

Glycosaminoglycans and mucopolysaccharidosis type III

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1. ABSTRACT

Mucopolysaccharidosis type III (MPS III), or Sanfilippo syndrome, is a lysosomal storage disease in which heparan sulfate is accumulated in lysosomes, as well as outside of cells, as the primary storage material. This disease is a complex of four conditions caused by dysfunctions of one of genes coding for lysosomal enzymes involved in degradation of heparan sulfate: *SGSH* (coding for heparan N-sulfatase) – causing MPS IIIA, *NAGLU* (coding for alpha-N-acetylglucosaminidase) - causing MPS IIIB, *HGSNAT* (coding for acetyl CoA alpha-glucosaminide acetyltransferase) - causing MPS IIIC, and *GNS* (coding for N-acetylglucosamine-6-sulfatase) – causing MPS IIID. The primary storage is responsible for some disease symptoms, but other arise as a result of secondary storage, including glycosphingolipids, and subsequent processes, like oxidative stress and neuroinflammation. Central nervous system is predominantly affected in all subtypes of MPS III. Heparan sulfate and its derivatives are the most commonly used biomarkers for diagnosis and prediction procedures. Currently, there is no therapy for Sanfilippo syndrome, however, clinical trials are ongoing for enzyme replacement therapy, gene therapy and substrate reduction therapy (particularly gene expression-targeted isoflavone therapy).

2. INTRODUCTION

Mucopolysaccharidosis type III (or MPS III), known also as Sanfilippo syndrome, is an inherited

lysosomal storage disease (LSD, for basic reviews, see refs. 1-3). As in all other mucopolysaccharidoses, glycosaminoglycan (GAG) storage is the primary cause of the disease. In MPS III, the only primarily accumulated GAG is heparan sulfate (HS). This compound is a polysaccharide, composed of variably sulfated repeating disaccharide unit (Figure 1). β -D-Glucuronic acid (GlcA) linked to N-acetylglucosamine (2-deoxy-2-acetamido- α -D-glucopyranosyl, GlcNAc) form the most common disaccharide unit in HS, making up about 50% of all disaccharide units in this GAG. Other carbohydrates that occur in HS are: α -L-iduronic acid (IdoA), 2-O-sulfo- α -L-iduronic acid (IdoA(2S)), 2-deoxy-2-sulfamido- α -D-glucopyranosyl (GlcNS), and 2-deoxy-2-sulfamido- α -D-glucopyranosyl-6-O-sulfate (GlcNS(6S)) (Figure 1).

Sanfilippo disease is, in fact, a common name for four different genetic disorders, caused by dysfunctions of four different genes, each coding for an enzyme involved in degradation of HS. Therefore, four different subtypes of the disease are distinguished, depending on the particular enzymatic deficiency: heparan N-sulfatase (*SGSH*) in MPS IIIA (OMIM #252900), alpha-N-acetylglucosaminidase (*NAGLU*) in MPS IIIB (OMIM #252920), acetyl CoA alpha-glucosaminide acetyltransferase (*HGSNAT*) in MPS IIIC (OMIM #252930), and N-acetylglucosamine-6-sulfatase

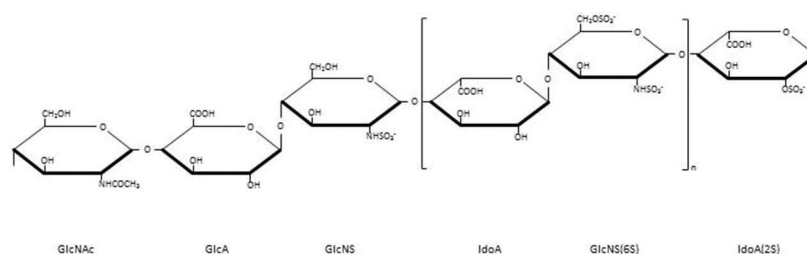


Figure 1. Structures of the most common disaccharide unit in heparan sulfate (HS), β -D-Glucuronic acid (GlcA) linked to *N*-acetylglucosamine (2-deoxy-2-acetamido- α -D-glucopyranosyl, GlcNAc), and other carbohydrates that occur in HS, α -L-iduronic acid (IdoA), 2-O-sulfo- α -L-iduronic acid (IdoA(2S)), 2-deoxy-2-sulfamido- α -D-glucopyranosyl (GlcNS), and 2-deoxy-2-sulfamido- α -D-glucopyranosyl-6-O-sulfate (GlcNS(6S)).

Table 1. Birth prevalence of MPS III

Region	MPS III incidence (per 100,000 live births)	Reference
USA (Utah)	0.5-1	(103)
Norway	0.27	(104)
British Colombia	0.28	(105)
Northern Ireland	0.36	(106)
Taiwan	0.39	(107)
Denmark	0.43	(104)
Sweden	0.67	(104)
France	0.68	(108)
Northern Portugal	0.84	(109)
Poland	0.86	(110)
Czech Republic	0.91	(111)
Greece	0.93	(108)
UK	1.11	(108)
Australia	1.37	(112)
Germany	1.57	(113)
The Netherlands	1.89	(114)

(GNS) in MPS IIID (OMIM #252940). A scheme for HS degradation, with indication of enzymes acting at particular stages and corresponding MPS III subtypes if activity of one of them is absent or severely decreased, is presented in Figure 2.

All four genes coding for enzymes defective in Sanfilippo disease are located on autosomes, thus, all subtypes of MPS III are inherited in the autosomal recessive manner, with male and female patients occurring in roughly equal frequency. The prevalence of this disease is roughly estimated as 1 per 100,000 live births, but it may differ in various geographical regions (Table 1). In every Sanfilippo disease subtype, various types of mutations can be found, including missense/nonsense substitutions, splicing substitutions, small

deletions/insertions/indels, large deletion/insertions/duplications, and complex rearrangements (Table 2).

3. CLINICAL PRESENTATION

Although the four subtypes of MPS III arise from dysfunction of different genes and enzymes, symptoms of patients suffering from each subtype are similar. Therefore, clinical presentations of Sanfilippo disease is usually described together for A-D subtypes (1-3).

The major symptoms of Sanfilippo disease are cognitive and neurological defects. Among them, developmental delay, cognitive decline, lack of speech or speech delay, sleep disturbances, seizures, hyperactivity, and aggression-like behavior are the most commonly occurring abnormalities. Somatic symptoms also occur in MPS III, however, they are less severe than in other types of mucopolysaccharidoses and appear very heterogeneous among patients. Mild facial dysmorphism may include a dolichocephalic skull shape with a short forehead, prominent eyebrows, an everted and thick lower lip, and an upturned upper lip with a protruding philtrum. Hirsutism, a low hair line, and very coarse, stiff hair are often present, and characteristic changes in hair morphology have been documented (4, 5). Some, but not all, patients display hepatomegaly, while splenomegaly occurs rarely. On the other hand, frequent ear and respiratory infections are common. Later in the course of the disease, some orthopedic syndromes may occur, like scoliosis, kyphosis, lumbar lordosis, hip dysplasia, and carpal tunnel syndrome.

MPS III patients appear normal at birth, and the first symptoms are recognized usually at the ages between 2 and 6 years. Their life span is, on average, between 2 and 3 decades, though some patients can survive longer (6-9).

It is possible to distinguish 3 phases of Sanfilippo disease, after several months or a few years of the lack of any specific symptoms (10). The first phase is characterized by slower or halted cognitive development, with speech deterioration or deficiency as

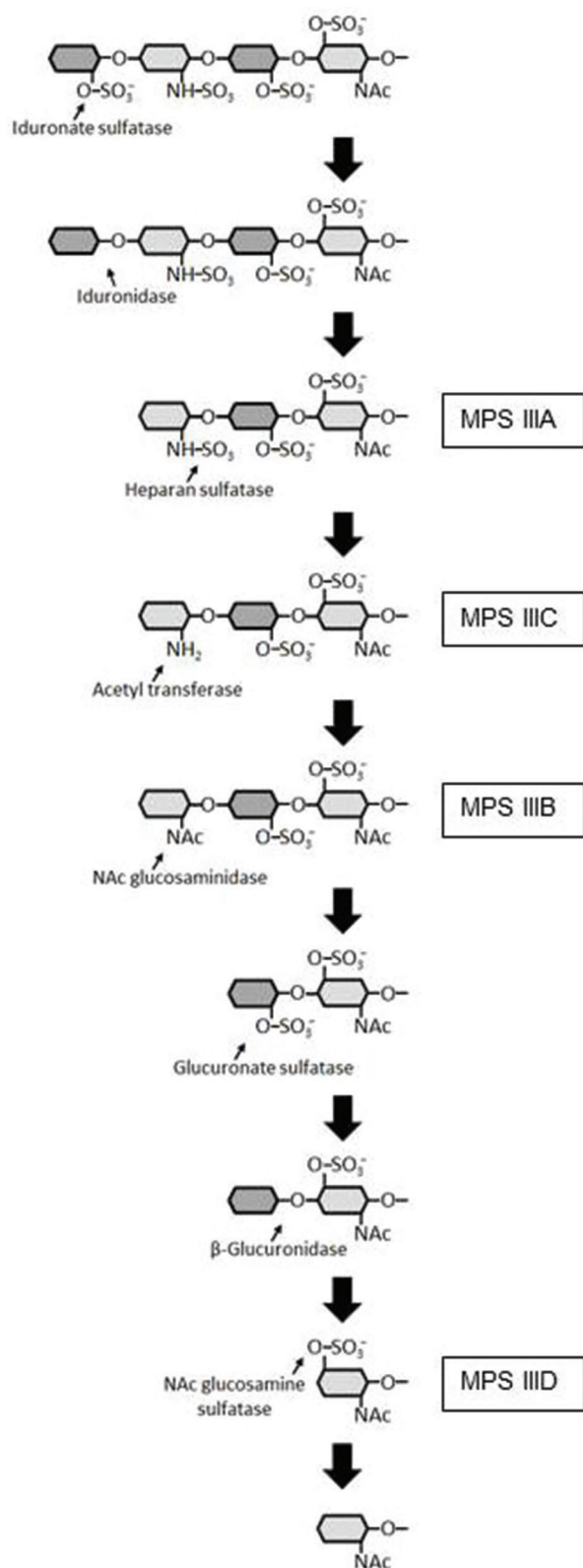


Figure 2. A scheme for heparan sulfate degradation, with indication of enzymes acting at particular stages and corresponding MPS III subtypes, caused by their dysfunctions.

the most severe sign. Although physical development is usually still normal at this stage of the disease, some behavioral problems may appear. The second phase emerges as progressive cognitive deterioration, sleeping disturbances (some patients may sleep as little as 2-3 hours per night), hyperactivity and extreme behavioral problems (impulsivity to such extent that patients have little or no regard for their own safety, aggression-like behavior which arises from hyperactivity rather than from the will to hurt other persons or animals, autistic behavior, and excessive anxiety). The third phase starts with motor functions declination which is followed by disappearance of behavioral problems when patients lose locomotion. Then, severe dementia, spasticity and swallowing difficulties appear. Patients die usually between second and third decade of life.

Recent years revealed some further interesting details regarding clinical presentation of MPS III patients. Patients suffering from Sanfilippo disease were compared to those with non-metabolic intellectual disabilities. Fewer behavioral difficulties as children aged, and more severe level of cognitive deficiency were found in the former group (11), indicating differences between the symptoms depending on their cause. Nevertheless, it is important to underline the variability in severity of symptoms occurring in MPS III patients. In some of them, only moderate mental retardation can be observed even at the end of the third decade of life (12). Sometimes the symptoms might be so uncharacteristic that Sanfilippo disease is misdiagnosed as idiopathic developmental delay, attention deficit/hyperactivity disorder (ADHD) or autism (2, 13-17). This concerns usually the first phase of the disease, as in the second and third phase the symptoms become more specific (18).

Detailed investigations of certain patients suffering from Sanfilippo disease indicated that some previously overlooked symptoms may be characteristic for this disorder. These include dental findings, like obliterated pulp chambers and root canals (19), osteonecrosis of the femoral head and hip dysplasia (20), precocious puberty in boys (21), and tachypnea (22). It is also important to mention that the use of advanced methods allowed to indicate specific changes in the brain, particularly diffuse hypomyelination with thinning of the corpus callosum, revealed by magnetic resonance imaging (23), to assess the sleep problems more precisely (24), to demonstrate compromised blood-brain-barrier integrity (25), and to find a significantly stunted growth (26) of MPS III patients.

Specific molecular mechanisms of diseases caused by mutations in a single gene can be understood on the basis on detailed genetic and biochemical studies. An example of such studies is a recent report demonstrating the crystal structure of glycosylated heparan N-sulfatase (also called N-sulfoglucosamine sulfohydrolase or

Table 2. Numbers of different mutation types responsible for causing four subtypes of Sanfilippo disease present in HGMD

Mutation type	Number of mutations			
	MPS IIIA (<i>SGSH</i>)	MPS IIIB (<i>NAGLU</i>)	MPS IIIC (<i>HGSNAT</i>)	MPS IIID (<i>GNS</i>)
Missense/nonsense substitutions	109	105	37	7
Splicing substitutions	2	5	14	3
Small (≤ 20 bp) deletions	17	23	5	4
Small (≤ 20 bp) insertions	9	13	5	4
Small (≤ 20 bp) indels	1	1	1	1
Large (> 20 bp) deletions	3	4	2	2
Large (> 20 bp) insertions/duplications	1	3	1	0
Complex rearrangements	0	0	1	2
Total	142	154	66	23
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SGSH) at 2 Å resolution (27). This enzyme is deficient in MPS IIIA, and the resolved structure provided insight into effects of pathogenic mutations in the *SGSH* gene on possible reasons of impaired activity and/or stability of its product.

Important information about a genetic disease pathomechanism can be obtained in studies on animal models, if they are available. Recent years did bring several interesting studies on mouse models of different subtypes of Sanfilippo disease. A novel MPS IIIA mouse model has been constructed, and female mutant homozygous mice were found to be hyperactive, to have a longer path length, to display rapid exploratory behavior, to spend less time immobile, and to display a reduced sense of danger than female wild-type animals (28). Intriguingly, no significant differences could be detected between male MPS IIIA and wild-type mice (28), which is in contrast to the lack of considerable differences between symptoms of male and female human MPS IIIA patients. Hyperactivity was also observed in the mouse model of MPS IIIB, however, the importance of choosing appropriate test has also been indicated (29).

The mechanism of impaired neuronal communication was studied with the use of adrenal chromaffin cells of MPS IIIA mice (30). In the affected cells, there were increased numbers of large and/or elongated chromaffin granules, resembling immature secretory granules, relative to wild-type cells. The number of exocytotic events was also reduced in MPS IIIA cells when compared to healthy one. These results suggested that the biogenesis and/or the cell surface docking and fusion potential of the vesicles is impaired in MPS IIIA which might contribute significantly to neuropathology (30).

Neuropathology was also evident in MPS IIIB mice, in both central and peripheral nervous systems (31). Regarding the latter system, lysosomal storage pathology was found in dorsal root ganglia affecting neurons, satellite cells, and Schwann cells. Moreover, MPS IIIB mice developed progressive impairments in sensory functions, with significantly reduced response to pain stimulation. Therefore, HS storage likely affects both afferent and efferent neural signal transduction (31). In accordance to this, neuroinflammation and neurodegeneration was evident in mucopolysaccharidosis III type C mouse model (32). Interestingly, mitochondrial defects could be found in brain neurons. These cells contained enlarged, structurally abnormal mitochondria and revealed impaired mitochondrial energy metabolism (32).

4. THE PRIMARY AND SECONDARY STORAGE IN MUCOPOLYSACCHARIDOSIS TYPE III, AND THE DISEASE MARKERS

Since MPS III is caused by deficiency in activity of one of enzymes involved in HS degradation (Figure 2), this GAG has been considered as the primary storage material. Indeed, significantly increased levels of HS in urine and plasma is routinely detected in patients suffering from Sanfilippo disease (1-3). The primary storage of HS was confirmed experimentally in MPS IIIB mouse model (33) and in MPS III patients (34).

To understand the molecular pathomechanism of MPS III, it is necessary to consider not only primary, but also secondary storage products, as well as their biochemical and cellular effects on various processes. In fact, we are now convinced that the mechanisms of mucopolysaccharidoses, including Sanfilippo disease, are much more complex than just accumulation of GAG(s) in lysosomes, dysfunction of these organelles

and then dysfunction of cells, as it was presumed when the primary causes of these disorders were recognized. It is well established that in MPS III, secondary storage of gangliosides, ceramides, galatosylceramides and sphingomyelin occurs in central nervous system. Recent work on the MPS IIIA mouse model indicated that the storage (both primary and secondary) occurs in both lysosomal and non-lysosomal compartments in the brain (35). The primary and secondary storage leads to characteristic cellular and physiological changes, like neuroinflammation, decreased and mislocalized synaptic vesicle associated membrane protein, VAMP2, and decreased post-synaptic protein, Homer-1 (36).

Since HS is accumulated both in lysosomes and outside the MPS III cells, the important question appeared whether extra-lysosomal storage can cause specific disturbance in cell physiology. Recent studies indicated that exposure of wild-type neural cells to exogenous soluble HS fragments activated integrin-based focal adhesions, which attach cells to the extracellular matrix (37). Defective cell polarisation and oriented migration in response to focal extracellular stimuli in affected cells were observed (37). Therefore, undegraded HS can not only affect cell functions due to its storage in lysosomes, but also influence cell physiology when present outside the cell. Microglial activation, neurodegeneration, and oxidative stress are other effects of the storage (38). It was demonstrated that HS activates microglial cells through toll-like receptor 4, and triggers neuroinflammation. However, the oxidative stress occurs in the brain of MPS IIIB mice independently from the neuroinflammation (38).

It is always the question if the findings in studies on mice reflect precisely the disease mechanisms occurring in humans. Therefore, development of other disease models is important to see if the observed effects are general or species-specific. The MPS IIIB emu model has been described recently (39). Importantly, it revealed the symptoms, as well as biochemical and cytological changes, very similar to those observed in mice and humans (39). This indicates that the disease mechanisms investigated on animal models of MPS III may be common also for humans.

Another important question is if the level of storage reflects the disease severity. If so, such parameter could be used for prediction of the course of MPS III and to monitor tested therapeutic methods. In the study with MPS III patients, it was demonstrated that plasma HS and urinary GAG levels were linearly associated with both an increased risk of loss of speech and loss of walking (40). Such a relationship was further confirmed in another study (12), indicating that HS levels in plasma and urine might be an appropriate biochemical markers in MPS III. Although other Sanfilippo disease markers have been proposed, including specific proteins, changes in

hair morphology assessed by electron microscopy, and disability, behavioral and cognitive tests (5, 41, 42), it appears that HS and its derivatives are optimal and the most useful biomarkers. Levels of this GAG, measured in dried blood spots, was suggested as a useful method for MPS III newborn screening (34).

To use HS as a biomarker, precise determination of its concentration in plasma and/or urine is necessary. Therefore, various approaches were reported to obtain precise and reproducible results when estimating HS levels in samples from MPS III patients. A liquid chromatography/tandem mass spectrometry method for determination and quantification of predominant disaccharide GAG units has been published recently (43). In fact, various modifications of modern biochemical and biophysical methods, allowing for accurate measurement of the amount of HS and its derivatives in tested samples, have been reported (44-53). Such methods can be extremely useful in both diagnostic procedures and in assessment of efficacy of various therapeutic approaches.

Although some differences between HS levels in different subtypes of MPS III have been reported (51), it appears that these differences are not significant, and that they result from kinds of mutations and/or rate of GAG synthesis rather than from particular disease subtype. On the other hand, concentrations of HS in urine and plasma decrease in age (44), thus, appropriate age-related reference values should be used when testing samples from patients.

The summary of recommendations for laboratory diagnosis of Sanfilippo disease has been presented recently. Determination of GAG, or more precisely – HS, levels was indicated as the first stage, followed by estimation of the enzyme activity and molecular tests to find mutations (54). Characterization of mutations in the SGSH gene, coding for N-sulfoglucosamine sulfohydrolase (an enzyme which is deficient in MPS IIIA), may be facilitated by a multiparametric computational algorithm for comprehensive assessment of genetic mutations in Sanfilippo syndrome type IIIA (55).

5. POTENTIAL THERAPEUTIC OPTIONS FOR SANFILIPPO DISEASE

Until now, no specific treatment has been registered for Sanfilippo disease. Nevertheless, there are several potential therapeutic options which are being investigated, and clinical trials for some of them were performed or are ongoing. MPS III is a special kind of mucopolysaccharidosis in which central nervous systems is mainly affected. Therefore, enzyme replacement therapy, which is currently used for treatment of MPS I, MPS II, MPS IVA and MPS VI, is considered problematic,

as intravenously administered recombinant enzyme cannot cross the blood-brain-barrier efficiently (3). Below, the most promising and/or probable potential therapies for MPS III are discussed briefly.

5.1. Enzyme replacement therapy

Enzyme replacement therapy (ERT) is based on delivery of an active, recombinant enzyme, which is lacking or deficient in patient's cells, into the body. The most common method for such delivery is intravenous injection. Although it works for MPS I, MPS II, MPS IVA and MPS VI, Sanfilippo disease is difficult to treat in this way, as somatic symptoms in this disorder are relatively mild, and the enzyme is not able to cross the blood-brain-barrier effectively (3). Nevertheless, attempts to use modified ERTs for MPS III are still ongoing.

One approach to delivery of the enzyme to the central nervous system is intrathecal, rather than intravenous, injection. Such a procedure was confirmed to be safe, when used for 6 months for recombinant human heparan N-sulfatase administration, in studies on cynomolgus monkeys (56). Then, the outcomes of repeated intrathecal injection of the recombinant human enzyme on pathological changes in the MPS IIIA dog brain were compared with those in animals treated via intra-cisternal or ventricular routes (57). It was found that the intra-spinal injection allowed the enzyme to penetrate into the brain. Moreover, a significant reduction in the primary substrate accumulation, as well as the secondary pathology, was observed in the MPS IIIA dog brain (57). The effect of the site of injection on the treatment efficacy was also studied on the mouse model of MPS IIIA. Ventricular and cisternal injection resulted in the enzyme delivery to brain and spinal cord regions. On the other hand, lumbar infusion allowed for a more restricted enzyme delivery (58). The problem is that while the ventricular route was the most effective, it is also the most invasive of the three methods studied. Experiments with the mouse model of MPS IIIA indicated that effectiveness of intra-cisternal cerebrospinal fluid enzyme replacement therapy strongly depends on the time of treatment initiation, with far the best results obtained when early intervention was performed (59). Currently, a clinical trial (extension of phase I/II), devoted to evaluation of administration of recombinant human heparan N-sulfatase (sulfamidase) in patients with Sanfilippo syndrome type A (MPS IIIA) is being conducted by the Shire company (clinical trial no. NCT01299727 or HGT-SAN-055; reference as on June 27, 2015: <https://clinicaltrials.gov/ct2/show/NCT01299727?term=sanfilippo&rank=1>).

Another possibility to deliver the enzyme to brain is construction of a fusion protein, composed of the enzyme and a factor which is actively transported through the blood-brain-barrier. A chimeric heparan N-sulfatase, obtained as a fusion of this enzyme with the signal peptide from iduronate-2-sulphatase

and the blood-brain barrier-binding domain from the apolipoprotein B (ApoB-BD), has been constructed (60). Different strategy was used for more efficient transfer of α -N-acetylglucosaminidase, an enzyme deficient in MPS IIIB, to cells. The recombinant enzyme was fused to the receptor-binding motif of insulin-like growth factor 2 to enhance its ability to enter cells using the cation-independent mannose 6-phosphate receptor. The fusion protein revealed an enhanced cellular uptake by MPS IIIB fibroblasts while maintaining the enzymatic activity (61). In subsequent studies, to bypass the blood-brain barrier, the fusion protein, in artificial cerebrospinal fluid, was administered intracerebroventricularly to the brain of MPS IIIB mice. Marked uptake of the administered enzyme was observed in many parts of the brain. Heparan sulfate was reduced to control level, and secondary storage of various compounds was decreased significantly (62).

5.2. Gene therapy

Since all types of Sanfilippo syndrome - like other mucopolysaccharidoses, and more generally, all lysosomal storage diseases – are monogenic disorders, gene therapy appears to be a very attractive therapeutic option. Nevertheless, although very promising results were obtained in studies on cellular and animal models, this approach is still experimental. On the other hand, first results of clinical trials with MPS III patients are already available. An excellent overview on the current stage of gene therapy for LSDs has been published recently (63), thus, in this article we will focus only on recent studies devoted to this kind of treatment of Sanfilippo disease.

The proof of concept of gene therapy for Sanfilippo syndrome type A was demonstrated in studies on MPS IIIA mice. After the systemic administration of an adeno-associated virus 9 vector bearing the sulfamidase gene, *SGSH*, under the control of a ubiquitous promoter, it was possible to obtain its expression in brain and in peripheral organs. A decrease in GAG storage in the brain and normalization of GAG levels in peripheral organs was observed. Moreover, the resolution of neuroinflammation and prolongation of survival of treated mice were evident (64). In subsequent studies, an AAV2/8 viral vector carrying the gene coding for a fusion protein consisting of heparan N-sulfatase, the signal peptide from iduronate-2-sulphatase, and the blood-brain barrier-binding domain from the apolipoprotein B (ApoB-BD), was injected to adult MPS IIIA mice (60). Efficient blood-brain-barrier transcytosis and restoration of heparan N-sulfatase activity in the brain of treated mice were demonstrated. Moreover, this treatment resulted in an improvement of brain pathology and recovery of a normal behavior of the animals (60). In studies on MPS IIIB mice, the animals were treated with intracranial AAV2/5-based vector bearing the *NAGLU* gene, intravenous lentiviral vector bearing *NAGLU* or the combination of both. All treatments resulted in significant improvements in motor function and hearing, as well as in a significantly

increased median life span (65). Other results suggested that targeting both the systemic and central nervous system disease of MPS IIIB early in life appears to be the most efficacious approach (65). The next step of research on MPS IIIB gene therapy was administration of the rAAV9-CMV vector bearing the human *NAGLU* gene (rAAV9-CMV-hNAGLU) to cynomolgus monkeys. Those studies demonstrated an effective and safe profile for systemic rAAV9-CMV-hNAGLU vector delivery in nonhuman primates (66).

The first clinical trial (phase I/II) of a gene therapy for Sanfilippo disease was performed with of an adeno-associated viral vector bearing the *SGSH* gene (67). Four MPS IIIA children received intracerebral injections. The vector was delivered bilaterally to the white matter anterior, medial, and posterior to the basal ganglia. Safety data indicated good tolerance, absence of adverse events related to the injected product, no increase in the number of infectious events, and no biological sign of toxicity related to immunosuppressive drugs. The clinical improvement of patients was, however, moderate (67). This discrepancy with results obtained in analogous studies conducted with MPS IIIA mice (64) might result from differences between murine and human metabolism, more efficient penetration of the enzyme in mice due to significantly smaller size of murine brain relative to the human organ, differences in the disease stages of investigated organisms at the time of the treatment initiation, or combination of these reasons. Next clinical trials with gene therapy for Sanfilippo disease have been announced (references as on January 25, 2016: <https://clinicaltrials.gov/ct2/show/NCT01474343>; <https://www.rareconnect.org/en/community/sanfilippo-syndrome/article/current-sanfilippo-research-programs-in-the-clinical-stage>).

Delivery of the therapeutic gene by vectors other than viruses is also considered. An example is the use of a plasmid vector for MPS IIIA gene therapy. Hydrodynamic delivery of a plasmid containing the *SGSH* gene into MPS-IIIA mice caused high serum levels of sulfamidase. GAG levels were corrected in visceral organs and reduced in the brain (68). This kind of therapy might be an alternative to virus-based gene therapy.

5.3. Substrate reduction therapy

Difficulties in delivery of a therapeutic enzyme to the brain have stimulated studies on alternative therapies for Sanfilippo disease. One of them is substrate reduction therapy (SRT). The objective of this method is to decrease the efficiency of synthesis of compounds that cannot be degraded in cells of affected organism (69).

The first successful attempt to impair HS synthesis was based on the use of (9-(2-carboxyphenyl)-6-diethylamino-3-xanthenylidene)-diethylammonium chloride, a compound known also as rhodamine B. Both production and storage of GAGs were decreased

in MPS IIIA fibroblasts after their treatment with this compound *in vitro* (70). When MPS IIIA mice were injected with rhodamine B, reduction in HS levels and behavioral improvement could be observed (71). Trans-generational exposure to low levels of rhodamine B did not adversely affect litter size and liver function in MPS IIIA mice (72). Nevertheless, it is assumed that higher doses might be toxic for humans (69). The mechanism of rhodamine B-mediated inhibition of GAG synthesis arises from reduction of the frequency of synthesis initiation rather than from shortening of the chains as the expression of genes responsible for the initiation and elongation of GAGs was down-regulated in the presence of this compound (73).

The antisense RNA-mediated technologies allow to decrease the amount of chosen mRNA levels, and thus to impair expression of particular genes. Therefore, the idea was presented to reduce efficiency of GAG synthesis due to down-regulation of genes coding for enzymes involved in the biosynthetic pathway. Indeed, by the use of siRNA or shRNA it was possible to significantly decrease the levels of GAGs in MPS III cells (74, 75). However, the general problems with stability and delivery of RNA molecules to patient's tissues makes this method still at the stage of pre-clinical experiments.

There is another possibility to lower the level of GAG synthesis. A specific isoflavone called genistein or 5, 7-dihydroxy-3- (4-hydroxyphenyl)-4H-1-benzopyran-4-one, has been demonstrated to inhibit GAG synthesis in MPS I, II, IIIA and MPS IIIB fibroblasts *in vitro* (76). Since this inhibition appeared to be due to impairment of the epidermal growth factor receptor activity, and further down-regulation of the signal transduction, which normally leads to stimulation of expression of genes coding for enzymes involved in GAG synthesis, this kind of potential substrate reduction therapy has been called 'gene expression-targeted isoflavone therapy' or GET IT (77). This treatment appeared particularly attractive for MPS III, as genistein can cross the blood-brain-barrier with efficiency of several percent. However, apart from positive results of early *in vitro* experiments and studies on animal models, many controversies appeared regarding GET IT. They were reviewed recently (78), therefore, here we will discuss them only briefly and present the most recent results.

The controversies started from the *in vitro* stage of the studies. Although early findings which indicated the inhibition of GAG synthesis in human MPS I, II, IIIA, and IIIB fibroblasts by genistein (76, 77, 79) were confirmed in studies on MPS IIIA, VII, and mucopolidosis type II, performed in other laboratories (80, 81), recently published report demonstrated opposite results in experiments with MPS I cells (82). Intriguingly, very recent paper showed again a negative effects of genistein on efficiency of GAG synthesis in MPS II cell cultures (83).

Moreover, microarray analyses, followed by quantitative RT-PCR, identified particular genes coding for enzymes necessary for GAG synthesis which were inhibited by genistein, while most of genes for GAG lysosomal hydrolases were stimulated by this isoflavone (84). This stimulation was apparently due to positive regulation of the transcription factor EB (TFEB), a master regulator for lysosomal biogenesis and function. Again, results for some genes were opposite to those published by another group (82 vs. 83 and 84), which is intriguing despite the fact that different cell types (MPS I vs. MPS II and normal cells) were used in those studies.

Results of experiments with genistein-treated MPS mice provided another controversy. Despite the very promising reports demonstrating significant reduction of GAG storage in peripheral tissues of MPS IIIB mice (85) and brain of MPS II mice (86), as well as correction of behavior in MPS IIIB mice (87), recent studies have shown unchanged GAG levels in MPS I mice receiving genistein (88). Moreover, some adverse effects were signaled, like decreased body length and femur length, as well as a scrotal hernia and/or scrotal hydrocele in males suffering from MPS I (88). To make the situation even more complicated, we would like to admit that using the same MPS mouse model (MPS I), we found a significant decrease in GAG levels in tissues of genistein-treated animals, while we did not observe any adverse effects, even those mentioned above (M. Malinowska *et al.*, manuscript in preparation).

Definitely, there must be some factors and/or experimental conditions which are not fully controlled and which differ between experiments. Most probably they influence the results obtained by various research groups. Since genistein acts by binding to EGFR and other membrane receptors, one might speculate that differences in the composition of sera used for *in vitro* cell cultures might result in changes of cellular responses to genistein. In fact, addition of an excess of EGF to the culture medium significantly modified the effects of genistein on GAG storage in MPS II, IIIA and IIIB cells (77). Furthermore, different abundance of the membrane receptors on the surfaces of cells in the mouse organism, caused by either differences in genetic backgrounds or local external conditions in animal facilities, might have effects similar to those described above.

Despite the differences in results of *in vitro* experiments and investigations with animal models, preliminary clinical studies were performed. In the works published to date, low doses of genistein (5-15 mg/kg/day, in contrast to 160 mg/kg/day used in experiments with mice), included in the soy isoflavone extracts, were employed. Different results of these studies increased the controversy about the efficacy of GET IT. In some studies, improvement of GAG storage and some clinical parameters (e.g. cognitive abilities, behavior, sleeping,

infections) have been reported in MPS II, IIIA and IIIB patients (89-92), while other researchers did not observe positive effects in MPS IIIA, IIIB and IIIC children (93). All these studies were open-label trials which further complicated the interpretation of the results. In the only randomized, crossover, placebo-controlled trial with a genistein-rich soy isoflavone extract (10 mg/kg/day of genistein), followed by an open-label extension study, published to date, a statistically significant decrease in plasma and urinary GAG levels in genistein-treated MPS IIIA, IIIB and IIIC patients was found, while no clinical changes were observed (94).

Definitely, a clinical trial with high dose of pure genistein is necessary to indicate if this compound can be effective in treatment of MPS III patients. Since high dose (150 mg/kg/day) of genistein was demonstrated to be safe during a long period treatment of children with MPS (no serious adverse effects in MPS II, IIIA, IIIB, and IIIC patients treated with at least 12 months were noted) (95), such a trial became possible. In fact, phase III, double blinded, randomized, placebo controlled clinical trial of high dose oral genistein aglycone in patients with Sanfilippo syndrome, has already been started (<http://public.ukcrn.org.uk/search/StudyDetail.aspx?StudyID=16209>; reference as on June 27, 2015; reference as on June 27, 2015).

5.4. Other therapies

Other therapies have also been tested for Sanfilippo disease. *N*-1-butyl-2-(hydroxymethyl) piperidine-3,4,5-triol or miglustat, is known as an inhibitor of glycosphingolipid synthesis, and is registered as a drug for treatment of Gaucher disease (96) and Nieman-Pick C disease (97). Since glycosphingolipids are secondary storage materials in MPS III, a clinical trial with the use of miglustat in Sanfilippo disease has been conducted. However, neither improvement nor stabilization in behavior problems in the treated patients occurred (98).

In contrast to some other mucopolysaccharidoses, it was reported that bone marrow or hematopoietic stem cell transplantation fails to prevent neurological deterioration in MPS III patients. This was also true when umbilical cord blood-derived stem cell transplantation was performed before appearance of symptoms in patients suffering from MPS IIIA and MPS IIIB (99). On the other hand, contrary to that, repeated administrations of human umbilical cord blood cells improved disease outcomes in a mouse model MPS IIIB (100).

Finally, *in vitro* treatment of MPS IIIA and MPS IIIB fibroblasts with either coenzyme Q₁₀ (CoQ₁₀) or a mixture of antioxidants (composed of α -tocopherol, N-acetylcysteine and α -lipoic acid), resulted in improved biochemical parameters of the cells (101). This preliminary study suggests that treatment of patients with CoQ₁₀ and/or antioxidants may be beneficial for patients suffering

Table 3. Summary of potential therapies for Sanfilippo disease under investigation

Therapy type	Drug/route of delivery	Results of studies	Limitations of therapies
Enzyme replacement therapy	Intrathecal injections; ventricular injections; cisternal injections	Enzyme penetrates into the brain; reduction in HS storage in brain; reduction of secondary pathology in the brain	Invasive delivery methods; repeated administration; effect in the brain depends on time of treatment initiation
Gene therapy	Fusion proteins; viral vectors or plasmid vectors delivery; systemic or intracranial administration	Expression of enzyme in the brain and peripheral organs; decrease of GAG storage in the brain; normalization of GAG levels in peripheral organs; resolution of neuroinflammation; prolongation of animal survival	Possible repeated administration; invasive delivery methods; moderate clinical improvement of patients in clinical trials
Substrate reduction therapy	Rhodamine B Genistein	Reduction of HS levels in liver and brain; reduced urinary GAG levels; behavioral improvement	Higher doses of rhodamine B possibly toxic for humans; possible adverse effects of genistein (particularly in males)
	Antisense RNAs (siRNA, shRNA)	Decrease in levels of GAGs in MPS III cells	Problems with the stability and delivery
Transplantation	Bone marrow or hematopoietic stem cells	Biochemical correction; fails to prevent neurological deterioration in MPS III patients	Invasive procedure; possible transplant rejections; graft-versus-host disease
	Umbilical cord blood-derived cells	Improved disease outcome in MPS IIIB mice; fails to prevent neurological deterioration in MPS III patients	Limited dose of useful stem cells in blood samples; slow engraftment
Other therapies	Miglustat	No improvement or stabilization occurred in MPS III patients	Possible adverse effects
	Coenzyme Q ₁₀ Mixture of antioxidants	Improved biochemical parameters of cells	Unknown
References are provided in the text			

from Sanfilippo disease. Moreover, since HS can also trigger neuroinflammation independently of the oxidative stress (38), one might suggest that anti-inflammatory agents could be potentially beneficial for MPS III patients.

6. CONCLUDING REMARKS

Mucopolysaccharidosis type III is a complex of four genetic conditions, each caused by mutations in one of genes coding for enzymes involved in degradation of heparan sulfate (1-10). Therefore, this glycosaminoglycan is accumulated in cells of patients (33-34). Such storage, accompanied with secondary storage of glycosphingolipids, and subsequent oxidative stress and neuroinflammation (35-40), leads to severe and progressive deterioration of functions of the central nervous system (11-32). Heparan sulfate, as the primary storage material, is used as the main biomarker of the disease, though other markers (specific proteins, hair morphology, neuropsychological parameters) have also been proposed (5, 41-55, 102). Although no therapy is currently available for MPS III, ongoing clinical trials with enzyme replacement therapy, gene therapy and substrate reduction therapy provide a hope for patients and their relatives (56-101). Potential therapies for Sanfilippo disease, with main results of published studies and possible limitations, are summarized in Table 3.

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