescence [181, 182]. In the case of FD, an endothelial cellular model, known as IMFE-1 cells, was generated in 2007 by Kaneski et al. [183] by including a human telomerase reverse transcriptase (hTERT) gene. These cells maintained the original phenotype of the FD patient, the main endothelial characteristics and pathological features. Thus, IMFE-1 cells were employed as a cellular model of FD to study gSRT [45]. The introduction of induced pluripotent stem cells (iPSCs) technology in 2005 by Yamanaka et al. [184] produced a reliable source of patient-derived cells. However, these monolayer cultures do not represent an exact tissue, and cellular response to therapeutic treatments might be erroneous due to the unnatural microenvironment [185]. In this context, three-dimensional (3D) culture systems have gained increasing interest as they are able to provide accurate models of organs or tissue physiology and associated disorders [186].

Preclinical studies with relevant animal models are a major prerequisite, not only as proof of effectiveness but also for safety and toxicity assessment, which is essential for translation to clinical trials [181, 187]. Animal models are classically obtained by replacing a particular region of the endogenous genome through homologous recombination. This technique depends on embryonic stem cells, which are genetically modified in vitro before transplantation to achieve differentiation. The availability of mouse embryonic stem cells has led to the production of several mouse models, but this approach is used less to obtain models of other species, such as non-human primates, because of the unavailability of suitable embryonic cells [188]. Currently, there are knockout murine models of different LSDs, including FD, GD, PD, and MPSs. In the case of PD, the most widely used preclinical model is the Raben knockout mouse (B6; 129-*Gaa*^{tm1Rabn}/J), which demonstrates survival into adulthood with muscle glycogen storage and progressive muscle weakness [189]. For substrate inhibition with gSRT for PD, *Gaa*$^{-/-}$ *mice* were employed since they presented a great glycogen accumulation, typically from PD [48, 49]. In the case of FD, the α-Gal A knockout mouse model (B6;129-*Gla*^{tm1Kul}/J) has also been employed since it has abolished the expression of *GLA* gene, mimicking the disease phenotype [190].

For the study of new therapeutic tools for LSDs, animal models with specific human pathogenic genetic variants are preferred. In this sense, the development of genome editing, especially CRISPR/Cas9 technology, has allowed for a potentially accurate and efficient alternative to the traditional method of transgenic model generation [191]. CRISPR/Cas9 allows genome modification or repair of somatic and germinal cells and avoids the need to use embryonic stem cells. This technique has led to the rapid

development of numerous novel disease models, including those involving small animals and non-human primates [188]. As an example, since 2020 there has been a knock-in murine (*Gaa*$^{c.1826dupA}$) model of PD generated by CRISPR/Cas9 technology that maintains the human phenotype, including the hypertrophic cardiomyopathy and skeletal muscle weakness of human infantile early-onset PD (IOPD) [189]. Since FD mouse models do not exhibit all of the typical symptoms seen in patients, a rat model has been developed by CRISPR/Cas9 in order to better recapitulate the neuropathic pain symptoms. The Fabry rat maintains the FD phenotype and allows for the elucidation of disease mechanisms and testing therapies to treat pain symptoms [192]. Among the identified articles in our systematic review, in one of them the CRISPR/Cas9 technology was employed to simulate the condition of FD in a kidney organoid by a complete and permanent knock-down of *A4GALT* gene expression [46].

4.4.3 Rare Disease Condition

The condition of rare disease contributes to the difficulty in the development of new treatments since clinical trials present challenges for several reasons [193, 194]. In addition, rare diseases are usually chronic conditions that require long-term and expensive treatments.

Patient recruitment is limited due to the small number of patients suffering from these conditions and the low awareness of the disease in society [195]. Moreover, since rare diseases are caused by different genetic mutations, identification of a homogenous population is not easy. Therefore, it is difficult to conduct randomized placebo-controlled clinical trials requiring hundreds of patients to clinically evaluate orphan drugs, particularly when the pediatric population is involved [196]. It is also important to note that rare disorders often affect vulnerable populations, such as children or individuals with severe, life-shortening conditions. This can raise ethical concerns about the conduct of clinical trials and the use of experimental therapeutics in these populations. As observed in Table S2 (OSM), in many of the current gene therapy clinical trials for LSDs, the study population includes children from several months to 18 years old. Frequently, clinical trials present a limited number of participants, frequently less than 20 people per study.

With the aim of promoting the development of new therapeutic options to treat rare diseases, different designations and processes have been implemented. Although the regulatory process for rare genetic disorders can also be complex, LSDs are one of those groups of diseases that have benefited from regulatory actions regarding orphan drugs [197]. The efficacy criteria for approving the marketing of orphan drugs do not differ much from those for other types of drugs, but some regulatory agencies have been more flexible in applying the evidence requirements [198]. In the case of the European Union, the EMA has provided instructions on the criteria and protocols for approving marketing authorization in exceptional situations, especially for rare diseases where complete evidence is lacking. Moreover, the designation of an orphan medicinal product, which must be approved by the Committee for Orphan Medicinal Products [199], is associated with total or partial fee reductions and 10-year marketing exclusivity [200, 201]. These incentives have stimulated the development of novel therapies in the field of rare conditions and the growth of orphan designations [202].

5 Conclusions and Future Perspectives

gSRT could be considered an alternative option for treatment of LDSs; however, it has been studied only at a preclinical level, with studies in sphingolipidoses, glycogen storage disorders, and MPSs sphingolipidoses. Nevertheless, it could be applied to all LSDs by selecting the target enzyme affected in each disease. The condition of rare diseases hinders the development of new therapies, and only 11 papers have been published addressing this goal, with the first paper published in 2006 and three new articles in 2023, all for FD, including the first one that used CRISPR/Cas9 technology in this group of diseases.

gSRT in LSDs has been implemented through a wide variety of mechanisms to downregulate gene expression, iRNA (siRNA and shRNA), ASOs, and CRISPR/Cas9, with siRNA as the most employed silencing technology. In the years to come, the number of genome-editing studies and products is expected to increase thanks to improvements in sequence-specific nuclease technology, which has led to the approval of the first ex vivo CRISPR/Cas9 therapy by the FDA and EMA (Casgevy®). Notwithstanding, the biopharmaceutical market of silencing nucleic acid-based products is expanding thanks to recently authorised siRNA and ASO therapeutics.

Nanomedicine is essential to solve the existing need to address the treatment of rare diseases through gene therapy and other new strategies, such as gSRT. To date, gene therapy clinical trials for LSDs have addressed only a gene supplementation strategy using DNA, either in vivo or ex vivo. Viral gene therapy is at the forefront of translational gene therapy, but safety problems (immunogenicity, concerns for readministration, and possible oncogenicity) as well as large-scale production limit its future prospects. gSRT could be an alternative to complementary therapy for LSDs, for which viral vectors are not considered the first option. Nanotechnology could be a worthwhile option to apply gSRT for reducing the stored substrate in specific organs affected in LSDs. Significant efforts have to be made in the field of nanotechnology to increase the

effectiveness of non-viral nanosystems. Recent milestones achieved with lipid nanoparticles such as the first siRNA marketed, ONPATTRO™ , and COVID-19 vaccine commercialization have raised the development of LNP-based therapies for nucleic acid delivery and will foster the advance of gSRT towards more advanced stages of the preclinical development.

Nervous system affectation is present in most LSDs but challenging to address due to the inability of different treatments to cross the BBB. Specific organ-targeting treatment would also be interesting in PD with the skeletal muscle especially affected and in FD for the heart and kidney. It is important to identify the key delivery points in order to design a suitable and effective vector able to deliver the nucleic acid to the target cell or organ. Nanosystems can be decorated for active targeting and other alternative strategies such as endogenous targeting by modifying lipid composition to modulate protein corona may be a more reliable option. Nevertheless, it is worth mentioning that targeting strategies to develop organ-specific nucleic acid delivery systems remain a challenge. Finally, intranasal administration of nanoparticle-based therapeutics should be taken into consideration for nucleic acid administration in CNS, including gSRT.

The gSRT for LSDs addresses unmet clinical needs, although its development is far from clinical use. In addition to the limited knowledge of the disease due to its rare disease status or the lack of animal models, more basic research and the involvement of the entire scientific community are needed for its progress. The advancement of nanotechnology together with gene-silencing and gene-editing technologies is essential for the development of gSRT products. Nanomedicine would enable the design of gSRT nanodelivery systems tailored to the type of nucleic acid, LSD, and target organ for effective and personalized treatments.

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