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Enzyme Replacement Therapy for Genetic Disorders Associated with Enzyme Deficiency

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Running Title: Enzyme-based therapy for genetic diseases

Abstract

Mutations in human genes might lead to loss of functional proteins causing diseases. Among these genetic disorders, a large class is associated with the deficiency in metabolic enzymes, resulting in both an increase in the concentration of substrates and a loss in the metabolites produced by the catalyzed reactions. The identification of therapeutic actions based on small molecules represents a challenge to medicinal chemists because the target is missing. Alternative approaches are biology-based, ranging from gene and stem cell therapy, CRISPR/Cas9 technology, distinct types of RNAs, and enzyme replacement therapy (ERT). This review will focus on the latter approach that since the 1990s has been successfully applied to cure many rare diseases, most of them being lysosomal storage diseases or metabolic diseases. So far, a dozen enzymes have been approved by FDA/EMA for lysosome storage disorders and only a few for metabolic diseases. Enzymes for replacement therapy are mainly produced in mammalian cells and some in plant cells and yeasts and are further processed to obtain active, highly bioavailable, less degradable products. Issues still under investigation for the increase in ERT efficacy are the optimization of enzymes interaction with cell membrane and internalization, the reduction in immunogenicity, and the overcoming of blood-brain barrier limitations when neuronal cells need to be targeted. Overall, ERT has demonstrated its efficacy and safety in the treatment of many genetic rare diseases, both saving newborn lives and improving patients' life quality, and represents a very successful example of targeted biologics.

Keywords: enzyme deficiency, genetic disease, recombinant proteins, cell internalization, mannose 6-phosphate, lysosomal storage disorders, metabolic diseases, biologics.

1. Introduction

Healthy life depends on many factors, including diet, activity, environment and, primarily, individual genetic heritage. Thus, it is not surprising that, as reported in DisGeNET platform (www.disgenet.org), there are 1,134,942 gene-disease associations and 369,554 variant-disease associations [1]. As reported in OMIM (Online Mendelian Inheritance in Man) database (www.omim.org), there are 5,683 single gene disorders and traits phenotypes involving 3,957 genes. Gene variants lead to many different phenotypes, including as prototypical examples: i) sickle cell disease associated with the point mutation Glu-Val at position 6 in the beta chain of hemoglobin, ii) cystic fibrosis associated with mutations in the cystic fibrosis transmembrane regulator ion channel, iii) lysosomal disorders due to deficiency of several enzymes with the accumulation of the corresponding substrates, iv) metabolic diseases associated with the deficiency of a key enzyme or protein, such as phenylalanine hydroxylase causing phenylketonuria, and v) several cancers. Many of these diseases are rare, as they have been detected in about 1:200,000 persons or, according to the European Union Regulation on Orphan Medicinal Products, no more than 1 person per 2,000 of the European population (www.orpha.net). The estimated number of persons affected by rare diseases is 350 million with a few cases for most of them [2]. Consequently, studies towards the development of a therapy were and are still mainly carried out with public and no-profit organization funds, being big pharmaceutical companies not reasonably interested in investing in diseases with a few patients and limited economical return. However, in the last years, a change in this view has occurred because public systems are supporting the very expensive rare disease therapies, often requiring weekly or fortnightly drug administration along all patient's life [2-4].

From the perspective of medicinal chemists, genetic diseases in which a protein is missing are very challenging because there is no direct target (Figure 1). Therefore, secondary or multiple targets have to be identified that might be inhibited or activated using small molecules. Other therapeutic strategies based on small molecules are i) the stabilization of misfolded proteins via pharmacological chaperones [5], ii) the reduction of substrate concentration via inhibition of its formation in the case of enzyme deficiency [6] and iii) the correction of gene transcription via stop codon read-through [7]. Alternative therapeutic strategies based on molecular and cellular biology are i) the replacement of the deficient gene using gene therapy [8, 9], ii) the replacement of defective cells via hematopoietic stem cell transplantation (HSCT) [10], iii) and gene-targeting nucleotides,

including the powerful CRISPR/Cas9 [11]. Some of these approaches are reviewed in other papers on this issue. Whenever a disease is associated with the deficiency of an enzyme, the same or a metabolically active enzyme might be exogenously supplied to patients. Therapeutic enzymes can also be exploited in non-genetic derived diseases (for an overview, [12], and another paper in this issue). In the present review, we will focus primarily on the so-called Enzyme Replacement Therapy (ERT), first investigated in the late '60s for the treatment of adenosine deaminase deficiency (ADA) and Gaucher disease and approved in 1990 and 1991, respectively (Table 1). The intravenous delivery of an enzyme, endowed with a delicate structure-function relationship, targeting a cell compartment is a quite challenging task. In fact, in order to be functional within the cellular environment, the protein should pass through different steps: endothelial permeation, organ adsorption, cell internalization via endocytosis and, eventually, compartment uptake. For some of these enzymes, the optimal cellular localization for an effective therapeutic action is within brain neural cells, but, unfortunately, the blood-brain barrier (BBB) prevents the desirable targeting of such enzymes. Strategies have been adopted to overcome these limitations, including direct brain delivery [13] and exploitation of nanoparticles or antibodies [14]. Given the low thermal stability of an active enzyme and its exposure to extra- and intracellular proteases, pharmacokinetic parameters have been closely monitored and bioavailability found to be generally low. Thus, in order to keep the level of the enzymatic function high enough to be close to normal and/or therapeutically effective, administration doses and frequency (from 1 mg to 20 mg per Kg of body weight, weekly delivered) need to be thoroughly investigated and defined for each disease [2]. Another key issue is related to antigenicity. Since most, if not all, enzymes used in ERT are nowadays obtained from recombinant DNA technology and not all are human isoforms, immune adverse reactions might occur producing antibodies against the delivered enzymes. Usually, antibody reaction was found not to represent the main obstacle to ERT. Nevertheless, some enzymes were chemically modified with polyethylene glycol (PEG) derivatives to avoid adverse immune reactions and, concomitantly, to protect them from proteases, prolonging their bioavailability.

Enzymes for ERT are usually expressed in Chinese hamster ovary (CHO), human cell lines or, for a few applications, in yeasts and transgenic plants. In the case of enzymes for lysosomal storage disorders, these expression systems are required because the glycosylation pattern with mannose 6-phosphate terminal units is mandatory for lysosome internalization. Other strategies were developed for specific targeting, such as a string

of aspartate residues that bind tightly hydroxyapatite for the delivery of asfotase alfa in the treatment of hypophosphatasia, or conjugation with monoclonal antibodies against insulin receptor for brain targeting of irudonidase in the therapy of mucopolysaccharidosis type I.

Overall, 11 distinct enzymes have been so far approved by the Food and Drug Administration (FDA) and/or European Medicines Agency (EMA) for lysosome storage diseases and few others for inborn errors in metabolism. Several reviews appeared in the last ten years, and many in 2020, reporting the vast amount of experimental and clinical studies carried out aiming at developing safe and effective ERT, with most of them focused on therapies for lysosome storage disorders [2, 3, 10, 15-18]. This intense reporting activity indicates that the field of ERT is mature and of growing interest and, therefore, enzymes represent an important part of biologics.

2. ERT for Lysosomal Storage Disorders

2.1 Gaucher disease

Gaucher disease (OMIM 230800) is the most common lysosomal storage disorder affecting 1:75,000 persons. The disease is caused by mutations of the human *GBA1* gene coding for beta-glucocerebrosidase (D-glucosyl-*N*-acylsphingosine glucohydrolase, EC 3.2.1.45) (Table 1). Beta-glucocerebrosidase is a monomeric glycoenzyme that in the lysosome degrades glucocerebroside in glucose and ceramide. The enzyme contains four glycosylation sites and 22 lysine residues. Enzyme deficiency leads to the accumulation of its substrates, glucosylceramide and glucosylsphingosine, which, in turn, causes organ and tissue damages. More than 80% of the 200 distinct gene mutations are single nucleotide changes [19]. Gaucher disease is classified into three types of which type I is the most common. Clinical features, natural history and life expectancy of the disease were previously reviewed [19, 20].

Two therapeutic strategies have been adopted: i) reduction of the concentration of glucocerebroside by inhibition of the synthetizing enzyme glucosylceramide synthase with miglustat, an imino sugar [21], and eliglustat, a glucocerebroside analog [22, 23] and ii) ERT based on the delivery of beta-glucocerebrosidase occurring via a unique, high-capacity pathway with specificity for macrophages, which are an important pathological focus in Gaucher disease [19] (Figure 2).

The glycoenzyme used initially for therapy and approved in 1991, alglucerase (Ceredase®, Genzyme, Cambridge, MA, USA), was purified from human placenta using a method involving extraction with cholate, salt fractionation, acid precipitation, butanol extraction, and hydrophobic chromatography [24]. The glycoenzyme was then modified by the action of specific exoglycosidases obtained from the jack bean to expose mannose terminal units, critical for lysosome uptake [25, 26]. In fact, phosphomonoesters of mannose 6-phosphate decorate many newly synthesized enzymes destined for the lysosome, allowing their uptake into cells by endocytosis mediated by mannose 6-phosphate receptors. Beta-glucocerebrosidase specifically enters the lysosome via the interaction with the lysosomal integral membrane protein 2 (LIMP-2) [27].

In 1994, beta-glucocerebrosidase was produced in recombinant form using CHO cells in tissue cultures and approved for therapy in 1995. The enzyme was named imiglucerase (Cerezyme®, Genzyme). As with alglucerase, this glycoenzyme was processed by sequential deglycosylation of its carbohydrate side chains to expose α-mannosyl residues using plant-derived exoglycosidase. The preparation of the recombinant enzyme for pharmaceutical use was described by Genzyme in the United States patent n. 5,549,892. Whereas alglucerase was partially contaminated by human chorionic gonadotrophin and formulated in a solution containing a mild solubilizing agent, imiglucerase was pure and lyophilized.

The efficacy of glucocerebrosidase from human placenta was first assessed on 12 patients [28]. Later, the efficacy of alglucerase was compared with imiglucerase on 15 patients and minimal differences were found, opening the way to the use of the recombinant form [29]. ERT with imiglucerase was monitored in order to identify a personalized therapeutic plan dependent on several factors, including disease severity and age [30]. Most patients initiated therapy on a fortnightly basis using imiglucerase doses ranging from 30 to 60 U/kg bodyweight per infusion [31], although also lower but more frequently administered doses were used [32]. A summary of clinical outcomes was reported [19]. Antibodies against the recombinant enzyme were detected in 12% of patients (32 out of 262) [33].

The exploitation of imiglucerase for ERT represented a hallmark in pharmaceutical biotechnology. Indeed, imiglucerase, produced by Genzyme, was a blockbuster biologics for an ultra-rare disease, opening a novel and economically rewarding field for recombinant proteins [19]. Upon Genzyme production difficulties due to contaminations in growth culture media, biosimilars were launched on the market, named velaglucerase alfa (VPRIV, Shire Human Genetics Therapy Inc., Cambridge, MA, USA) in 2010, taliglucerase alfa (Elelyso,

Pfizer, New York, NY, USA) in 2012 and an imiglucerase biosimilar, Abcertin® (ISU Abxis, Seongnam, Korea), in 2014.

Velaglucerase alfa is produced in a cell line derived from a human fibrosarcoma and displays the natural amino acid sequence and several mannose units. Interestingly, the glycosylation pattern is modified during the cell culture by selectively inhibiting the glycoprotein processing mannosidase 1 with kifunensine. This method leads directly to an extensive array of terminal mannose sugars, thus further modifications are not required. However, the higher degree of mannose decoration does not seem to confer a higher uptake with respect to imiglucerase [34], resulting in similar efficacy [35, 36].

Taliglucerase alfa is a recombinant enzyme produced in carrot cell cultures. For purification goals, short tags of 3 and 7 amino acids were added at the N- and C-termini, respectively, to favor targeting the vacuole of the plant cell. The enzyme displays terminal mannose residues on a complex glycan pattern, containing also xylose and fucose which are uncommon in mammalian glycoproteins [37]. Early clinical investigations demonstrated the safety and efficacy of the treatment with taliglucerase alfa [38] that were further confirmed by six studies on 33 patients [39] and a recent multicenter, open-label, expanded-access study on 58 patients [40]. Only a study has appeared for the imiglucerase biosimilar Abcertin® reporting data showing a good safety profile [41].

BBB hampers the delivery of therapeutic enzymes to treat central nervous system pathologies. Recently, using a Gaucher mouse model, velaglucerase alfa was formulated within saposin C and dioleoylphosphatidylserine nanovesicles and found to cross the BBB and to be delivered selectively to neuronal tissues. As a result, significant amelioration in brain inflammation and neurological phenotypes was observed [42]. This strategy might pave the way to ERT for other neurological diseases.

2.2 Fabry disease

Fabry disease (OMIM 301500) is a lysosomial disorder affecting 1-5:10,000 live births. The disease is caused by mutations of *GLA* gene that codes for alpha-galactosidase A (EC 3.2.1.22), with reported about 900 gene mutations (Table 1). Alpha-galactosidase is a homodimer, composed of two 50 kDa subunits, that catalyzes the breakdown of globotriaosylceramide in galactosylceramide with the removal of the α-galactose unit. The enzyme exhibits poor stability both at neutral and acidic pH in either phosphate buffer or human serum [43,

44]. Lack of alpha-galactosidase function leads to the accumulation of the substrate in cells, causing neurological, cutaneous, renal, cardiovascular, cochleovestibular and cerebrovascular manifestations. Specifically, since the disease is associated with progressive heart damages, including hypertrophy, heart failure and cardiac death, several clinical strategies have been proposed and reviewed [45-49].

The pharmacological treatment of Fabry disease is based on i) *ex vivo* and *in vivo* gene therapy [50-52]; ii) mRNA delivery [53]; iii) chaperone therapy [54]; iv) substrate reduction therapy with lucerastat [55, 56], migalastat [57] and venglustat [58], and v) ERT with agalsidase alfa (Replagal®, Shire Human Genetics Therapy Inc., Lexington, MA, USA), agalsidase beta (Fabrazyme®, Genzyme) [59, 60] pegunigalsidase alfa (PRX-102, Protalix Biotherapeutics, Carmiel, Israel and Chiesi Farmaceutici, Parma, Italy) [61] and an agalsidase beta biosimilar [62, 63] (Figure 2).

Fabry disease was one of the first disorders to be treated with an enzyme replacement therapy using alpha-galactosidase extracted from either placenta cells [64] or spleen and blood [65]. The former enzyme preparation was found to be the most effective. As for other ERTs, natural sources were replaced by recombinant forms as soon they became available. Specifically, agalsidase beta was produced from genetically engineered CHO cells [66] and agalsidase alfa from a genetically engineered human foreskin fibroblast cell line [59, 60]. The safety and efficacy of agalsidase beta, administered at a dose of 1mg per Kg of body weight, was evaluated in a multicenter, randomized, double-blind, placebo-controlled trial and subsequent open-label study, enrolling 58 patients [66]. Upon six months, clearance of microvascular endothelial deposits of globotriaosylceramide was observed in most patients [66]. In 2003 agalsidase beta was approved by FDA.

Agalsidase alfa was purified with a procedure that involves six chromatographic steps [60]. The safety and pharmacokinetics of the enzyme were evaluated on 10 patients [60]. More recently [44], pegunigalsidase alfa (PRX-102) was produced in the ProCellEx® plant cell-based protein expression system and expressed in a BY2 tobacco cell culture. Upon purification, the enzyme was reacted with a homo-bifunctional PEG2000 cross-linker with the aim of increasing its bioavailability. This procedure leads to the formation of a stable PEGylated homodimer [44]. Pegunigalsidase alfa showed higher stability, retaining 80% activity upon ten days of incubation under lysosomal conditions, whereas agalsidase alfa and agalsidase beta activities were lost upon two days. Furthermore, pegunigalsidase alfa pharmacokinetics, measured in Fabry mouse model, showed a $t_{1/2}$ of 581 min, a value 10-fold higher than agalsidase alfa and agalsidase beta [44]. Pegunigalsidase alfa was

found to lead to a significant reduction of globotriaosylceramide in analyzed organs [44]. More recently [61], it was demonstrated that PEGylation *per se* does not improve enzyme stability and activity. However, the exploitation of crosslinking PEG reagents with different spacers led to the formation of a covalently stabilized homodimer, that did show superior functional and bioavailability properties both in solution and in mice models, and reduced immunogenicity [61]. The safety of pegunigalsidase alfa was evaluated in a 1-year phase 1/2 clinical trial on 16 Fabry patients obtaining encouraging results [67], with further clinical trials that are ongoing.

To enhance alpha-galactosidase bioavailability, thus ERT efficacy, nanoparticles containing the enzyme, serum albumin and 30Kc19, a cell-penetrating protein originated from the hemolymph of the silkworm *Bombyx mori* [68], were produced [69]. In fibroblasts of Fabry patients, it was found an enhanced cellular uptake, increased enzyme stability, and a higher degree of globotriaosylceramide processing [69].

An agalsidase beta biosimilar was identified in *Nicotiana benthamiana* and named A1.1 (gene accession ID GJZM-1660), using a fluorescent modified substrate of the enzyme, alpha-galactosyl-cyclophellitol-aziridine, coupled to biotin. This plant enzyme is monomeric, deprived of N-glycans, active in processing 4-methylumbelliferyl-α-D-galactopyranoside in solution, and able to degrade globotriaosylceramide in fibroblasts [70]. It might be suitable for optimization toward ERT exploitation.

2.3 Pompe disease

Pompe disease (OMIM 232300) is a lysosomal storage disorder caused by the deficiency of functionally active acid alpha glucosidase (also named acid maltase) (EC 3.2.1.20), coded by *GAA* gene (Table 1). There are about 600 *GAA* gene mutations, the five most frequently detected being 32-13 T>G (75% of patients), 1935 C>A (Asp645Glu) (frequent in the Taiwanese population), del 525, del exon18, and Arg309 (common in Netherlands population), 2560 C>T (Arg854X) (common in American black population). The disease affects about 1:40,000 newborns [71]. Alpha glucosidase enzyme catalyzes the degradation of glycogen with the formation of glucose. Enzyme deficiency causes the accumulation of glycogen in all tissues. In skeletal muscle, smooth muscle and cardiac muscle excessively high levels of glycogen cause tissue damages leading to “soft muscles”, generating cardiac pathologies, walking disability and reduced respiratory function. Pompe disease is classified either as classic infantile, childhood and adult, or, alternatively, infantile-onset and late-onset

disease. When untreated, Pompe disease leads to death within one year from birth, whereas studies have shown that earlier is the treatment (within five months), the better is the outcome. However, myopathies slowly develop [72-74]. Studies on clinical features, pathophysiology and therapy of Pompe disease have been reviewed over the years [71, 75, 76].

Pompe disease therapy is based on i) small-molecule chaperone, ii) gene therapy, iii) substrate reduction therapy and iv) ERT using recombinant human acid alpha-glucosidase, named alglucosidase alfa (Lumizyme®, Genzyme), marketed as Myozyme® outside the United States, approved by FDA in 2006 and re-approved in 2010, upon changes in enzyme production (Figure 2).

A small-molecule chaperone, AT2220, was identified as an enhancer of alpha galactosidase folding, stabilizing the enzyme and increasing the catalytic action, even of the mutated enzyme [77]. AT2220 was also shown to improve the catalytic efficiency of the recombinant enzyme [77]. Clinical studies are ongoing for the assessment of the efficacy of a gene therapy consisting of a virus-mediated delivery of human galactosidase [78]. Inhibition of glycogen synthase by short hairpin RNA or antisense oligonucleotides led to a reduction of lysosomal glycogen in skeletal muscle of Pompe mouse models [79].

In the 1960s, Pompe disease was the first lysosomal storage disorder for which ERT was tentatively exploited. Alpha-galactosidase was obtained from *Aspergillus niger* and human placenta, but no clinical benefits were observed due to the lack of the mannose 6-phosphate unit enabling the enzyme interaction with the receptor for efficient endocytosis. Enzyme obtained from bovine testis and human urine was found to be more effective, but not enough to cure the disease. The issue was that a high dosage was needed, of the order of 20 mg/Kg every other week. This regime was achieved only when human alpha-glucosidase was obtained in the recombinant form. The enzyme was produced either in CHO cells [76, 80] or in milk of transgenic rabbits [81]. For production in CHO cells, *GAA* complementary DNA was used whereas, for the production in milk, the entire acid alpha-glucosidase gene was placed in an expression vector under the control of the bovine αS1-casein promoter. The enzyme from CHO is called alglucosidase alfa and is a 110 kDa precursor protein containing mannose 6-phosphate that upon internalization is proteolyzed in two different forms of 70 and 76 kDa, exhibiting higher catalytic activity [76]. Both products were tested in animals and fibroblasts from patients and showed similar cellular uptake and, at doses higher than 10 mg/Kg, were significantly effective in decreasing glycogen levels in several organs, except, not surprisingly, the brain [71].

As for other enzymes used for ERT, an issue was the degree of cellular uptake. Investigations were carried out aimed at engineering the enzyme either by remodeling the carbohydrate pattern of the enzyme to favor receptor interaction [82, 83] or by developing a chimeric enzyme linked to a portion of insulin-like growth factor 2 allowing glycosylation-independent cellular uptake [84]. The former product is called avalglucosidase alfa or neoGAA. A phase 1, open-label, multicenter, multinational, ascending dose study for evaluating safety, bioavailability and efficacy was carried out [85] and an open-label phase 2 clinical study is ongoing and expected to end on December 2021 (NCT02032524).

Recently, a further form of human recombinant glucosidase was developed, ATB200 (Amicus proprietary rhGAA) [76]. ATB200 exhibits a higher mannose 6-phosphate content than alglucosidase alfa, including both mono- and bis-phosphorylated forms. When ATB200 was administered to a mouse model of Pompe disease in combination with AT2221 (miglustat), the therapeutic index was higher than the standard treatment with alglucosidase alfa [76]. By exploiting a glycosidase from *Cellulosimicrobium cellulans* that "uncaps" N-glycans, an improved alpha-glucosidase with higher levels of mannose 6-phosphate, and thus higher therapeutic efficacy, was obtained [86].

In order to increase the targeting and uptake of recombinant glucosidase, a powerful strategy is the conjugation with an antibody [87]. A chimera, called VAL-1221 (Valerion Therapeutics, Boston, MA, USA), was produced with the enzyme fused to the 3E10 Fab fragment. For internalization, the product exploits both the mannose 6-phosphate receptor and the equilibrative nucleoside transporter 2 (ENT-2), maintaining its activity at both neutral and low lysosomal pH [88]. The therapeutic efficacy of VAL-1221 was evaluated in a phase 1/2 clinical study in comparison with Myozyme®/Lumizyme® in 12 patients. Results indicate that VAL-1221 is safe with no adverse effects [89].

2.4 Mucopolysaccharidoses

Mucopolysaccharidoses (MPSs) are lysosomal storage diseases originated by the deficiency of enzymes required for the breakdown of mucopolysaccharides, in particular glycosaminoglycans (GAGs) [90, 91]. Depending on the type of lacking enzyme, MPSs are classified in different classes (from MPS I to IX) and often different clinical phenotypes can be observed within the same class [92]. Many of the enzymes responsible for MPSs are lysosomal sulfatases, which catalyze mucopolysaccharides degradation by hydrolysis

of sulfate esters. ERT has been widely applied to the treatment of MPSs [93, 94] (Figure 3). We review here the main clinical manifestations, biochemical properties of the deficient enzymes, and the therapeutic approaches only for MPSs for which ERT is presently applied, which are MPS I, II, IVA, VI, and VII (Table 1).

2.4.1 Mucopolysaccharidosis type I (Hurler and Hurler-Scheie or Scheie forms)

Mucopolysaccharidosis type I (MPS I, OMIM 607014) is an autosomal recessive genetic disease caused by the deficiency of alpha-L-iduronidase (EC 3.2.1.76), coded by the *IDUA* gene. This enzyme is an acid hydrolase responsible for the removal of α-L-iduronic acid residues of the GAGs dermatan and heparan sulfate. Its deficiency leads to a lysosomal accumulation of the mucopolysaccharides, exerting a toxic effect on several tissues and organs [92]. Alpha-L-iduronidase belongs to the family of glycoside hydrolases exhibiting two Glu residues in the active site which favor the nucleophilic attack on substrate mucopolysaccharides with consequent hydrolysis [95].

The disease can manifest as three different clinical sub-types: Hurler syndrome, Hurler-Scheie syndrome and Scheie syndrome. A continuum spectrum of different phenotypes can be found, ranging from the most severe (Hurler) to the mildest (Scheie) disease. Hurler syndrome manifests with growth and intellectual disability, hepatosplenomegaly, hydrocephalus, and death occurs within the first decade of life if untreated [96].

ERT has been applied to treat MPS I (Figure 3) [97, 98]. Laronidase (Aldurazyme®, BioMarin Pharmaceutical Inc., San Rafael, CA, USA), a recombinant alpha-L-iduronidase produced in CHO cells and developed for the treatment of MPS I was first approved by FDA in 2003 [99]. Laronidase is internalized and delivered inside target cells by mannose 6-phosphate receptor [100]. This recombinant enzyme is generally well tolerated and serious adverse events are uncommon [101].

Laronidase is not recommended for severe Hurler forms since this formulation is unable to cross the BBB. A genetically engineered fusion protein of alpha-L-iduronidase with a monoclonal antibody against insulin receptor named AGT-181 has been developed in order to allow penetration of the enzyme through the BBB [102, 103]. An alternative treatment for MPS I is HSCT, preferred to ERT for the severe forms of the disease [104]. CRISPR/Cas9 genome editing has also been recently proposed [105]. Therapeutic approaches applied so far are listed in a recent review on MPS I [106].

2.4.2 Mucopolysaccharidosis type II (Hunter syndrome)

The Hunter syndrome, or mucopolysaccharidosis type II (MPS II, OMIM 309900), is a genetic recessive X-linked disease caused by mutations in the *IDS* gene encoding for iduronate 2-sulfatase (EC 3.1.6.13), an enzyme that cleaves the sulfate group on the glycosidic moiety of dermatan and heparan sulfate [92, 107]. MPS II is one of the most common mucopolysaccharidoses, showing a prevalence of about 0.2 to 1.07 per 100,000 newborns [108]. The disease affects mainly males and can occur with different phenotypic severity: the more severe phenotype is associated with central nervous system deterioration and survival is less than two decades, while milder forms show a longer life expectancy with little or no cognitive impairment [109]. Iduronate-2 sulfatase activity towards a series of substrates derived from dermatan and heparan sulfate has been thoroughly characterized [110].

Since 2006, ERT is available for the treatment of Hunter syndrome thanks to the development of the recombinant enzyme named idursulfase (Elaprase®, Shire Human Genetics Therapy Inc.) [111] (Figure 3). Idursulfase is a glycoprotein with a MW of about 76 kDa (525 amino acids) containing two disulfide bonds and eight N-linked glycosylation sites. The post-translational modification of Cys59 to formylglycine is necessary for enzyme activity. The mannose 6-phosphate groups on the glycosylation sites are important for cell internalization and lysosome targeting. Moreover, idursulfase is modified with sialylated glycans responsible for a prolonged half-life of the protein in blood [112]. The enzyme is produced in human cells generated by transfecting HT-1080 human fibrosarcoma cell lines [113].

Clinical studies regarding ERT based on idursulfate injections have been reviewed elsewhere [3, 108]. The enzyme is well tolerated although adverse events can occur, such as headache, abdominal or chest pain, arthralgia, pruritic rash and swelling at the infusion site [114]. Idursulfase has been also used to treat MPS II in very young children (age 1.4-7.5 years old) showing a similar safety and efficacy profile with respect to previous clinical studies on an older group of patients (age >5 years old) [115].

Recently, a similar recombinant enzyme, named idursulfase beta (Hunterase®, Green Cross Corporation, Yongin, South Korea), produced in CHO cells, has been developed in South Korea for ERT of MPS II [116]. Differently for MPS I, HSCT is not recommended as a treatment option for MPS II, since the neurocognitive impairment is not reduced by this therapeutic strategy [117]. Another alternative proposed treatment is

genistein, a soy derived isoflavone, which was shown to reduce GAGs synthesis [118]. Genome editing using CRISPR/Cas9 is also a potential future strategy for the treatment of MPS II [105].

2.4.3 Mucopolysaccharidosis IVA (Morquio A syndrome)

Mucopolysaccharidosis IVA (MPS IVA, OMIM 253000) is an autosomal recessive disease caused by a genetic defect in the gene *GALNS* coding for N-acetylgalactosamine 6-sulfatase (EC 3.1.6.4), an enzyme necessary for the catabolism of chondroitin-6-sulfate and keratan sulfate [119]. When the enzymatic deficiency occurs, these GAGs accumulate mainly in bone, cartilage, and extracellular matrix. MPS IVA manifests as a degenerative systemic skeletal dysplasia within the age of 2, showing, such as other MPSs, a range of different phenotypes, from mild to severe. The clinical features of MPS IVA are identical to those of MPS IVB, caused by the deficiency of beta-galactosidase. N-acetylgalactosamine-6-sulfate sulfatase was firstly characterized in the ’80s [120]. The enzyme is formed by two subunits, of 40 and 15 kDa each, linked by a disulfide bond [121].

ERT to treat Morquio A syndrome was firstly approved in 2014 [122, 123] (Figure 3). The recombinant enzyme, elosulfase, also named BMN 110 (Vimizim®, BioMarin Pharmaceutical Inc.), is produced in CHO cells [124]. The product is a glycosylated protein identical to the natural enzyme, formed of 496 amino acids with a MW of 55 kDa and is a dimer in solution [125]. The N-linked glycosylation sites are also the same as the natural enzyme, and one of the oligosaccharide chains contains mannose 6-phosphate, recognized by receptors on cells for endocytosis of the exogenous enzyme [122]. The efficacy of ERT on MPS IVA is still on debate since it was shown that a 2.0 mg/Kg/week dose improves endurance during locomotory activity and respiratory function [126], although the inability of the enzyme to penetrate the avascular bone and cartilage lesions has been highlighted as a limitation of the therapy [127, 128]. Similar issues regarding the efficacy have also been raised for HSCT applied to treat MPS IVA [104].

2.4.4 Mucopolysaccharidosis VI (Maroteaux-Lamy syndrome)

Mucopolysaccharidosis VI (MPS VI, OMIM 253200) is a progressive autosomal recessive disease with multisystem involvement determined by mutations in the gene *ARSB* coding for arylsulfatase B, also called N-acetylgalactosamine 4-sulfatase (E.C.3.1.6.12) [129-131]. The enzyme is responsible for the cleavage of the

C4-sulfate group in *N*-acetylgalactosamine 4-sulfate at the non-reducing ends of dermatan sulfate and chondroitin 4-sulfate [92]. The disease phenotype can vary widely among MPS VI patients, in a continuum spectrum of severity. In most patients, the skeleton is the most affected organ [129]. In the severe forms, the accumulation of GAGs, especially dermatan sulfate, causes bone dysplasia, joint restriction, organomegaly, cardiac disease, corneal clouding, and a reduced life expectancy. Arylsulfatase B is a 43 kDa protein formed upon processing of a 66 kDa precursor [132].

ERT for the treatment for MPS VI is available since 2005 and is based on galsulfase (Naglazyme®, BioMarin Pharmaceutical Inc.), a recombinant arylsulfatase B produced in CHO cells [129, 133] (Figure 3). Such as enzymes previously described for other MPSs, galsulfase utilizes the mannose 6-phosphate receptor for intracellular localization. The safety profile of this recombinant enzyme is considered acceptable [131, 134]. HSCT was used since 1984 for the treatment of Maroteaux-Lamy syndrome but ERT represents the preferred therapy nowadays, since it ameliorates several aspects of the disease. However, ERT is not able to solve bone deformity, heart valve impairment, or spinal cord compression. Therefore, alternative approaches have been proposed, such as intrathecal administration of galsulfase or gene therapy [135].

2.4.5 Mucopolysaccharidosis VII (Sly syndrome)

Mucopolysaccharidosis VII (MPS VII, OMIM 253220) is caused by genetic mutations of the exoglycosidase β-glucuronidase, a glycosidic hydrolase that cleaves β-D-glucuronic acid residues from the non-reducing termini of GAGs bringing to the accumulation of chondroitin sulfate, dermatan sulfate, and heparan sulfate [136-138]. Among the clinical features of MPS VII there are intellectual disability, organ dysfunction, short stature, and a shortened life span [139]. β-glucuronidase is a tetrameric enzyme with monomers formed of 651 amino acids and a MW of about 78 kDa. The sequence contains a signal peptide of 22 amino acids and four N-linked glycosylation sites. The crystal structure of β-glucuronidase was solved in 1996 and a higher resolution structure was reported later [140, 141]. A review on the structure, function and clinical applications of β-glucuronidase was previously published [142]. β-glucuronidase is not only involved in GAGs degradation, but also has a role in the interconversion of various metabolites such as pentose, glucuronate, chlorophyll, porphyrin, starch, and sucrose.

MPS VII is treated by ERT with the recombinant form of the enzyme, called vestronidase alfa (Mepsevii®, Ultragenyx Pharmaceutical Inc, San Rafael, CA, USA), produced in CHO cells and available since 2017 (Figure 3). A report on the pharmacological activity of the enzyme has been recently published [143] and safety and efficacy have been assessed, indicating that the drug provides sustained improvement over time with the continuation of the treatment, and no life-threatening adverse events [144, 145]. A chemically modified version of vestronidase alfa was obtained by the treatment with sodium metaperiodate, followed by reduction with borohydride (PerT-GUS) [146]. This product was developed in order to favor the transit of the enzyme through the BBB. It was observed that perT-GUS is more effective in clearing the neuronal accumulation of GAGs than vestronidase alfa [147]. Alternative therapeutic approaches for Sly syndrome are HSCT [104] and gene therapy [148].

2.5 Lysosomal acid lipase deficiency (Wolman disease and cholesteryl ester storage disease)

Wolman disease and cholesteryl ester storage disease (OMIM 278000) are two forms of lysosomal acid lipase deficiency (EC 3.1.1.13), an enzyme involved in the degradation of cholesterol in lysosomes, encoded by the *LIPA* gene (Table 1). Lysosomal acid lipase deficiency induces accumulation of triglycerides and cholesterol esters in lysosomes in several different tissues. Residual enzyme activity is found in cholesteryl ester storage disease patients, while the activity is completely abolished in Wolman disease patients. The prevalence of lysosomal acid lipase deficiency is estimated between 1 in 40,000 to 300,000 newborns. The clinical manifestation of cholesteryl ester storage disease is milder than that of Wolman disease, the latter showing hepatosplenomegaly, calcification of adrenal glands, severe malabsorption and failure to thrive, and death within one year of life [149]. Lysosomal acid lipase was originally purified as two bands, one of 41 kDa (322 amino acids) and one of 56 kDa (372 amino acids), which was considered a longer precursor of the active 41 kDa protein [150, 151]. As other lysosomal enzymes, also lysosomal acid lipase is N-linked glycosylated.

Sebelipase alfa (Kanuma®, Alexion Pharmaceuticals, Boston, MA, USA) is a recombinant lysosomal acid lipase used for ERT for the treatment of Wolman and cholesteryl ester storage diseases since 2015 (Figure 4) [152-154]. The enzyme is produced in egg whites of transgenic hens. Sebelipase alfa has five N-linked glycosylated sites and mannose 6-phosphate moieties allow cell targeting. Clinical trials demonstrated that the drug is useful to reduce the hepatic damage and lipid abnormalities caused by lysosomal acid lipase deficiency

and enhance the life expectancy for Wolman disease patients [155-157]. Different therapeutic strategies are applied to lysosomal acid lipase deficiency, depending on the severity of the disease, ranging from dietary management and lipid-lowering medication to liver transplantation [152]. In particular, the reduction of cholesterol levels has been pursued using inhibitors of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, i.e. statins, in combination with drugs that reduce cholesterol and bile acids absorption such as ezetimibe and cholestyramine (Figure 4). HSCT has also been attempted, but results, differently from ERT, have not been encouraging [152].

2.6 Alpha-mannosidosis

Alpha-mannosidosis (OMIM 248500) is a rare disease occurring in 1 over 300,000-700,000 newborns, reported in 1967 [158]. The disorder is caused by mutations of a single human gene, *MAN2B1*, localized in chromosome 19 that codes for α-mannosidase (EC 3.2.1.24) (for general review, see [159-163]) (Table 1). The enzyme is an exoglucosidase that catalyzes the hydrolysis of the terminal mannose unit linked via α1,2, α1,3 and α1,6 bonds in a variety of α-D-mannosides. The mannosidase activity is required for the catabolism of N-linked carbohydrates released during glycoprotein turnover. Enzyme deficiency leads to the accumulation of unbranched neutral oligosaccharides or glycopeptides that form cytoplasmic vacuoles in the central nervous system and parenchymatous organs.

The disease shows a variety of clinical manifestations depending on severity, including intellectual disability, immunodeficiency, recurrent infections, impaired hearing, and Hurler-like skeletal changes [160]. Alpha-mannosidosis is classified as mild form (type I) with onset after age 10, moderate with onset before age 10 (type II), being the majority of cases, and severe with onset in early infancy (type III). At least 155 variants have been detected bearing single or multiple mutations and are associated with different severity of the disorder, without any apparent correlation between genotype and phenotype [159, 164, 165]. To evaluate potential therapeutic approaches, a mouse model of a mild form of the disorder was developed by disrupting the encoding gene via homologous recombination [166].

Alpha-mannosidase is synthesized as a single polypeptide chain that is broken down into smaller subunits with MWs depending on cellular localization. Subunits can be linked by disulfide bridges. The active enzyme is a dimer [167, 168] and contains zinc ions essential for activity. Alpha-mannosidase exists as two isozymes, A

and B, with similar biochemical and stability properties, distributed with different ratios in different tissues. The alpha-mannosidase optimal pH for activity is 4.5. The three-dimensional structure of bovine α-mannosidase was solved, revealing an architecture based on four domains with one alpha/beta domain that contains the active site and three all-beta domains [169]. Structure availability allows the localization of pathological mutations, discriminating between mutations affecting folding and mutations impairing activity. Most mutations are unique to each patient and relatives. However, three mutations account for about 35% of the diseases [170]. Specifically, the human R750W, H200L, and H200N mutations affect folding impairing the enzyme transport in the lysosome, whereas R22H and H72L lead to inactive lysosomal enzymes [162].

The therapy for alpha-mannosidosis is based on gene therapy, bone marrow transplantation, and ERT. However, the two former approaches are still under investigation due to observed drawbacks [171, 172], whereas the latter has been approved by EMA in 2018 successfully applying a recombinant human α-mannosidase, named velmanase alfa (Lamzede®, Chiesi Farmaceutici) [173-175] (Figure 4).

Alpha-Mannosidase for ERT was initially purified either from bovine kidney or secretions of mammalian cells producing the recombinant mouse or human enzyme [176]. The enzyme purified from bovine kidney was a mixture of polypeptides (11-48 kDa) and exhibited a low mannose 6-phosphate content leading to about 6% of enzyme interaction with an affinity column. The recombinant enzyme was obtained from the secretions of mannose 6-phosphate-receptor deficient mouse fibroblasts and CHO cells, respectively, primarily in their precursor forms [176]. The mannose 6-phosphate index for the mouse form was about 74%, whereas that of the human form was only 4% [176]. The efficacy of these forms was evaluated in a mouse model and found an 80-90% reduction of storage substrate with the human form, which resulted to be more effective than the mouse form [176-178]. In 2012, a recombinant human alpha-mannosidase (velmanase alfa) was produced from CHO cells by Zimenex, a Danish biotech company, later acquired by Chiesi Farmaceutici [173]. Phase 1/2 clinical trials using velmanase alfa were carried out on 10 patients receiving for 12 months multiple intravenous doses obtaining positive results [173, 174]. Results of a phase 3 multicenter, double-blind, randomized, placebo-controlled trial on 25 patients indicated the efficacy of velmanase alfa ERT infused at 1 mg/Kg in the treatment of alpha-mannosidosis, especially in patients younger than 18 years old, with consistent clinical improvements, reduction of serum oligosaccharide levels and increase in IgG titers [174, 175].

2.7 GM1 gangliosidosis

GM1 gangliosidosis (OMIM 230500) is a lysosomal storage disorder affecting 1 in 100,000 newborns. The disease is due to mutations of the *GLB1* gene that codes for beta-galactosidase (EC 3.2.1.23) (Table 1). This enzyme hydrolyzes the terminal galactose moiety from different organic and protein molecules. The active enzyme is a dimer with an equilibrium monomer-dimer dependent on pH, low pH values favoring the dimer and neutral pH values establishing a monomer-dimer equilibrium. Beta-galactosidase is expressed as an 85-kDa precursor that is post-translationally glycosylated generating an 88 kDa form. Upon the transfer to the lysosome, the enzyme is cleaved to the mature form of 64 kDa [179, 180]. Over 160 gene mutations have been reported [181]. Lack of beta-galactosidase activity leads to accumulation of GM1 gangliosides and related glycoconjugates causing lysosomal swelling, cellular damage, and organ dysfunction due to progressive destruction of neurons in the brain and spinal cord [182]. The disease is classified into three types depending on the time of onset, being lethal in the infantile and juvenile forms. The severity of the disease is strongly dependent on the degree of remaining galactosidase activity. Patients exhibit enzyme activity lower than 5% of the normal level. It was found that about 15% of enzyme activity is enough to alleviate symptoms. This finding opened the way to ERT for GM1 gangliosidosis, as gene therapy seems not to be fruitful due to accumulation of the inactive enzyme in the endothelial reticulum [183]. As for other lysosomal diseases, substrate reduction agents were evaluated either in animal models or in a few patients. Miglustat, a galactosidase inhibitor, was used to slow down disease progression in patients with juvenile and adult GM1 gangliosidosis [184] and, similarly, lucerastat was used [185]. N-octyl 4-epi-β-valienamine, a chemical chaperone, was also evaluated on a mouse model observing positive effects [186].

Recent studies have been carried out aiming at evaluating the effects of ERT using recombinant human beta-galactosidase [183]. The enzyme produced in CHO cells was delivered to patient fibroblasts via the mannose 6-phosphate receptor, as demonstrated by delivery inhibition in the presence of 1 mM mannose 6-phosphate, and intracellularly processed with the removal of a 20 kDa moiety. Doses of the enzyme as low as 3 nM were delivered and found to be effective in lowering substrate concentration. A single dose was injected in the brain of the disease mouse model to overcome BBB and found to obtain a bilateral distribution. ERT prolonged for 8 weeks with weekly administration led to a reduction of beta-galactosidase substrates levels and reversion of secondary neuropathological markers [183].

2.8 Metachromatic leukodystrophy

Metachromatic leukodystrophy (OMIM 250100) is caused by mutations in the *ARSA* gene, with more than 200 reported mutations [187, 188]. The gene codes for arylsulfatase A (EC 3.1.6.8), an enzyme that catalyzes the hydrolysis of the acidic sphingolipid 3-O-sulfogalactosylceramide, also known as sulfatide (Table 1). In the absence of arylsulfatase, sulfatide accumulates in the lysosomes of glia cells and neurons causing metachromatic staining features and progressive demyelination in the peripheral and central nervous system resulting in motor and cognitive deteriorations. A few metachromatic leukodystrophy patients exhibit mutations in the *PSAP* gene. This gene codes for proteins, such as saposin B, that cooperates with arylsulfatase A in hydrolyzing sulfatides. Metachromatic leukodystrophy is reported to occur in 1 in 40,000 to 160,000 individuals worldwide. The disease is classified into three clinical subtypes depending on the age of the patient at symptom onset: late-infantile, juvenile, or adult.

Arylsulfatase A (isoform I) contains 507 amino acids (MW 53 kDa) and exists in an alternative splicing isoform II composed of 423 amino acids. The enzyme exhibits three N-linked carbohydrates and a bound calcium ion. The three-dimensional structure was determined [189].

To achieve full catalytic activity, sulfatases undergo a post-translational oxidation of an active-site cysteine to C-alpha formylglycine catalyzed by the formylglicine-generating enzyme. Thus, not surprisingly, C69A variant in arylsulfatase A is inactive [190] and mutations in formylglicine-generating enzyme are associated with multiple sulfatases deficiency, a still untreated disease [191].

As for other lysosomal storage diseases, several therapeutic strategies have been proposed. The efficacy of HSCT in the treatment of metachromatic leukodystrophy was evaluated in a mouse model, detecting significant improvements [192-194] and gene therapy was applied also in a mouse model [195, 196]. However, the most investigated approach has been ERT. Recombinant human arylsulfatase A (MW of 53 kDa) was obtained in CHO cells [197]. The recombinant enzyme contains the naturally linked carbohydrates and the correct mannose 6-phosphate units [197]. ERT exploiting recombinant arylsulfatase A was evaluated in a mouse model observing storage reduction in peripheral tissues as well as, surprisingly, also in spinal cord and brain [197]. However, overall, ERT showed lower efficacy than in other lysosomal diseases likely due to reduced uptake [198], even when the delivery was mediated by either nanoparticles [199] or peptide vectors [200]. To

overcome the issue of BBB permeability, attempts were carried out for the intrathecal delivery of a recombinant arylsulfatase A named TAK-611 (Takeda Pharmaceutical Co., Tokyo, Japan, previously indicated as SHP611 or HGT1110) both in animal models and humans [13], using a human enzyme produced via a modified process [201]. Specifically, the process changes led to an increased level of sialic acid and mannose 6-phosphate and higher purity, without significantly altering the enzyme activity. A comparison of toxicology, pharmacokinetics, and bioavailability of the enzyme obtained from the two processes and administered in a mouse model indicated no substantial differences [201].

2.9 Acid sphingomyelinase deficiency (Niemann Pick disease)

Acid sphingomyelinase deficiency (known as Niemann Pick disease, types A and B, OMIM 257200 and 607616, respectively) is associated with mutations in the *SMPD1* gene with a frequency of 1:250,000 ([202, 203] and references therein). More than 180 gene mutations were identified [202]. *SMPD1* gene codes for acid sphingomyelinase (EC 3.1.4.12), a phospholipase that catalyzes the breakdown of sphingomyelin, an abundant component of cellular membranes, especially myelin nerve cells (Table 1). The products of acid sphingomyelinase catalytic action are phosphocholine and ceramide, the latter being a relevant metabolite in the ceramide-mediated signaling pathway [204, 205]. In patients with acid sphingomyelinase deficiency, sphingomyelin accumulates within lysosomes in different organs especially in ganglion cells of the central nervous system causing cell death. The disorder leads to multisystemic clinical manifestations such as hepatosplenomegaly, interstitial lung disease, and bone marrow infiltration along with central nervous system involvement in the more severe phenotypes [206]. The clinical phenotype ranges from a severe infantile form with neurologic degeneration resulting in death usually by 3 years of age (type A) to a later-onset non-neurologic form (type B) that is compatible with survival into adulthood. Mouse models of the disease were obtained, allowing the first drug testing [207, 208].

The three-dimensional structure of acid sphingomyelinase produced from HEK cells was determined and compared with the enzyme obtained in CHO cells, being the latter the recombinant form, olipudase alfa, used in ERT (see below) [209]. No significant differences were observed. The enzyme consists of a multidomain architecture: a saposin domain, a proline-rich linker, a metallo-dependent phosphatase catalytic domain, and an ill-defined C-terminal domain. The enzyme also contains two zinc ions, separated by 3.5 Å, whose role in

the catalytic function has not yet been unveiled. There are also 6 N-linked glycosylation sites. About 90 mutations associated with the disease were mapped in the structure and found that 82% are located in the catalytic domain, resulting in a 21.6% mutation rate in the protein sequence. In contrast, the mutation rates in the saposin, proline-rich linker, and C-terminal domains are less than 11% [209]. One group of mutations is centered into the catalytic domain, thus impacting directly on the enzyme activity, whereas the other group of mutations is scattered in the protein matrix impacting on folding.

ERT for acid sphingomyelinase deficiency is based on the administration of the recombinant enzyme produced in CHO cells, olipudase alfa (Sanofi Genzyme, Cambridge, MA, USA) [210]. The recombinant form showed identical catalytic properties of the natural form, including the optimal acidic pH profile and zinc-dependence [210]. ERT was first evaluated in a mouse model observing some positive results but also no improvement for central nervous system markers [211]. On these bases, the authors concluded that ERT with olipudase alfa might be only effective for type B disease. Indeed, clinical studies on 11 type B patients did show that olipudase alfa was effective, although some adverse effects were recorded [202, 212]. A parallel clinical study was carried out on 5 adults with non-neuronopathic type B disease observing positive results [213].

A 42-month efficacy study on the administration of olipudase alfa has been recently reported showing an improved pro- and anti-atherogenic lipid profile [206]. This study expands and confirms a previous 30-month safety and efficacy study [214, 215].

In order to increase the amount of enzyme delivered into lysosome and avoid limitations to internalization due to non-ideal glycosylation profile (i.e. mannose 6-phosphate recognition pattern) of recombinant sphingomyelinase, the enzyme was coupled to 100 nm nanocarriers bearing anti-intracellular adhesion molecule (ICAM)-1. The rationale of this approach is that the transmembrane glycoprotein ICAM-1 is highly expressed under inflammatory conditions typically associated with lysosomal storage diseases. Results indicated that the delivery system was effective in enzyme internalization [216]. An alternative delivery strategy was based on the loading of recombinant sphingomyelinase into liposomes. Several formulations were attempted to identify a composition that showed a higher encapsulation efficiency of the recombinant human enzyme (21%) and cellular uptake by fibroblasts and macrophages [217].

3. ERT for metabolic diseases

3.1 Adenosine deaminase deficiency and severe combined immunodeficiency disease

Adenosine deaminase (ADA) deficiency is a rare genetic autosomal disease (OMIM 608958, 102700) primarily associated with immune system impairment, showing an incidence between 1:200,000 and 1:600,000 newborns. ADA deficiency, firstly identified in 1972 [218], causes the insurgence of a severe combined immune deficiency (SCID) involving both humoral and cell-mediated immunity, which accounts for about 15% of the known cases of SCID. If not promptly treated, ADA-SCID is fatal in the first year of life because of the insurgence of common infections. For this reason, the disease has been comprised in the newborn screening for SCID programs [219].

ADA (EC 3.5.4.4) takes part in the purine metabolism, catalyzing the irreversible deamination of adenosine or 2'-deoxyadenosine to inosine or 2'-deoxyinosine and ammonia (Figure 5). The substrate adenosine plays an important homeostatic role as an intermediate metabolite in the nucleic acids and energetic metabolisms and extracellular signaling. ADA is encoded by the *ADA* gene located on chromosome 20q13.12, composed of twelve exons, generating a 41-kDa monomeric protein with a known three-dimensional structure [220] (Table 1). The human genome encodes also for other two adenosine deaminases, namely ADA2 and ADA-like (ADAL or ADA3), that are structurally and functionally not related to ADA [221-224]. The impairment in the homeostasis of adenosine and 2'-deoxyadenosine triggered by the absence of a functional ADA activity interferes with the normal immune response, thymopoiesis, and DNA synthesis and repair. Thus, the main consequences are limited V(D)J recombination, hypogammaglobulinemia, lymphotoxicity, severe lymphopenia of T, B, and natural killer cells ([225-228]; [229] and references therein; [230] and references therein). Other relevant consequences are developmental delay, skeletal dysplasia, hearing and cognitive impairment, hepatic failure, and respiratory distress [231, 232].

The first ERT proposed for the treatment of ADA-SCID was the transfusion to patients of irradiated red blood cells from healthy donors [233]. The therapy was successful but presented several possible drawbacks, pushing the development of an alternative ERT approach (Figure 5). With this purpose, Davis and coworkers proposed PEG-ADA (pegademase), the PEGylated form of bovine adenosine deaminase, that shares 93% of identity with human ADA [234]. The enzyme was derivatized with 5 kDa monomethoxypoly(ethylene glycol) (mPEG) on lysine residues, and the modification decreased protein immunogenicity and incremented ADA half-life [235]. Pegademase was the first PEGylated protein approved by FDA in 1990 (Adagen®, Enzon

Pharmaceuticals, Inc., Cranford, New Jersey, USA) (Table 1) [236]. Starting from early childhood, pegademase is administered weekly or biweekly by intramuscular injection, with a half-life varying from three to more than six days; ADA activity levels need to be periodically monitored [237].

In 2018, FDA approved the alternative recombinant ADA elapegadamase-lvlr (ELA-ADA, Revcovi™, Leadiant Biosciences, Inc, Gaithersburg, MD, USA), a recombinant bovine ADA expressed in *Escherichia coli*, labeled initially as EZN-2279 (NCT01420627) (Table 1). ELA-ADA carries the C74S mutation and is modified with 13 mPEG succinimidyl carbonate chains on lysine residues. The use of the recombinant protein improves the product quality and availability and reduces the theoretical risk of transmission of bovine spongiform encephalopathy (BSE), still showing *in vitro* efficacy comparable to pegademase and potentially even better *in vivo* results [238].

The early administration of ADA ERT improves immune and metabolic abnormalities. Several studies tracked the effects of prolonged pegademase administration, demonstrating the drug safety and efficacy in recovering patient conditions during at least one decade after the beginning of the treatment [239-244]. However, the therapy efficacy decreases over years and becomes only partially beneficial, probably because of the age-related decline of thymopoiesis [239] and occasionally as a consequence of the development of anti-bovine ADA antibodies [243, 245]. Therefore, ADA ERT is generally an interim treatment for ADA-SCID, because of its long-term unaffordable costs ($ 200,000-300,000 per year), the temporal proximity between administrations, and the recommended metabolic monitoring. When possible, its employment is better applied as a palliative approach aimed at the amelioration of the physiological context of patients before HSCT or gene therapy [246].

HSCT has been for a long time the first-line choice for ADA-SCID treatment, although associated with significant risks; the success of the therapy spans from 81-86% in case of a transplant from sibling or familiar donors (possible in less than 25% of ADA-SCID cases) to 43% in case of haploidentical donors [247]. Concurrently, gene therapy has been successfully pursued for the treatment of ADA-SCID, thanks to the pervasive presence of ADA in human tissues, to the nonessential fine regulation of the expression profile and the survival advantage of enzyme-expressing cells [248]. In 2016, autologous CD34+ cells transduced to express ADA (Strimvelis™, Glaxo-SmithKline, London, UK), developed by a joint work of Tiget and S. Raffaele Institutes (Milan, Italy) and GSK (NCT03478670) [249-252], was the first *ex vivo* gene therapy

approved by EMA. Strimvelis™ is indicated for the treatment of ADA-SCID patients inadequate to receive ERT or HSCT. The survival rate after ten years is 100%, with long-term benefits and sustained improvement of health conditions.

3.2 Hypoxanthine-guanine phosphoribosyltransferase deficiency and Lesch-Nyhan syndrome

Hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency is a rare X-linked genetic disorder of purine metabolism, involving the *HPRT1* gene (Xq26), firstly observed in the 1960s [253-256] (Table 1). The disorder has an incidence of 1:380,000 newborns with recessive inheritance and affects male subjects, whereas females are generally asymptomatic [256]. Normally, HPRT (also known as HGPRT, EC 2.4.2.8) participates in the purine salvage pathway catalyzing the transfer of the 5-phosphoribosyl group between α-D-5-phosphoribosyl-1-pyrophosphate and hypoxanthine or guanine to form inosine monophosphate or guanosine monophosphate [257, 258] (Figure 5). The enzyme is expressed in all body tissues and its activity is clinically detected in erythrocytes. The genetic defect of HPRT manifests in mild to severe and even fatal disorders, depending on its gravity. It is associated with a major syndrome, namely Lesch-Nyhan syndrome (OMIM 300322, 300323; [259]) – the most severe form – and Lesch-Nyhan attenuated variants, characterized by a partial absence of the enzymatic activity (*HPRT1*-related neurologic dysfunction and *HPRT1*-related hyperuricemia) [260, 261]. Several hundreds of mutations in *HPRT1* have been associated to the insurgence of pathological phenotypes, consisting in neurological and physiological defects with different severity [262, 263]. At cerebral level, neurological symptoms show a late onset and the damage due to the dopaminergic system and basal ganglia seems to originate before birth, during the development of the nervous system [264, 265]. The most common manifestations include intellectual disability, impaired motility, dystonia, dysarthria, dysphagia, and self-mutilation, the last typical of Lesch-Nyhan syndrome [259, 266]. On the metabolic side, the absence or the malfunction of HPRT limits hypoxanthine and guanine recovery and deviates them to degradation, with a concomitant upregulated demand for *de novo* synthesis. In healthy subjects, uric acid is filtered in kidneys, where in the proximal tubule is partially excreted and over 90% is reabsorbed. Its physiological concentration in the blood is about 400 μM (6.8 mg/dl), not far from its limit of solubility. An imbalance in uric acid concentration, due to an augmented catabolite production and/or reduced excretion, known as hyperuricemia, forces the precipitation of monosodium urate crystals in the synovial fluids in joints

and tendons and the formation of renal stones: the persistence of this condition elicits the insurgence of a chronic inflammation state and gout. Lesch-Nyhan syndrome patients have a life expectancy limited to twenty-thirty years, the main consequence of symptoms associated with hyperuricemia, particularly renal failure.

Several cellular and animal models for the investigation of this complex disease were developed [267-270], even though they are more useful for metabolic studies than for neurological and behavioral evaluation.

The reported attempts of HPRT replacement exploiting normal erythrocytes transfusions and bone marrow transplantation failed [271-274] and, to date, neurologic and metabolic symptoms are treated separately.

The reduction of neurological manifestations associated with the disease was investigated in several clinical trials (NCT00935753; NCT01065558; NCT01751802; NCT00004314), but no relevant results have been obtained so far [275]. However, some symptoms can be managed by the administration of psychoactive or antipsychotic drugs.

Hyperuricemia-related symptoms are generally treated with simple therapies (alkalization of urines by bicarbonate, hyperhydration) or the administration of drugs aimed at the reduction of uric acid production or its increased excretion. Part of the former group are the first-line drug allopurinol (approved in 1965) and febuxostat (commercialized from the late 2000s) inhibiting xanthine oxidase, the enzyme that catalyzes the conversion of hypoxanthine to xanthine and then to uric acid. Despite the lowering in hematic urate concentration, the consequent accumulation of poorly soluble xanthine could induce lithiasis. On the other hand, uricosurics (e.g. probenecid, lesinurad, benzbromarone, sulfinpyrazone) enhance the excretion of uric acid, generally acting on urate transporters responsible for its reabsorption. These drugs, however, demonstrated limited benefits and several severe side effects at renal and hepatic levels, with consequent controlled commercialization and administration [276]. In this context, an alternative therapy, conceived for the treatment of hyperuricemia associated with the tumor lysis syndrome (TLS), was developed, consisting in the administration of the enzyme urate oxidase or uricase (UOx) (EC 1.7.3.3) [277]. UOx is a homotetramer of 140 kDa and catalyzes the oxidation of urate, in the presence of oxygen and water, to 5-hydroxyisourate (HIU) and hydrogen peroxide. HIU spontaneously degrades through an intermediate species to racemic allantoin, a more soluble molecule than urate, that can be easily excreted by the kidney. It has been demonstrated that hydrogen peroxide derived from UOx-mediated urate degradation is buffered by normal erythrocyte metabolism [278]. Nevertheless, to overcome serious side effects, patients carrying glucose-6-

phosphate dehydrogenase deficiency or other metabolic disorders known as related to hemolytic anemia cannot assume any form of UOx [279].

In a first attempt in the ’70s, the non-recombinant fungal enzyme from *Aspergillus flavus* cultures (Uricozyme®, Sanofi, Paris, France; PDB 1R56) was successfully employed in clinical applications. Nonetheless, its use was hampered by low yields of extraction and the insurgence of allergies (0% to 4.5%), probably caused by impurities and its natural hyperglycosylation [280-282]. For these reasons, Uricozyme® was replaced by rasburicase, the recombinant protein from *A. flavus* expressed in a genetically modified *Saccharomyces cerevisiae* strain (Table 1). Rasburicase is distributed in Europe, the USA, and Japan since the 2000s with the name of Fasturtec®, Elitek®, and Rasuritek® (Sanofi), respectively, and is primarily administered to prevent the TLS in oncologic patients [283] and for gout treatment. The recombinant enzyme was successfully tested for the alleviation of gouty symptoms in Lesch-Nyhan disease patients, improving hyperuricemia and being well tolerated [275, 284, 285]. Several studies and clinical trials carried out on cancer patients confirmed its effectiveness and its favorable safety with respect to Uricozyme® [286-288]. A single rasburicase dose, administered daily as an infusion of 1.5 mg of rasburicase for up to five days, degrades the circulating uric acid in four hours. It was demonstrated that rasburicase administration stimulates the production of anti-rasburicase antibodies (ras-Ab), able to block the catalytic activity [289], yet recent studies emphasize that diminishing the number of administered doses decreases the adverse effect insurgence without affecting the therapeutic outcome [283, 290]. A detailed description of rasburicase properties, pharmacokinetics, pharmacodynamics, and clinical trials was reported [291-297].

Rasburicase is predominantly degraded by peptide hydrolysis and only minimally excreted by the kidney [294] with a half-life of 19 hours. Thus, for the application in prolonged therapeutic sets, a PEGylated UOx variant was introduced to extend the half-life (214 hours) and reduce the immunogenicity of the drug. Pegloticase (Puricase®/Kristexxa®, Savient Pharmaceuticals, East Brunswick, NJ, USA) is a recombinant porcine/baboon variant uricase derivatized with 10-kDa mPEG with a total mass of 540 kDa (Table 1) [298]. However, clinical studies evidenced that pegloticase stimulates an immune response, elicited by the mPEG methoxy moiety [299]. Antipegloticase antibodies reduce the half-life of the circulating drug but are not associated with allergic reactions [300], even if their presence seems linked to infusion reactions [301].

Pegloticase therapy consists of a biweekly single-dose of 8 mg of enzyme administered by infusion. It is employed in cases of tophaceous gout, where xanthine oxidase inhibitors are not tolerated or ineffective. Among reported side effects there are gout flares and cardiovascular adverse events. To date, no clinical data are available on pegloticase administration to Lesch-Nyhan disease patients [275], yet it could present more side effects than rasburicase based on the results obtained for gout-suffering patients. A detailed description of pegloticase properties, pharmacokinetics, pharmacodynamics, and clinical trials was reported [302-305].

Non-primate organisms naturally produce the more soluble (S)-allantoin as the end-product, through the degradation of uric acid operated by three distinct hepatic enzymes: UOx, HIU hydrolase, and 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU) decarboxylase [306]. To date, it is not clear if the transient intermediate species forming from urate degradation are toxic for the organism. In fact, mice lacking HIU hydrolase activity developed hepatocellular carcinoma, raising some questions and concerns about HIU and OHCU toxicity after prolonged exposure [307]. Based on these evidence, Percudani and colleagues proposed an alternative approach to UOx administration, where all the three enzymatic activities are supplied as a PEGylated complex [308, 309].

3.3 Phenylketonuria

Phenylketonuria (OMIM 261600) is a genetic disease caused by the deficiency of phenylalanine hydroxylase (EC 1.14.16.1), the enzyme that catalyzes the conversion of phenylalanine in tyrosine, coded by the *PAH* gene (Table 1). High levels of phenylalanine in the blood are toxic for the brain causing cognitive dysfunction, memory impairment, and psychiatric problems. A strict diet depleted of phenylalanine followed all life long is the first line of therapy for phenylketonuria. However, while diet therapy is effective in young patients, older patients tend to abandon it with critical outcomes. For this reason, a complementary ERT strategy has been pursued [310, 311]. The direct exploitation for ERT of human phenylalanine hydroxylase is hampered by the need for the cofactor tetrahydrobiopterin for enzyme activity [312]. An alternative strategy was pursued based on the use of phenylalanine ammonia lyase, an enzyme converting phenylalanine to ammonia and trans-cinnamic acid, an approach also used to deplete cancer cells of phenylalanine (Figure 6). Recombinant phenylalanine ammonia lyase was purified from several expression systems, including *E. coli*, yeast, *Petroselinum crispum*, and *Anabaena variabilis* cyanobacterium, and different PEGylation strategies were

applied to minimize immunogenicity while preserving activity [313-316]. The most promising enzyme was found to be from *A. variabilis* [315]. Mutations were also introduced in the recombinant enzyme, the more effective in preventing aggregation being C503S and C565S [315]. ERT for phenylketonuria was approved in 2018 and is based on pegvaliase (Palynziq®, BioMarin Pharmaceutical), a recombinant *A. variabilis* PEGylated phenylalanine ammonia lyase. The enzyme from *Rhodosporidium toruloides* was also proposed to be used to remove phenylalanine from protein food lysates [317]. Results of the first 1.5 years of clinical application of pegvaliase on 46 patients indicate that about 70% achieved a lowering of phenylalanine levels, with some minor adverse effects [318]. This investigation extended a previous long-term phase 3 clinical study on 261 patients that showed the efficacy of pegvaliase ERT [319]. Issues of pegvaliase treatment are associated with antibody response against the non-human protein, requiring co-treatment with antihistamines and, eventually, epinephrine [320, 321].

Further ongoing studies are aimed at improving the catalytic activity of *A. variabilis* phenylalanine ammonia lyase via direct evolution [322]. The strategy is to grow *E. coli* K-12 strain on a minimal medium in the presence of phenylalanine. As this strain cannot grow using phenylalanine as a unique nitrogen source, colonies expressing phenylalanine ammonia lyase forms with high catalytic efficiency are randomly produced generating the preferred ammonium nitrogen source. These colonies were identified and single or multi amino acid mutations were determined. It was found that M222L and L4P/G218S increased k_{cat} with respect to the wild enzyme, whereas only M222L showed also about 2-fold enhancement of k_{cat}/K_m [322]. This study highlights the possibility of generating more highly efficient phenylalanine lyase for ERT. Furthermore, for increasing the enzyme bioavailability, thus reducing the frequency of administration, pegvaliase was encapsulated in mesoporous silica nanoparticles coated with poly(allylamine) and poly(acrylic acid)-bowman birk (protease inhibitor) conjugate [323].

An alternative strategy for the treatment of phenylketonuria is based on gene editing using liver-tropic recombinant AAV2/8 vectors for delivering CRISPR/Cas9 machinery that allows correction of the mutated gene [324].

3.4 Hypophosphatasia

Hypophosphatasia (OMIM 241500, 241510, 146300) is a metabolic bone disease caused by the deficiency of the *ALPL* gene coding for tissue-nonspecific alkaline phosphatase isozyme (EC 3.1.3.1) [325-329] (Table 1). The membrane-bound enzyme catalyzes the dephosphorylation of monophosphate esters and pyridoxal 5'-phosphate (PLP). Its deficiency leads to elevated blood and urine levels of PLP, phosphoethanolamine, and pyrophoshate. Consequently, decreased intracellular levels of PLP are detected because only when dephosphorylated it can be imported into the cell and rephosphorylated and act as a coenzyme. Low levels of PLP cause epileptic seizures. Furthermore, increased levels of pyrophosphate interfere with bone biomineralization because they prevent hydroxyapatite crystal formation. As a result, increased cases of rickets are observed [330]. Hypophosphatasia is classified in six forms depending on the age of onset and severity [326]. The frequency is 1:100,000-450,000 of newborns.

Alkaline phosphatase is a dimer with a MW for each monomer of 80 kDa and contains zinc, calcium, and magnesium ions. The enzyme is associated with the membrane via a glycosylphosphatidylinositol moiety that is attached to an aspartate residue upon removal of 29 C-terminal amino acid residues [326]. The three-dimensional structure has not yet been obtained but a homologous model was built based on human alkaline phosphate and *E. coli* orthologue [326]. Nowadays, 411 mutations of the enzyme have been detected (http://www.sesep.uvsq.fr/03_hypo_mutations.php) and most of them, 302, were mapped in the structure [326]. Severe disease forms were found to be associated with mutations at the catalytic site, dimer interface and protein key structural elements. Treatment for hypophosphatasia has been pursued via gene therapy [331]. A mouse model of hypophosphatasia was developed [332-334] allowing testing therapeutic strategies first in mice [335].

In 2015 ERT with a recombinant enzyme, asfotase alfa (Strensiq™; Alexion Pharmaceuticals, Inc.) was approved in the USA, Europe, Canada, and Japan [329, 336] (Figure 6). The enzyme produced in CHO-DG44 cells was modified to obtain a soluble form by removal of the C-terminal hydrophobic tail and the concomitant insertion of the Fc region of human IgG for an easy purification step. In addition, 10 aspartate residues were inserted into the Fc region to favor binding to hydroxyapatite, thus targeting the enzyme to bones [335, 337]. Indeed, the recombinant enzyme binds 32 fold tighter to hydroxyapatite than the soluble bovine kidney form. Two disulfide bonds between Fc moieties led to a MW of asfotase alfa of 370 kDa, indicative of a tetrameric form [335]. Clinical investigations were carried out over a period of at least seven years monitoring the efficacy

and safety of asfotase alfa administration, showing that the treatment improves skeletal mineralization, respiratory, growth, cognitive, and motor functions with limited adverse effects [325, 328, 329, 336, 338-340].

4. Conclusions

The extensive studies carried out over more than forty years have led to establishing ERT as a mature weapon to cure diseases caused by gene mutations coding for metabolically critical enzymes. This achievement was made possible by the advent of recombinant enzymes, which has significantly improved the possibility of exploring diversified dose strategies. In addition, the delivered enzymes have been optimized by decoration with PEG or conjugation with targeting proteins. Some issues still remain on ERT efficacy and adverse immune reactions, but, in most cases, are negligible. Thus, ERT has saved many lives and has improved life quality of many patients affected by rare and ultra-rare diseases and is a clear-cut example of the therapeutic power of targeted biologics.

List of Abbreviations

Food and Drug Administration, FDA; European Medicines Agency, EMA; Enzyme Replacement Therapy, ERT; Hematopoietic Stem Cell Transplantation, HSCT; blood-brain barrier, BBB; Chinese hamster ovary, CHO; polyethylene glycol, PEG; Mucopolysaccharidoses, MPSs; glycosaminoglycans, GAGs; molecular weight, MW; 3-hydroxy-3-methylglutaril-CoA, HMG-CoA; monosialotetrahexosylganglioside, GM1; intracellular adhesion molecule, ICAM; adenosine deaminase, ADA; severe combined immune deficiency, SCID; monomethoxypoly(ethylene glycol), mPEG; hypoxanthine-guanine phosphoribosyltransferase, HPRT; tumor lysis syndrome, TLS; uricase, UOx; 5'-hydroxyisourate, HIU; 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline, OHCU; pyridoxal 5'-phosphate, PLP.

Conflict of Interest

The authors declare no conflict of interests.

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Table 1. List of genetic diseases treated with ERT.

Lysosomal Storage Disorder	**OMIM**	**Defective gene**	**Natural enzyme**	**EC**	**Substrate**	**Biologics**	**Source/expression system**	**Year first approval by FDA/ EMA**
Gaucher	230800	*GBA1*	beta-glucocerobrosidase	3.2.1.45	glucocerebroside	alglucerase imigluceras e velagluceras e alfa taliglucerase Abcertin® imigluceras e biosimilar	human placenta CHO human fibrosarcom a carrot cell CHO	1991
Fabry	301500	*GLA*	alpha-galactosidase A	3.2.1.22	globotriaosylceramide	agalsidase alfa agalsidase beta PRX-102 pegunigalsidase alfa agalsidase beta biosimilar	fibroblast cell line CHO ProCellEx® plant cell ProCellEx plant cell *Nicotiana benthamian a*	2003
Pompe	232300	*GAA*	acidic alpha glucosidase	3.2.1.20	glycogen	alglucosidase alfa	CHO	2006

						avalglucosidase alfa ATB200		
Mucopolysaccharidosis type I	607014	*IDUA*	alpha-L-iduronidase	3.2.1.76	dermatan sulfate, heparan sulfate	laronidase	CHO	2003
Mucopolysaccharidosis type II	309900	*IDS*	iduronate 2-sulfatase	3.1.6.13	dermatan sulfate, heparan sulfate	idursulfase idursulfase beta	HT-1080 human fibrosarcoma cell lines CHO	2006 NA
Mucopolysaccharidosis type IVA	253000	*GALNS*	N-acetylgalactosamine 6-sulfatase	3.1.6.4	chondroitin-6-sulfate, keratan sulfate	elosulfase alfa	CHO	2014
Mucopolysaccharidosis type VI	253200	*ARSB*	N-acetylgalactosamine 4-sulfatase	3.1.6.12	dermatan sulfate, chondroitin-4-sulfate	galsulfase	CHO	2005
Mucopolysaccharidosis type VII	253220	*GUSB*	beta-glucuronidase	3.2.1.31	chondroitin sulfate, dermatan sulfate, heparan sulfate	vestronidase alfa	CHO	2017
Lysosomal acid lipase deficiency	278000	*LIPA*	lysosomal acid lipase	3.1.1.13	cholesterol esters, triacilglycerols	sebelipase alfa	egg whites of transgenic hens	2015

alpha-Mannosidosis	248500	*MAN2B1*	alpha-mannosidase	3.2.1.24	terminal mannose moiety	velmanase alfa	CHO	2018
GM1 gangliosidosis	230500	*GLB1*	beta-galactosidase	3.2.1.23	terminal galactose moiety	beta-galactosidase	CHO	NA
Metachromatic leukodystrophy	250100	*ARSA*	arylsulfatase A	3.1.6.8	acidic sphingolipid 3-O-sulfogalactosylceramide	arylsulfatase A	CHO	NA
Acid sphingomyelinase deficiency	257200/607616	*SMPD1*	acid sphingomyelinase	3.1.4.12	sphingomyelin	olipudase alfa	CHO	NA
Metabolic disease								
ADA deficiency	608958, 102700	*ADA*	adenosine deaminase	3.5.4.4	adenosine, deoxyadenosine	pegademase elapegadamase	bovine enzyme expressed in *E. coli*	1990, 2018
HPRT deficiency	300322, 300323	*HPRT1*	hypoxanthine-guanine phosphoribosyltransferase	2.4.2.8	alpha-D-5-phosphoribosyl-1-pyrophosphate, hypoxanthine, guanine	rasburicase	*A. flavus* enzyme expressed in *S. cerevisiae*	2001 (2009) *
Phenylketonuria	261600	*PAH*	phenylalanine hydroxylase	1.14.16.1	phenylalanine	pegvaliase	*A.variabilis*	2018

Hypophosphatasia	241500, 241510, 146300	*ALPL*	tissue-nonspecific alkaline phosphatase isozyme	3.1.3.1	monophosphate esters, PLP	asfotase alfa	CHO-DG44	2015

NA, not yet approved by FDA or EMA. *Rasburicase was approved in 2001 by FDA for pediatric use only for the treatment of TLS and from 2009 is administered also for the initial treatment of hyperuricemia in oncogenic adult patients. Its employment for the treatment of hyperuricemia in HPRT deficiency and LND patients is reported [275, 284, 285].

Figure Legends

Figure 1. Therapeutic strategies for the treatment of genetic diseases linked to enzyme deficiency.

Figure 2. ERT and substrate reduction approach for lysosomal Gaucher disease, Fabry disease, and Pompe disease. Natural enzymes are indicated in black boxes, ERT enzymes are indicated in green boxes and small molecules inhibitors used for substrate reduction are indicated in red boxes.

Figure 3. ERT for lysosomal mucopolysaccharidoses (MPS). GAGs stands for glycosaminoglycans. Natural enzymes are indicated in black boxes and ERT enzymes are indicated in green boxes.

Figure 4. ERT for lysosomal acid lipase deficiency (Wolman disease and cholesteryl ester storage disease) and alpha mannosidosis. Natural enzymes are indicated in black boxes, ERT enzymes are indicated in green boxes and small molecules inhibitors used for substrate reduction are indicated in red boxes.

Figure 5. ERT for adenosine deaminase (ADA) deficiency and hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency. XO is xanthine oxidase. Natural enzymes are indicated in black boxes, ERT enzymes are indicated in green boxes and small molecules inhibitors used for substrate reduction are indicated in red boxes.

Figure 6. ERT for phenylketonuria and hypophosphatasia. Natural enzymes are indicated in black boxes, ERT enzymes are indicated in green boxes.

Figure 1

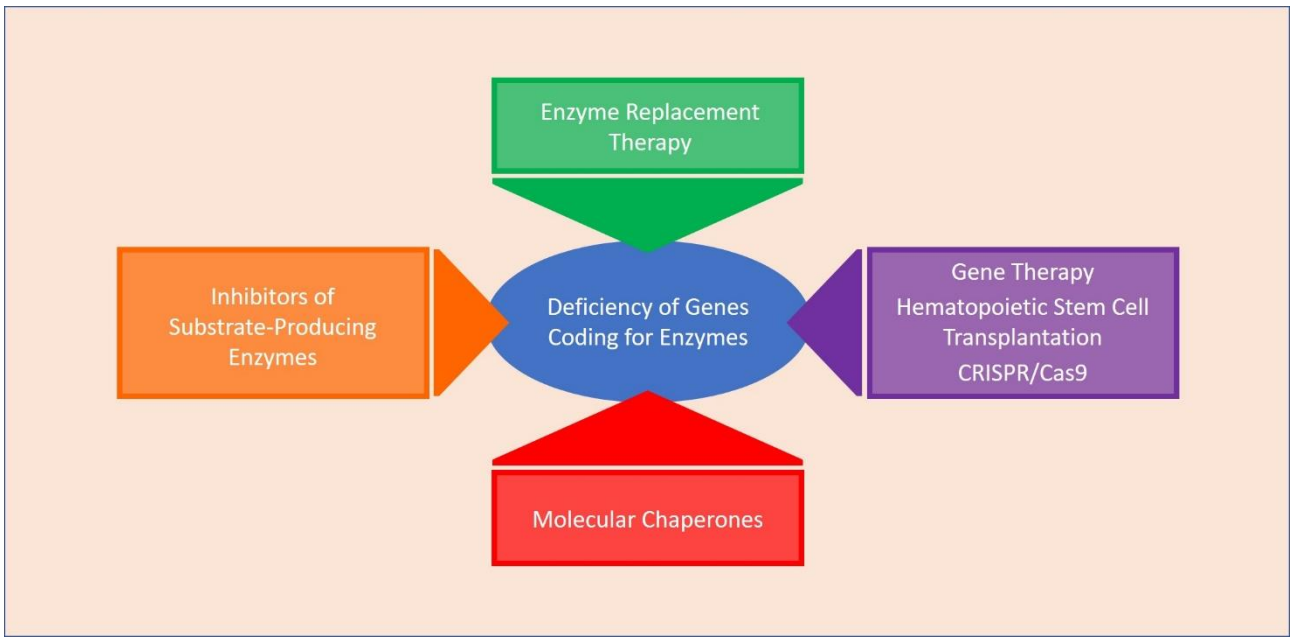

Enzyme Replacement Therapy
Inhibitors of Substrate-Producing Enzymes
Deficiency of Genes Coding for Enzymes
Gene Therapy
Hematopoietic Stem Cell Transplantation
CRISPR/Cas9
Molecular Chaperones

Figure 2

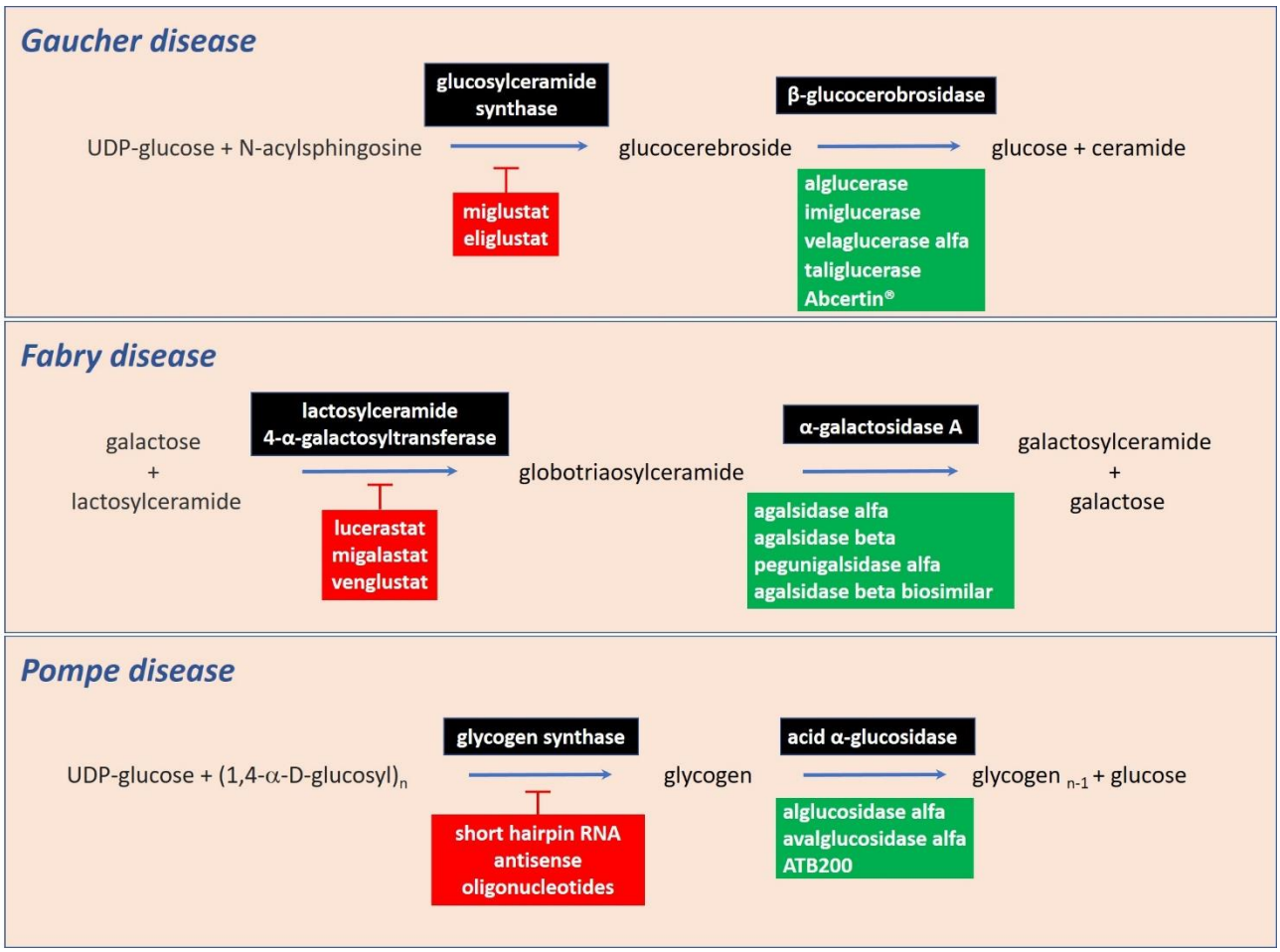

Gaucher disease
glucosylceramide synthase
β-glucocerobrosidase
UDP-glucose + N-acylsphingosine
glucocerebroside
glucose + ceramide
miglustat
eliglustat
alglucerase
imiglucerase
velaglucerase alfa
taliglucerase
Abcertin®
Fabry disease
lactosylceramide 4-α-galactosyltransferase
α-galactosidase A
galactose
+
lactosylceramide
globotriaosylceramide
galactosylceramide
+
galactose
lucerastat
migalastat
venglustat
agalsidase alfa
agalsidase beta
pegunigalsidase alfa
agalsidase beta biosimilar
Pompe disease
glycogen synthase
acid α-glucosidase
UDP-glucose + (1,4-α-D-glucosyl)n
glycogen
glycogen n-1 + glucose
short hairpin RNA antisense oligonucleotides
alglucosidase alfa
avalglucosidase alfa
ATB200

Figure 3

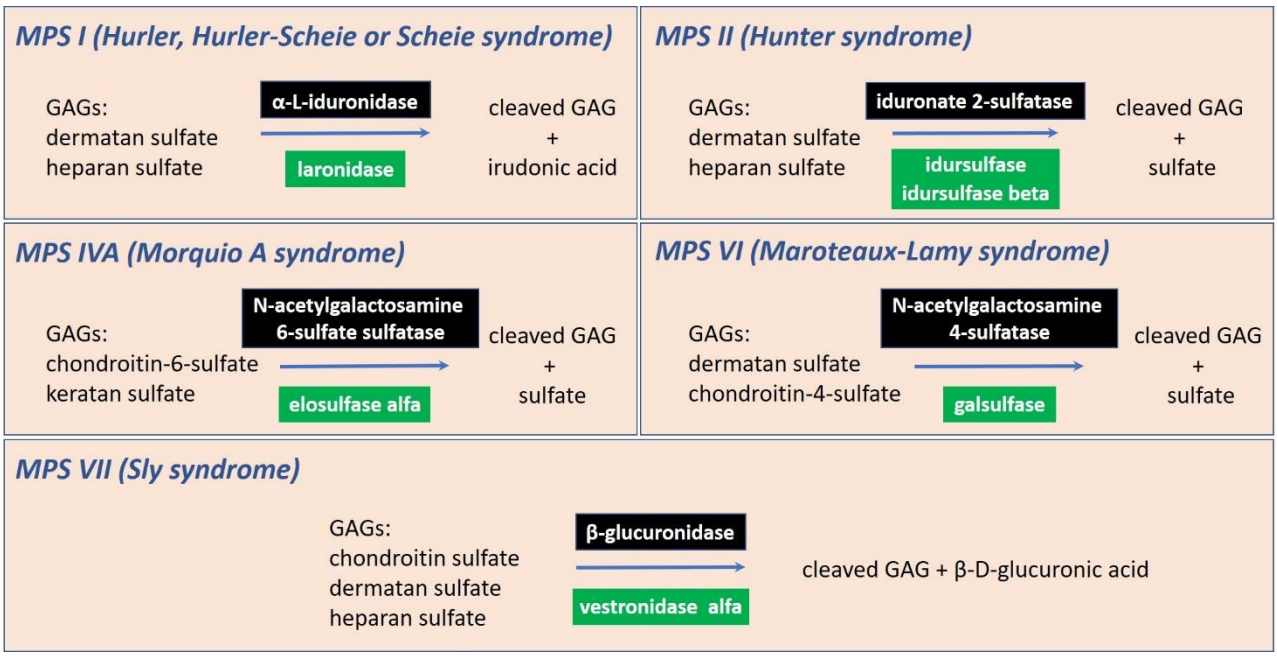

MPS I (Hurler, Hurler-Scheie or Scheie syndrome)
GAGs:
dermatan sulfate
heparan sulfate
α-L-iduronidase
laronidase
cleaved GAG
+
irudonic acid
MPS II (Hunter syndrome)
GAGs:
dermatan sulfate
heparan sulfate
iduronate 2-sulfatase
idursulfase
idursulfase beta
cleaved GAG
+
sulfate
MPS IVA (Morquio A syndrome)
GAGs:
chondroitin-6-sulfate
keratan sulfate
N-acetylgalactosamine
6-sulfate sulfatase
elosulfase alfa
cleaved GAG
+
sulfate
MPS VI (Maroteaux-Lamy syndrome)
GAGs:
dermatan sulfate
chondroitin-4-sulfate
N-acetylgalactosamine
4-sulfatase
galsulfase
cleaved GAG
+
sulfate
MPS VII (Sly syndrome)
GAGs:
chondroitin sulfate
dermatan sulfate
heparan sulfate
β-glucuronidase
vestronidase alfa
cleaved GAG + β-D-glucuronic acid

Figure 4

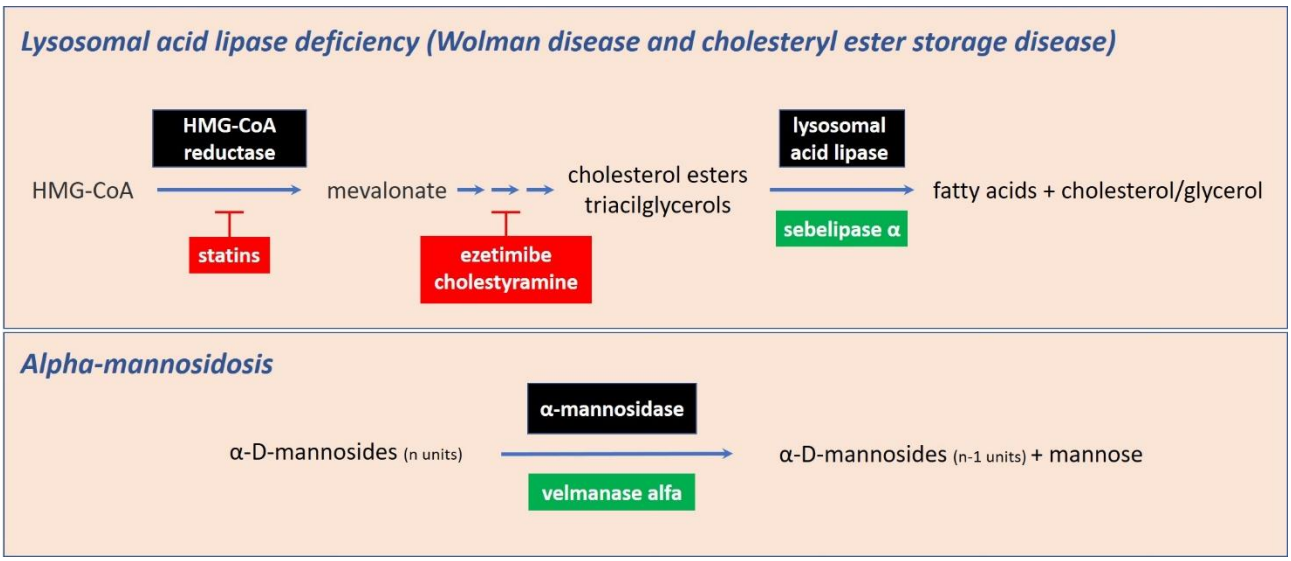

Lysosomal acid lipase deficiency (Wolman disease and cholesteryl ester storage disease)
HMG-CoA reductase
HMG-CoA
mevalonate
cholesterol esters
triacilglycerols
lysosomal acid lipase
fatty acids + cholesterol/glycerol
statins
ezetimibe
cholestyramine
sebelipase α
Alpha-mannosidosis
α-mannosidase
α-D-mannosides (n units)
α-D-mannosides (n-1 units) + mannose
velmanase alfa

Figure 5

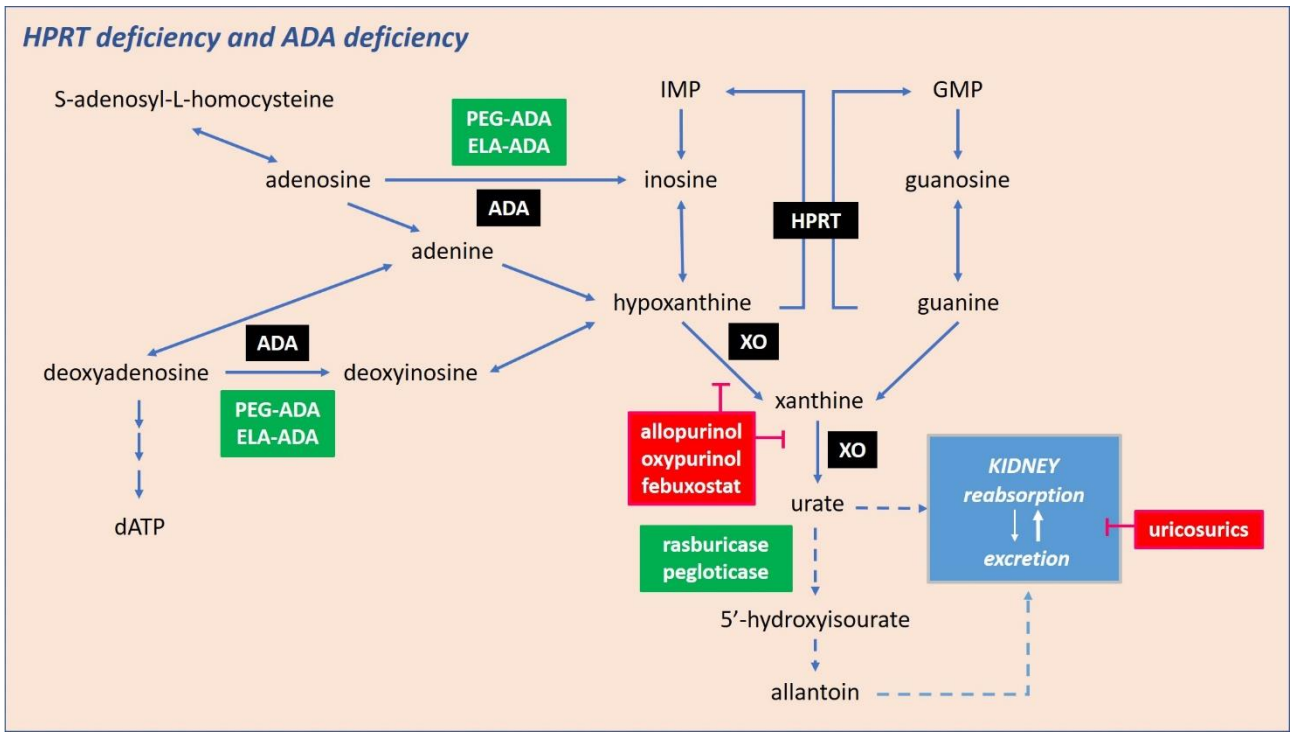
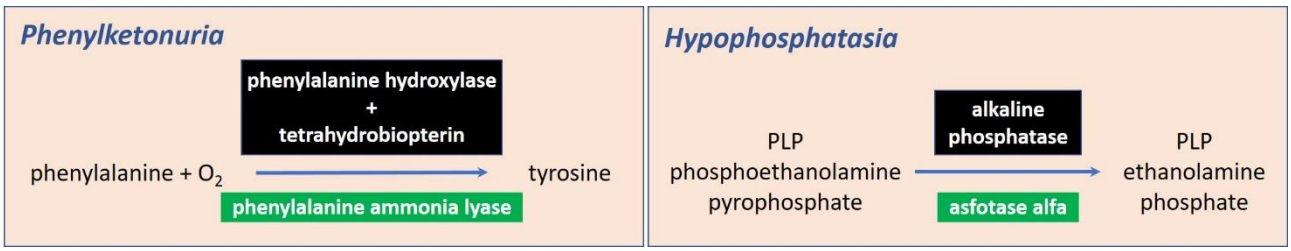

HPRT deficiency and ADA deficiency
S-adenosyl-L-homocysteine
PEG-ADA
ELA-ADA
IMP
GMP
adenosine
inosine
guanosine
ADA
HPRT
adenine
hypoxanthine
guanine
ADA
XO
deoxyadenosine
deoxyinosine
PEG-ADA
ELA-ADA
xanthine
allopurinol
oxypurinol
febuxostat
XO
KIDNEY
reabsorption
excretion
uricosurics
urate
dATP
rasburicase
pegloticase
5'-hydroxyisourate
allantoin

Figure 6


Phenylketonuria
phenylalanine hydroxylase
+
tetrahydrobiopterin
phenylalanine + O_2
tyrosine
phenylalanine ammonia lyase
Hypophosphatasia
alkaline
phosphatase
PLP
phosphoethanolamine
pyrophosphate
PLP
ethanolamine
phosphate
asfotase alfa