EXPERT OPINION ON ORPHAN DRUGS, 2017
VOL. 5, NO. 2, 131–141
http://dx.doi.org/10.1080/21678707.2016.1266933

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REVIEW

Galactosialidosis: historic aspects and overview of investigated and emerging treatment options

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ABSTRACT

Introduction: Galactosialidosis is a glycoprotein storage disease caused by mutations in the *CTSA* gene, encoding lysosomal protective protein/cathepsin A (PPCA). The enzyme's catalytic activity is distinct from its protective function towards β-galactosidase (β-GAL) and neuraminidase 1 (NEU1), with which PPCA forms a complex. In this configuration the two glycosidases acquire their full activity and stability in lysosomes. Deficiency of PPCA results in combined NEU1/β-GAL deficiency. Because of its low incidence, galactosialidosis is considered an orphan disorder with no therapy yet available.

Areas covered: This review gives a historic overview on the discovery of PPCA, which defined galactosialidosis as a new clinical entity; the evidence for the existence of the PPCA/NEU1/β-GAL complex; the clinical forms of galactosialidosis and disease-causing *CTSA* mutations. *Ppca*$^{-/-}$ mice have proven to be a suitable model to test different therapeutic approaches, paving the way for the development of clinical trials for patients with galactosialidosis.

Expert opinion: Improved understanding of the molecular bases of disease has sparked renewed incentive from clinicians and scientists alike to develop therapies for rare conditions, like GS, and has increased the willingness of biotech companies to invest in the manufacturing of new therapeutics. Both ERT and gene therapy may become available to patients in the near future.

ARTICLE HISTORY
Received 12 September 2016
Accepted 28 November 2016

KEYWORDS
PPCA; CTSA; galactosialidosis; therapy; lysosomal storage disease

1. Galactosialidosis and PPCA: historical aspects

Galactosialidosis (GS) was first classified as a variant of GM1-gangliosidosis, a glycosphingolipid storage disease due to an isolated deficiency of the lysosomal β-GAL [1,2]. However, the high residual β-GAL activity measured in GS fibroblasts (~15–20% of control values) could not account for the severe, early onset presentation of the symptoms in patients. Subsequently, the identification by Wenger et al. [3] of undetectable NEU1 activity, in addition to the partial deficiency of β-GAL, in fibroblasts from a patient described as variant of GM1-gangliosidosis defined GS as a separate clinical entity. At that time the disease was thought to be caused by a primary deficiency of NEU1. Yet, co-culturing of fibroblasts with a combined NEU1/β-GAL deficiency with fibroblasts with an isolated NEU1 deficiency partially corrected NEU1 activity in the former cells, raising the possibility that a third gene product was involved in GS. The identity of this 'corrective factor' secreted in the medium and capable of restoring NEU1 and β-GAL activities in GS fibroblasts was eventually discovered fortuitously because of its physical association with β-GAL [4,5]. An antiserum raised against purified β-GAL precipitated this enzyme with three previously unknown proteins of 54-, 32-, 20 kDa from human fibroblasts [5]. Remarkably, these three proteins were absent in fibroblasts of a GS patient with a severe clinical presentation. It was soon apparent that the three proteins were the products of a single gene and that the 54-kDa polypeptide was in fact the uncleaved precursor of the 32- and 20-kDa proteins [5]. The 54-kDa precursor was also the form present extracellularly that once taken up by GS cells restored NEU1 and β-GAL activities and their lysosomal stability. These findings gave the first proof that deficiency of a 'protective protein' secondarily affected both glycosidases, and reinforced the biochemical evidence that the three proteins physically associate to form a multienzyme complex.

This discovery established GS as a disease distinctive from GM1-gangliosidosis and sialidosis. After the cloning of the protective protein cDNA, Galjart et al. [6] demonstrated that some of the early onset cases of GS lacked the protective protein mRNA and identified the homology of the protein with serine proteases. Independently, the group of Erdös [7] confirmed that the protective protein was indeed a serine protease with carboxypeptidase/deamidase/esterase activity (see below). Together, these findings proved unequivocally the nature of the primary defect in GS.

2. GS: clinical phenotypes

GS is a prototypical lysosomal storage disease (LSD) of glycoprotein catabolism, which is inherited as an autosomal recessive trait. The disease is rare, although its prevalence is currently unknown. GS is one of the few LSDs caused by a primary defect in one of the lysosomal cathepsins. However, the secondary severe loss of NEU1 activity probably accounts for most of the overt clinical manifestations seen

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Article highlights

- History of the discovery of galactosialidosis primary defect
- Galactosialidosis clinical phenotypes and *CTSA* mutations
- Protective protein cathepsin A as a multifunctional enzyme
- Mouse models with *CTSA* deficiency
- Experimental and preclinical therapies in galactosialidosis

This box summarizes key points contained in the article.

in patients and for the disease pathogenesis. GS primarily affects cells of the reticuloendothelial system and can be suspected in children with features typical of a lysosomal disorder, such as coarse facies, macular cherry-red spots, vertebral changes, foam cells in the bone marrow, and vacuolated lymphocytes in peripheral blood [8]. Sialyloligosaccharidiuria is diagnostic of the disease but it is indistinguishable from that observed in patients with sialidosis, caused by an isolated deficiency of NEU1 (sialidase). In fact, patients with GS and those with sialidosis share clinical and biochemical features that are attributed at least in part to the loss of NEU1 function in both diseases [8]. Based on the age of onset and the severity of the symptoms, patients with GS are usually classified into three clinical types.

2.1. Early infantile GS

The *early infantile type* of GS presents with signs of the disease between birth and 3 months of age; these include non-immune hydrops fetalis, neonatal edema, coarse facies, inguinal hernias, proteinuria, and telangectasias. In addition, these patients develop visceromegaly, skeletal dysplasia, renal and cardiac failure, and variable neurological involvement. Ocular abnormalities, including corneal clouding and fundal changes (cherry-red spot), and heart involvement with cardiomegaly and thickening of the septum have been seen in a number of patients [8–11]. These very severe patients die within the first year of life likely because of heart and kidney failure [8]. Importantly, early infantile type of GS may be associated with fetal loss.

2.2. Late infantile GS

The *late infantile type* of GS comprises a distinct group of patients characterized by mild or absent cognitive disability. Patients usually present with phenotypic alterations and symptoms within the first 2 years of life and they slowly progress into adulthood. These include coarse facies, hepatosplenomegaly, dysostosis multiplex, especially of the spine, and growth retardation associated with muscular atrophy. Heart involvement, with thickening of the mitral and aortic valves, and hearing loss are recurrent features. Cherry-red spots and corneal clouding can occur in some patients, while seizures and overt neurologic signs are very rare. Many of the patients diagnosed with the late infantile phenotype survive into adulthood and are still alive. Therefore, they may develop additional kidney and pulmonary complications [8,12].

2.3. Juvenile/Adult

The majority of GS patients belong to the *juvenile/adult type*. The reason for the dual designation is that their age of onset and clinical course vary greatly and patients present with a broad and continuous spectrum of severity of their symptoms [8,13–15]. They are mostly of Japanese origin. In contrast to the late infantile types, patients with the juvenile/adult form of GS have a more severe clinical presentation. Besides characteristic features such as coarse facies, vertebral changes, cherry-red spots, and corneal clouding, patients develop severe neurologic manifestations, including myoclonus, cerebellar ataxia, generalized seizures, progressive cognitive impairment, and mental retardation. Absence of visceromegaly, angiokeratoma, and long survival are features frequently observed in this group of patients [8,16].

As it is the case for sialidosis [17], a few examples of atypical patients with confirmed diagnosis of GS, but no oligosaccharidiuria have been reported [18,19]. These findings highlight the fact that GS should be taken into consideration for some of the undiagnosed cases, even in the absence of this diagnostic marker of the disease.

2.4. Disease-causing CTSA mutations

The human *CTSA* locus is situated on chromosome 20q13.1 in a complex genomic region with two overlapping genes both at the 3′ and 5′ end. Using genomic DNA, cDNA, or whole-exome sequencing, a total of 27 mutations have been identified in the *CTSA* gene, including small deletions/insertions, missense mutations, splicing variants, and only one nonsense mutation (refer to http://www.hgmd.cf.ac.uk/ac/index.php and http://www.ncbi.nlm.nih.gov/clinvar) (Table 1). As foreseeable, the most heterogeneous pool of *CTSA* mutations have been found in patients with the severe, early infantile form of GS, although some recurrent mutations seem to point to a founder effect in specific ethnic groups [20–23]. Instead, the group of patients with the late infantile form of the disease is characterized by two recurrent, missense mutations, resulting in two amino acid substitutions, Phe458Val and Tyr267Asn, that appear to be pathognomonic for this clinical phenotype. The extent of severity and type of symptoms in these patients may vary depending on whether these mutations are present in homozygosity or compound heterozygosity [20].

By far, the majority of *CTSA* mutations reported in the literature have been identified in Japanese patients [8,20,21]. These patients share a common single base substitution at the donor splice site of intron 7, causing aberrant splicing of the pre-mRNA and the skipping of exon 7 (SpDEx7) [22]. This mutation apparently segregates with the juvenile/adult type of the disease. However, the patients' clinical presentation may be more or less severe, depending if it occurs in compound heterozygosity with missense mutations found in early infantile cases, or in homozygosity, although exceptions have been reported also within this group of patients.

Table 1. CTSA disease-causing mutations identified in galactosialidosis patients.

Codon number	Type of mutation	Aminoacid change	Reference
37	Deletion	C111delG	[24]
67	Missense	GLN-ARG	[21]
69	Missense	SER-TYR	[25]
83	Missense	TRP-ARG	[21]
95	Deletion	c.284delC	Unidad de Diagnostico y Tratamiento de Errores Congenitos del Metabolismo
103	Missense	GLY-SER	[26]
103	Missense	GLY-VAL	[12]
108	Base substitution	SER-LEU	[21]
116	Missense	HIS-ARG	[23]
150	Missense	VAL-MET	[25]
189	Deletion	c.564delTT	[27]
217	Deletion	C649delC	[28]
254	Missense	LEU-PRO	[25]
259	Missense	CYS-ARG	[23]
267	Missense	TYR-ASN	[25]
296	Deletion	C887_888delAT	[25]
347	Insertion	C904insC	[26]
406	Nonsense	GLN-STOP	[23]
413	Missense	TYR-CYS	[21]
424	Missense	MET-THR	[25]
442	Missense	ARG-TRP	[12]
457	Missense	GLY-SER	[25]
458	Missense	PHE-VAL	[25]
471	Missense	LYS-GLU	[20]
	Splicing	IVS3 ds + 1 G-T	[24]
	Splicing	IVS7 ds + 3 A-G	[22]
	Splicing	IVS8 ds + 9 C-G	[27]

Expression and structural studies have examined the impact of different mutations on the biochemical and structural properties of PPCA [25,29] Recently, using an *in silico* program, Caciotti et al. [23] have predicted the effects of specific *CTSA* mutations on the functional properties of the PPCA protein, proposing this method as a reliable tool for future studies on novel mutations.

In general, we can assert that there is a strong correlation between the type and combination of amino acid substitutions and the clinical outcome. However, we need to keep in mind that other genetic, environmental, and dietary factors could play a crucial role in the expression and penetrance of specific clinical manifestations in patients.

3. PPCA biosynthesis

Human PPCA is synthesized as a 480-amino acid pre-proform containing a canonical hydrophobic signal peptide of ~46 residues and nine cysteines. The signal peptide includes a stretch of leucine repeats that can vary in number from individual to individual, which caused discrepancies in the numbering of the PPCA amino-acids; so a careful assessment of any PPCA amino acid sequence is highly recommended in order to ascertain that the correct number is assigned to individual amino acids, especially when mutations identified in GS patients need to be assigned to the protein sequence (see http://www.hgmd.cf.ac.uk/ac/index.php). After removal of the signal peptide, the precursor is N-linked glycosylated at sites Asn163 and Asn351, and forms a homodimer soon after synthesis in the endoplasmic reticulum. The acquisition of the mannose-6-phosphate recognition marker on the Asn117 oligosaccharide chain allows for the correct routing and lysosomal localization of the PPCA precursor. A small pool of phosphorylated precursor can also be recovered extracellularly, but this secreted form can be re-internalized by cells via mannose-6-phosphate receptor-mediated endocytosis and routed to the lysosomes where it becomes catalytically active as the *de novo* synthesized enzyme [5,6].

In the endosomal/lysosomal compartment the inactive 54 kDa precursor undergoes an initial endoproteolytic cleavage that generates a 34- and 20-kDa inactive, two-chain intermediate. This proteolytic step is followed by the removal of a 2-kDa 'linker' or 'excision' peptide from the C-terminus of the large chain, which ultimately gives rise to the mature and fully active, two-chain enzyme of 32- and 20-kDa, covalently linked by disulfide bridges [30]. Tryptic peptide sequencing of the mature enzyme purified from placenta revealed that the excision peptide encompasses residues Met331-Arg344 [6]. The structural basis of PPCA maturation and catalytic activation has been revealed through the determination of the 3D structure of the precursor and the mature enzyme [29,31]. Maturation of the PPCA precursor can also be recapitulated *in vitro* by limited proteolysis with trypsin (Figure 1) [6].

Mouse PPCA [32] is 87% identical at the amino-acid level to the human counterpart, and shares similar biochemical and kinetic properties. In fact, murine PPCA can substitute for the human enzyme in correcting both NEU1 and β-GAL activities when taken up by patients' fibroblasts [32].

4. PPCA – a serine carboxypeptidase identical to cathepsin A

Analysis of the primary structure of human PPCA revealed strong sequence similarity to serine carboxypeptidases,

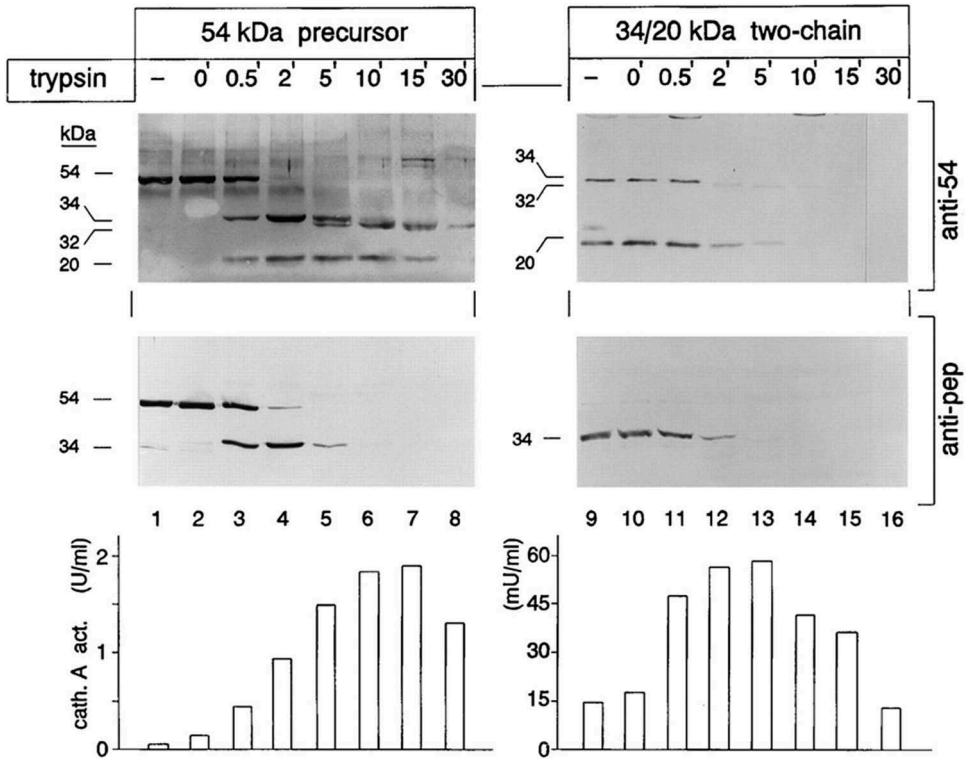

Figure 1. Limited proteolysis with trypsin of 54-kDa precursor and 34- and 20-kDa reconstituted two-chain protein. Aliquots of medium concentrates containing the 54-kDa precursor and 34- and 20-kDa associated protein were incubated at 37°C with 1 mg of trypsin in the presence of bovine serum albumin (1 mg/ml) for the indicated periods of time. Reactions were stopped with 3 mg of trypsin inhibitor. Samples in lanes 1 and 9 were untreated. At time 0 (lanes 2 and 10), the samples were treated with trypsin inhibitor prior to the addition of trypsin. A portion of each sample was separated by SDS-polyacrylamide gel electrophoresis, followed by electroblotting and immunostaining with anti-54 and anti-pep antibodies. Cathepsin A activity toward the acylated dipeptide benzyloxycarbonyl-phenylalanyl-alanine was measured in each aliquot. One milliunit (mU) of activity is defined as the enzyme activity that releases one nanomole of alanine/minute. Adapted from [30] with permission of JBC.

suggesting that the protein had an intrinsic enzymatic activity. By homology with yeast serine carboxypeptidases, the serine in the active site of PPCA was mapped within a domain of six amino acids (GESYA(G)G) found in all serine carboxypeptidases [6]. A second conserved domain of four amino acids (HMVP) is located in the 20-kDa chain and includes His475. It was postulated that this histidine and the aspartic acid at position 418 activate the serine in the active site of the protein; hence, the three amino-acids, Ser196, His475, and Asp418, were proposed to make up the catalytic triad of an active PPCA enzyme [29,31].

A serine carboxypeptidase activity for the mature PPCA was first demonstrated in purified preparations of the complex, using a dipeptide substrate Z-Phe-Leu commonly used for the yeast carboxypeptidase CPY [33]. These authors named the enzyme *carb-L* and showed that it was deficient in GS patients [34]. However, the biochemical properties of PPCA and its substrate specificity closely correlated with those of a previously identified lysosomal carboxypeptidase, cathepsin A. It was soon demonstrated that indeed PPCA and cathepsin A had the same identity and that an antibody against human PPCA immunoprecipitated a cathepsin A activity that was less than 1% of the control values in GS fibroblasts.

5. PPCA, a pleiotropic enzyme

In spite of its proven cathepsin A activity, the enzyme is still referred to as PPCA because catalytic and protective functions are seemingly independent from one another. PPCA mutants generated by substitution of the Ser196 or His475 active site residues loose cathepsin A activity but still retain their ability to interact and 'protect' NEU1 and β-GAL when taken up by GS fibroblasts [35].

In absence of functional PPCA, NEU1 is retained in the endosomal compartment while the pool of β-GAL reaching the lysosomes is rapidly degraded [36]. Formation of the PPCA/NEU1/β-GAL complex [5,6,37–41] has a reciprocal advantage for the three enzymes that in such configuration acquire their active and stable conformation in lysosomes [36]. However, only a fraction of PPCA and β-GAL activities is found in the complex, which instead contains all of the measurable NEU1 catalytic activity, hinting on potential functions of PPCA outside the multienzyme complex. It is nonetheless genetically and biochemically proven that PPCA functions as an indispensable chaperone/auxiliary transport protein, especially for NEU1, that is catalytically inert without PPCA. The two proteins likely interact already in an early biosynthetic compartment. The mode of their interaction is particularly intriguing because amino acid domains within NEU1, which are important for its interaction with PPCA, also serve as binding sites for other NEU1 molecules. Thus, it appears that PPCA prevents self-aggregation of NEU1 molecules, suggesting that heterodimerization between NEU1 and PPCA helps them to acquire the most suitable conformation for proper intra-organellar routing and full activation/stability in lysosomes [42].

Aside from its cathepsin A activity at acidic pH, PPCA also functions as deamidase and esterase at neutral pH. *In vitro*, the enzyme can deamidate selected neuropeptides, such as substance P and neurokinin and can function as carboxypeptidase on oxytocin-free acid, bradykinin, and endothelin I [7,43–48]. All enzymatic activities of PPCA were shown to be drastically reduced in lymphoblastoid cells and fibroblasts from numerous GS patients of Japanese origin [45]. In addition, it was also demonstrated that an enzyme hydrolyzing the C-terminus of endothelin I was deficient in tissues from a GS patient [46], a finding that implicates cathepsin A activity in the degradation of endothelin I in human tissues. A recent study has investigated the impact of cathepsin A upregulation on the murine cardiac proteome, using a mouse model with restricted expression of the protein in cardiomyocytes. Interestingly, increased levels of cathepsin A induced the upregulation of cathepsin B, D, and Z, and the downregulation of numerous protease inhibitors, as well as several antioxidative stress proteins [49]. The same group has further demonstrated that inhibition of cathepsin A activity has cardioprotective properties and proposed the use of this approach for the treatment of heart failure after myocardial infarction [50].

All together, these data suggest that PPCA has pleiotropic activities that may go beyond its lysosomal functions and, if so, may contribute to the complex phenotype of GS patients. An indication that reinforced this notion came from experiments aimed to purify the lysosomal-associated membrane protein 2a (LAMP2a), one of the isoforms of LAMP2 generated through alternative splicing, which is involved in the process of chaperone-mediated autophagy or CMA [51]. CMA entails the chaperone-dependent selection of soluble cytosolic proteins that are destined for degradation in lysosomes. Under specific conditions, mostly associated with cellular stress, cytosolic proteins embedding a specific stretch of amino acids (KFERQ) directly translocate across the lysosomal membrane using LAMP2a as acceptor receptor, and gain access to the lysosomal lumen for degradation. The unique features of this type of autophagy are the selectivity of the protein substrates targeted to lysosomes and their mode of translocation into the organelle that does not require vesicular trafficking [52]. All substrates of CMA contain the KFERQ or KFERQ-like target motif that is recognized by the heat shock protein Hsc70, which, in turn, binds to the cytosolic tail of LAMP2a during the translocation across the lysosomal membrane. It was shown that in lysosomal preparations isolated from rat liver PPCA co-purified with LAMP2a, indicating that cathepsin A alone or in complex controls the turnover of LAMP2a [51]. In fact, a reduction in the rate of LAMP2a degradation was demonstrated in both human and mouse PPCA-deficient fibroblasts, which in contrast displayed increased levels of CMA [51].

6. Mouse models with PPCA deficiency

6.1. PPCA$^{-/-}$ animal model

A mouse homozygous for a null mutation at the *Ppca (Ctsa)* locus was one of the first genetically engineered animal models for an LSD [53]. These mice have a clinical and pathological presentation that closely recapitulates the early onset forms of GS (Figure 2). Tissues and cells isolated from mutant mice have no cathepsin A activity and severe secondary deficiency of Neu1, while β-Gal is partially reduced only in some cell types [53,54]. Extensive morphological changes are recognizable already in the first weeks of life, with severe vacuolation and lysosomal expansion in some cells of most systemic organs and the central and peripheral nervous system. As the disease primarily affects the reticuloendothelial system, pathologic changes are first detected in tissues and organs of epithelial origin. Similarly to patients with the severe form of the disease, mutant mice present with severe early-onset nephropathy, associated with edema and proteinuria, in addition to oligosaccharidurie. Time-dependent splenomegaly and heart involvement are also characteristic features of the disease. Homozygous knockouts are infertile, because of structural changes in the blood–epididymal barrier, resulting in altered sperm motility [55], and have a reduced lifespan of ~6–9 months, although gender differences have been documented. The complete loss of Neu1 activity explains why many phenotypic abnormalities in *Ppca*$^{-/-}$ mice are similar to those seen in the *Neu1*$^{-/-}$ model, although on close examination features that are unique for one or the other disease model have been identified [56]. The most overt difference is seen in the cerebellum; early in life *Ppca*$^{-/-}$ mice acquire acute and progressive ataxia that is associated with regional loss of cerebellar Purkinje cells and impaired motor coordination (Figure 3) [56]. This phenotype is not observed in the *Neu1*$^{-/-}$ mice at least not until the end of their lifespan (5–7 months). Because the expression levels of PPCA in Purkinje cells are greater than those of Neu1, it is possible that these neurons are more sensitive to the loss of cathepsin A activity than of Neu1 activity, but more rigorous testing is needed to corroborate this hypothesis.

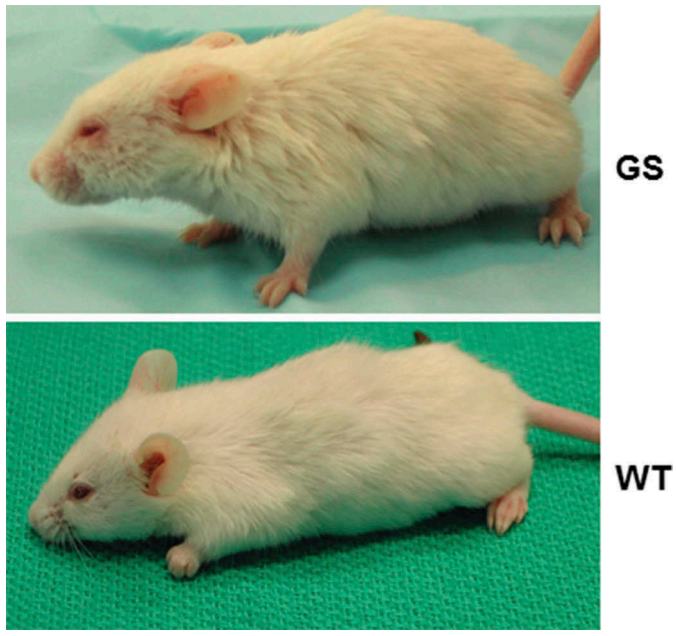

Figure 2. Gross phenotypic appearance of a PPCA$^{-/-}$ mouse at 7 months of age, compared to a wild-type littermate. The affected mouse has a broad face, disheveled coat, and swollen limbs and eyelids. Mouse model was generated in Zhou et al. 1995.

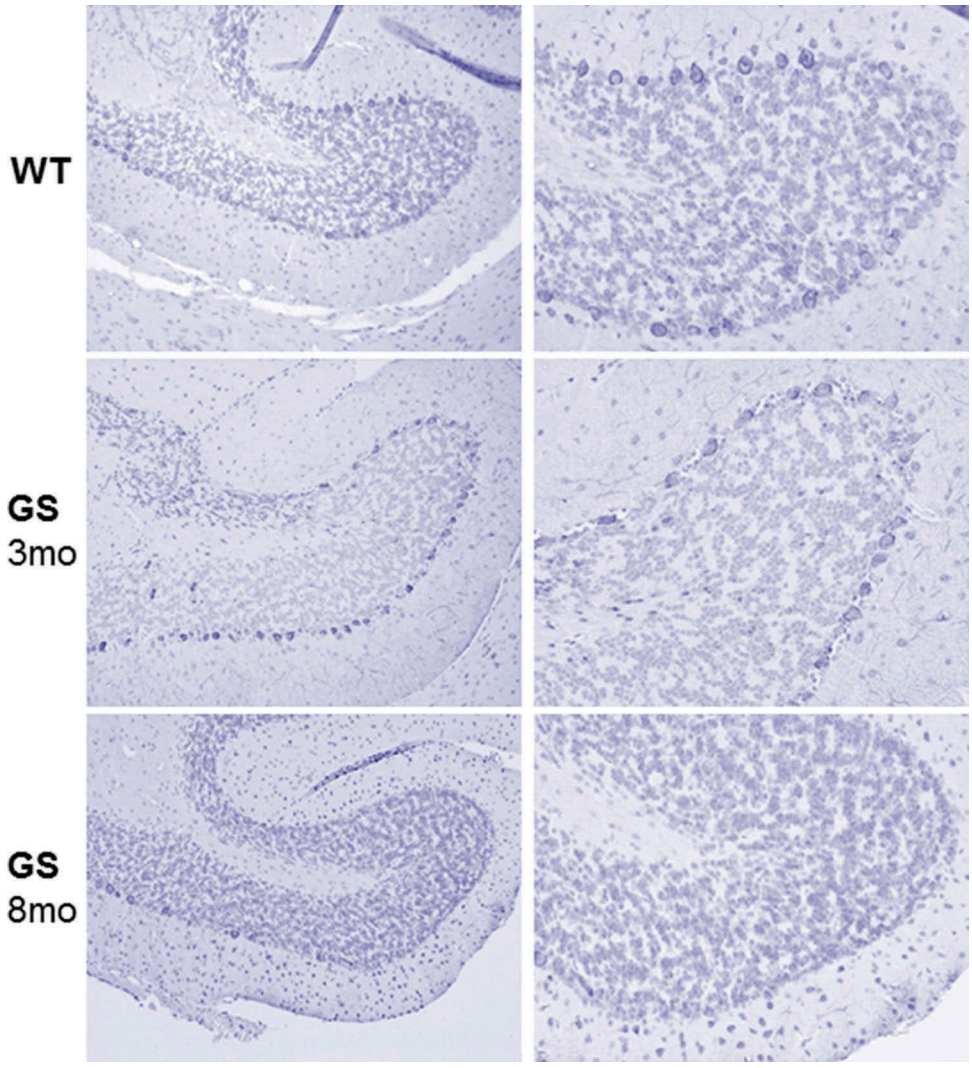

Figure 3. Low magnification images of a cerebellar lobe show progressive loss of Purkinje cells in the *PPCA*$^{-/-}$ mouse. Sections were immunostained with an antibody against the PEP 19 marker. Size bar 100 µm. Adapted with permission from [56].

6.2. *CathA*$^{S190A-Neo}$ animal model

A knock-in mouse model (*CathA*$^{S190A-Neo}$) was generated, using a targeting construct carrying a point mutation that resulted in serine to alanine amino acid substitution at the catalytic site of the Ppca protein, followed by a PGK-Neo cassette inserted in intron 7 of the *Ppca* gene [47]. Although the mutation targeted the *Ppca* locus, the *CathA*$^{S190A-Neo}$ mice displayed a drastically reduced activity of Neu1 in most tissues, due to destabilization of *Ppca* mRNA by the Neo cassette. Contrary to the *Ppca*$^{-/-}$ mice, the *CathA*$^{S190A-Neo}$ mice are apparently vital and fertile, develop normally, and have a normal lifespan, suggesting that 10% NEU1 activity is sufficient to promote normal development and growth. Upon removal of the Neo cassette from intron 7, the same authors successfully generated mice (*CathA*^{S190A}) with an isolated deficiency of cathepsin A, but with intact protective properties toward NEU1; *CathA*^{S190A} mice have normal Neu1 activity. At the histopathological level, these mice show abnormalities in elastin-rich tissues, such as skin, arteries, and lung, and their skin fibroblasts have impaired deposition of insoluble elastin [47]. In addition, *CathA*^{S190A} mice have significantly higher levels of both diastolic and systolic blood pressure than their wild-type siblings. The latter phenotype could be the results of impaired processing of endothelin-1, a potent vasoconstrictive peptide, due to loss of cathepsin A activity. In fact, cultured embryonic brain cells isolated from *CathA*^{S190A} mice secrete higher levels of endothelin-1 and have reduced degradation rate of endothelin-1 in blood and tissues [47]. These results demonstrate that cathepsin A plays an important role in blood pressure regulation and development of the elastic fibers which may influence the complex phenotype observed both in the GS mouse model and GS patients.

7. Investigated and emerging therapies for GS

7.1. Investigated therapies in the GS mouse model

A number of therapeutic approaches have been developed and implemented, using the knockout mouse model of GS. The first experimental therapy was conducted on the newly generated *Ppca*$^{-/-}$ mice. The strategy, which was very innovative at that time, consisted on transplanting mutant mice with bone marrow isolated from transgenic mice, in which the

expression of a human PPCA minigene was driven by the promoter and 'locus control region' of the β-globin gene [53]. These transgenic mice overexpressed the human PPCA exclusively in their erythroid precursors and secreted it abundantly into the circulation. Remarkably, this secreted precursor was present in sufficient amount in the serum of transgenic mice to correct NEU1 and β-GAL activities when taken up by deficient patients' fibroblasts [53]. Transplantation of *Ppca*$^{-/-}$ mice with the transgenic bone marrow led to complete reversal of their systemic pathology, normalization of urinary oligosaccharides and increased cathepsin A and Neu1 activities, although correction of the CNS pathology was only partial [53]. Treated mice lived longer than affected mice. This unexpectedly positive outcome gave the first indication that bone marrow transplantation (BMT) could be beneficial for GS, provided that a compatible allogeneic donor BM is available.

These initial findings spearheaded the testing of a similar therapeutic approach designed to target more efficiently the brain disease. It was hypothesized that BM-derived monocytes, tissue macrophages and microglia would constitute a more appropriate population of cells to be used as the source of the therapeutic enzyme [57]. For this reason, the human colony stimulating factor 1 receptor was chosen to drive expression of the human PPCA restricted to the monocyte/macrophage population of transgenic mice [57]. Bone marrow isolated from these mice and transplanted into the *Ppca*$^{-/-}$ mice afforded widespread correction of the systemic phenotype, indicating that cell-specific overexpression of PPCA had sufficient therapeutic potential. Most importantly, 1-year post-transplantation in the brain of treated mice numerous PPCA-positive, BM-derived repopulating microglia were likely responsible for the amelioration of the brain morphology and the improved motor coordination [57].

Because of the successful outcome of BMT with PPCA overexpressing BM cells, an *ex-vivo* BM-mediated gene therapy strategy was later tested for the treatment of GS. Stable hematopoietic progenitors cells isolated from *Ppca*$^{-/-}$ animals were transduced with a murine stem cell virus (MSCV)-based bicistronic retroviral vector, overexpressing PPCA and the GFP marker, and transplanted back into *Ppca*$^{-/-}$ mice [58]. Transplanted mice showed sustained and long-term expression (up to 10 months) of the transgene, which was accompanied by a marked reduction of disease pathology in all visceral organs (Figure 4) and resolution of the edematous phenotype characteristic of the disease. In the brain, this therapeutic approach resulted in a general improvement of the brain architecture with significant reduction of the number of neural cells containing storage material. In addition, treated mice showed delayed loss of cerebellar Purkinje cells and, in turn, enhanced motor coordination [58].

Together, these results suggest that BM-mediated *ex-vivo* gene therapy could be sought for the treatment of GS.

The beneficial therapeutic effect of targeting monocyte/macrophage cells for reverting or ameliorating the systemic disease in *Ppca*$^{-/-}$ instigated the testing of enzyme replacement therapy (ERT), addressing specifically monocyte/macrophage population of cells, one of the primary affected cells in GS. For this approach, a recombinant human PPCA was generated in insect cells using a baculovirus expression system [59]. Glycoproteins expressed in insect cells carry truncated trimannosyl core glycans, which can bind to cells, such as macrophages, that express the mannose receptor. Insect cell-produced recombinant enzyme can be used effectively to treat LSDs with primary involvement of the reticuloendothelial system like GS. Intravenous administration of either PPCA alone or a combination of PPCA and NEU1, 1-month-old *Ppca* mice resulted in complete correction of the systemic phenotype. Two weeks after ERT, treated mice showed normalized cathepsin A activity in many of the systemic organs and reduction of lysosomal storage (Figure 5) [59].

7.2. Emerging therapies and preclinical trials

In recent years, a preclinical study was designed for the treatment of GS, targeting specifically the late infantile group of patients with the non-neuropathic form of the disease. The strategy was based on the use of a recombinant adeno-associated viral (rAAV) vector expressing human PPCA under the control of a liver-specific promoter (scAAV2/8LP1PPCA) [60]. The assumption was that overexpression of the enzyme restricted to the liver would minimize potential toxicity and still afford correction of systemic disease via sustained secretion of the precursor protein into the circulation followed by its uptake by cells of neighboring or distant organs. A large cohort of 30-day-old *Ppca*$^{-/-}$ mice was tail vain injected with increasing doses (low-, intermediate-, and high dose) of the rAAV and analyzed 4-month after treatment. Regardless of the viral dose used, treated mice appeared indistinguishable from WT mice; they had normal fur, coordination and gait, moved actively, and were no longer edematous. Interestingly, treated mice showed reversal of their infertility because they were able to produce normal size litters. Immunohistochemical and biochemical analysis of tissues showed that the levels of PPCA expression in treated mice correlated well with the dose of administered virus. Increased expression of PPCA was paralleled by rescue of the endogenous activity of Neu1 and normalization of urinary oligosaccharides. The rescue of PPCA and Neu1 activities resulted in a dramatic improvement of tissue architecture in all visceral organs tested. Remarkably, mice injected with intermediate or high dose of rAAV showed normal PPCA expression in the choroid plexus, accompanied by clearance of lysosomal storage [60]. No adverse side effects have been identified and no difference in heart and lung morphology has been observed. So far, all investigative and preclinical therapeutic approaches applied to the GS mouse model have been extremely successful and have encouraged the development of an AAV-mediated gene therapy for the treatment of the mild late infantile group of GS patients with no neuropathic signs. These findings also hold promise for the treatment of GS with an ERT regimen.

8. Expert opinion

PPCA is an unusual lysosomal enzyme, which functions as a molecular chaperone for NEU1 and β-GAL, while maintaining an intrinsic carboxypeptidase/deamidase/esterase activity toward a selected number of bioactive peptides. Its differential

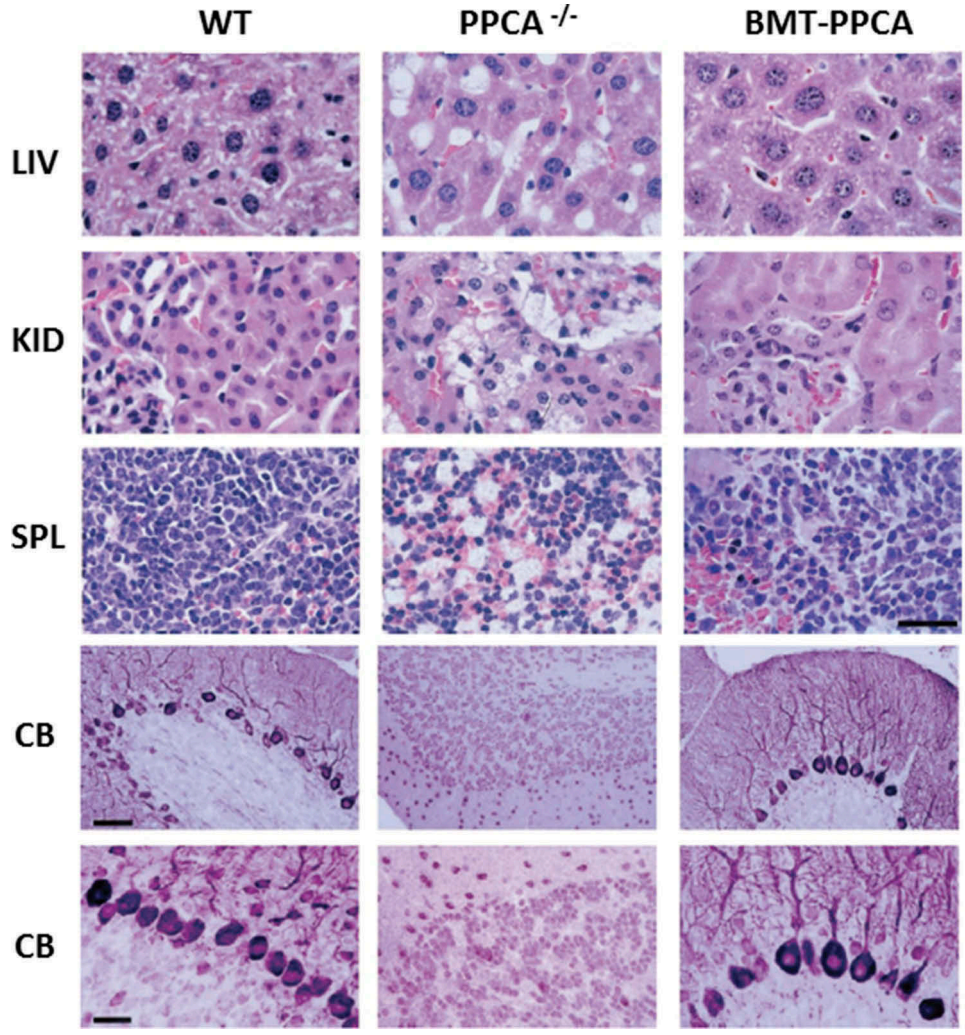

Figure 4. Histology of systemic organs and cerebellum from BM-transplanted GS mice. Top panels: Organs from *PPCA* $^{-/-}$ mice transplanted with total $^{-/-}$ bone marrow (BM) transduced with the Murine Stem Cell Virus-PPCA (MSCV-PPCA); BMT-PPCA retrovirus were isolated at different time points after treatment. Hematoxylin and eosin-stained tissue sections of the liver (LIV), kidney (KID), and spleen (SP) from a BMT-PPCA–treated *PPCA* $^{-/-}$ mouse sacrificed 9 months after treatment, and from age-matched wild-type and *PPCA* $^{-/-}$ mice revealed the complete restoration of normal tissue morphology with BM expressing PPCA, compared to the extensive vacuolation present in the *PPCA* $^{-/-}$ control mouse. Size bar corresponds to 30 μm. Lower panels: Serial sections of the cerebellum from a 9-month-old GS mouse transplanted with MSCV-PPCA–marked BM cells were stained with anti-PEP19 antibody (marker of Purkinje cells). Note the dramatic loss of Purkinje cells in an age-matched GS mouse and the significant number of these cells that are retained in the treated animal. Size bars correspond to 60 μm and 30 μm. Adapted from [58] with permission of the American Society of Hematology.

tissue distribution and level of expression in different cell types likely reflect the physiological need for the protein to either complex with the two glycosidases or to remain a free dimer, depending on the range and type of substrates to be degraded or processed. Obviously, the availability of pools of free and assembled enzymes that are committed to diverse functions and potentially be differentially regulated creates a highly dynamic degradative/processing system. Although, based on current evidence, it is the PPCA's protective function, not its enzymatic activity, at the basis of lysosomal dysfunction in GS patients, the possibility remains that some of the patients' clinical symptoms and complications are related to the loss of cathepsin A activity. Thus, in spite of recent advancements in the field, a full understanding of PPCA's pleiotropic functions awaits the identification of the complete range of substrates that are the physiological targets of this enzyme *in vivo* and could be exploited as prognostic or diagnostic biomarkers.

The availability of a faithful animal model for GS has been instrumental for testing investigative therapies that have been overall very successful in correcting the clinical manifestations characteristic of the disease in patients, including partial reversal of the brain pathology. The favorable outcome of these preclinical studies supports the notion that glycoprotein storage diseases, like GS, are particularly responsive to different types of treatment from ERT to gene therapy. In addition, the biochemical characteristics of PPCA, its long half life as secreted inactive precursor (zymogen) in circulation, its efficient internalization from cells at multiple sites and its scalable purification make it an extremely versatile therapeutic product. Based on these considerations and the renewed interest of pharmaceutical companies to develop therapy for orphan diseases, it is foreseeable that a clinical therapeutic protocol for GS will become available in the near future with a good chance of being successful.

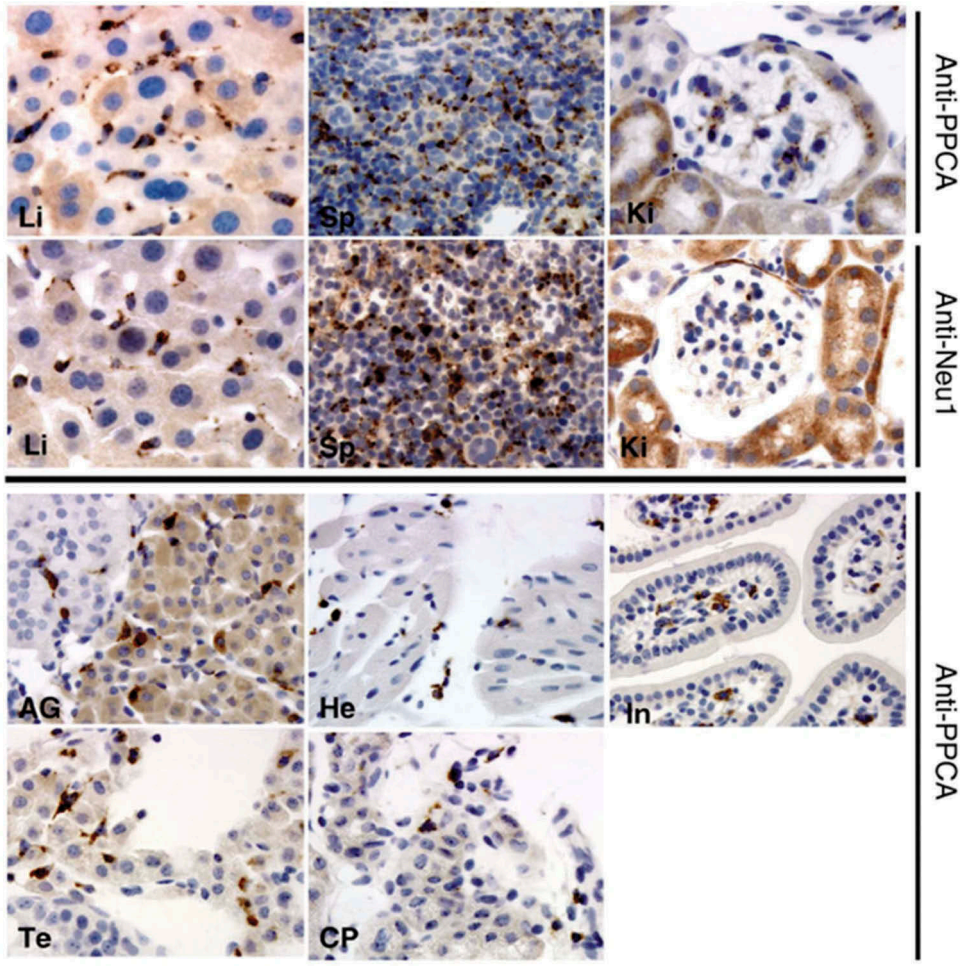

Figure 5. Immunostaining of tissue sections from mice after dual-enzyme replacement therapy (ERT). (a) Numerous cells expressing both PPCA and Neu1 were detected with anti-PPCA and anti-Neu1 antibodies in liver (Li), spleen (Sp), and kidney (Ki). (b) Cells expressing only PPCA were detected in adrenal gland (AG), heart (He), intestine (In), testis (Te), and choroid plexus (CP). Punctuated staining demonstrates internalization of corrective enzyme mostly by Kuppfer cells in the liver and resident macrophages in other tissues. Magnification = x400. Adapted from [59] with permission of FASEB.

Acknowledgments

We apologize if we have omitted some of the outstanding contributions to this field of research because of space constraints.

Funding

This work was funded in part by NIH [Grant Numbers: RO1DK095169 and NIH RO1GM104981], the Assisi Foundation of Memphis, Ultragenyx Pharmaceutical and the American Lebanese Syrian Associated Charities (ALSAC).

Declaration of interest

A.d'Azzo holds the Jewelers for Children Endowed Chair in Genetics and Gene Therapy. The authors have no other relevant affiliations or financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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