Unfolding mucopolysaccharidosis type III

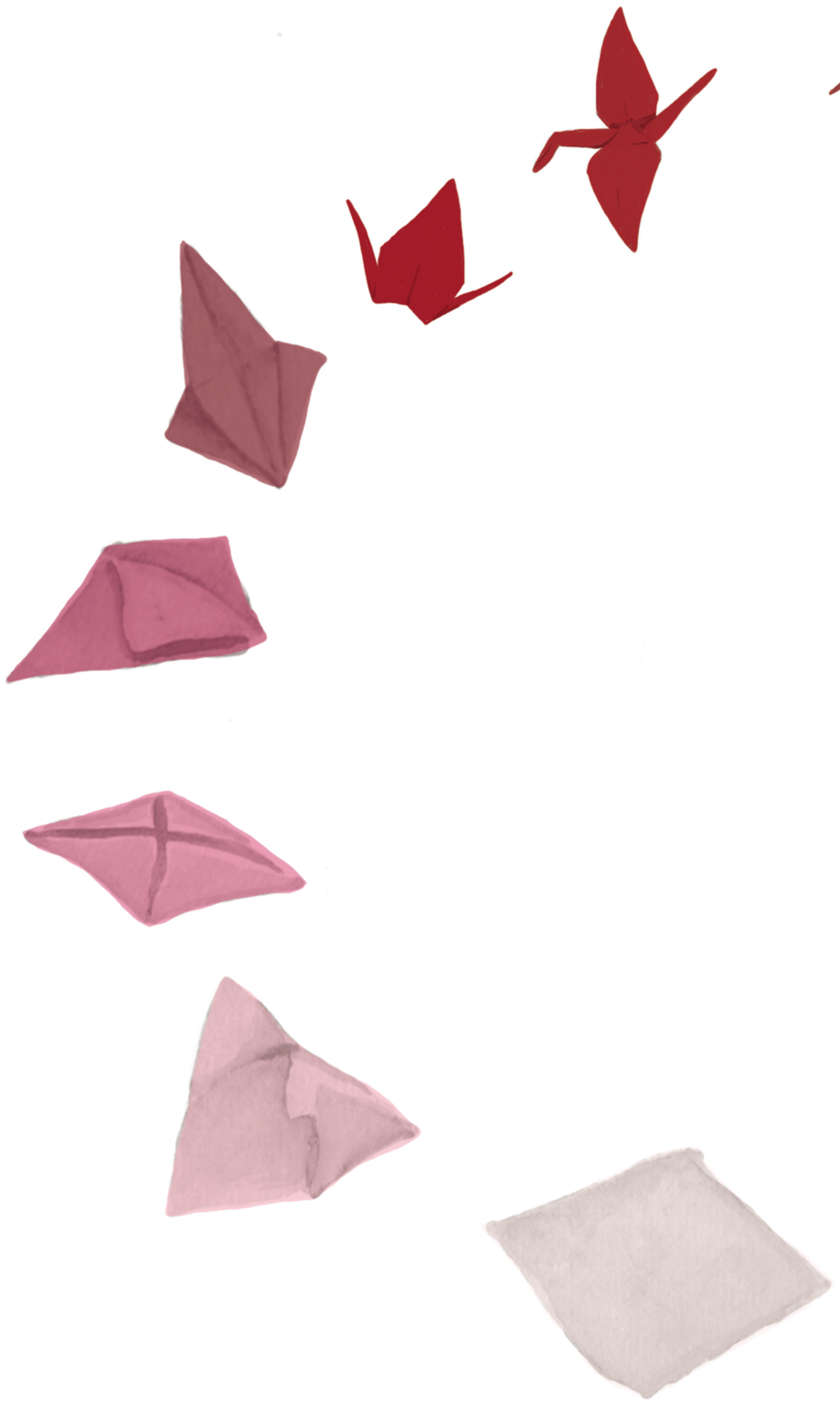
Pathophysiology, diagnosis and treatment

Meijer, O.L.M.

Publication date
2018

Document Version
Final published version

License
Other



Unfolding mucopolysaccharidosis type III

Olga Meijer

Unfolding
Mucopolysaccharidosis type III
Pathophysiology, diagnosis and treatment

Olga Meijer

Unfolding mucopolysaccharidosis type III:

pathophysiology, diagnosis and treatment

Olga Meijer

Unfolding mucopolysaccharidosis type III: pathophysiology, diagnosis and treatment

Thesis, Academic Medical Center, University of Amsterdam, The Netherlands

Cover	Lisa Wiersma – www.lisawiersma.nl
Lay out	Nicole Nijhuis and Yvonne Meeuwsen, Gildeprint
Printing	Gildeprint – www.gildeprint.nl
ISBN	978-94-6233-868-5

Unfolding mucopolysaccharidosis type III:

pathophysiology, diagnosis and treatment

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. ir. K.I.J. Maex
ten overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op vrijdag 23 februari 2018, te 12:00 uur

door Olga Laurentia Maria Meijer
geboren te Groningen

PROMOTIECOMMISSIE

Promotor:	prof. dr. F.A. Wijburg	AMC - UvA
Copromotor:	dr. N. van Vlies	Intravacc Bilthoven
Overige leden:	Prof. dr. C.E.M. Hollak	AMC - UvA
	Prof. dr. B.T. Poll-The	AMC - UvA
	Prof. dr. A.J. Verhoeven	AMC - UvA
	Prof. dr. R.J.A. Wanders	AMC - UvA
	Prof. dr. H.R. Waterham	AMC - UvA
	Prof. dr. R.A. Wevers	Radboud Universiteit Nijmegen

Faculteit der Geneeskunde

The brain is wider than the sky,
For, put them side by side,
The one the other will include
With ease, and you beside

Emily Dickinson, no. 632 (1862)

TABLE OF CONTENTS

Introduction

Chapter 1

General introduction

GENERAL INTRODUCTION

This chapter aims to provide a brief overview of the history, clinical presentation, pathophysiology, diagnosis and treatment of mucopolysaccharidosis type III (MPS III or Sanfilippo disease) and to address the current challenges regarding these topics.

Introduction

Mucopolysaccharidosis type III (MPS III or Sanfilippo disease) is a lysosomal storage disorder (LSD) caused by a deficiency in one of the enzymes involved in the lysosomal degradation of the glycosaminoglycan (GAG) heparan sulfate (HS). Four MPS III subtypes are distinguished based on differences in the underlying enzyme deficiency: MPS IIIA (heparan *N*-sulfatase (SGSH); OMIM #252900), MPS IIIB (*N*-acetyl-α-glucosaminidase (NAGLU); OMIM #252920), MPS IIIC (acetyl CoA:α-glucosaminide *N*-acetyltransferase (HGSNAT); OMIM #252930) and MPS IIID (*N*-acetylglucosamine 6-sulfatase (GNS); OMIM #252940). MPS III belongs to a group of seven mucopolysaccharidoses (MPSs), which are caused by different lysosomal enzyme deficiencies and all result in the accumulation of GAGs (table 1) [1].

MPS III or Sanfilippo disease is named after the American pediatrician Sylvester J. Sanfilippo. In 1963 he first recognized MPS III as a separate MPS entity after he had seen several patients with MPS I-Hurler-like symptoms but a more pronounced neurological decline and a distinct urinary GAG excretion profile [2,3]. After the Nobel price-winning discovery of the lysosome by de Duve *et al.* in 1955 [4–6], these disorders were later recognized to be lysosomal storage disorders and it was by the extensive work of Neufeld and Kresse that the underlying disease causing mechanism was shown to be a lysosomal enzyme deficiency [7]. They first characterized the enzyme of which the deficiency results in MPS IIIA, followed by the discovery of the enzymes responsible for MPS IIIB (1972) [8,9], MPS IIIC (1978) [10] and MPS IIID (1980) [11].

Clinical presentation

The four MPS III subtypes are clinically indistinguishable. Compared to the other MPSs, somatic disease in MPS III is relatively mild. However, in MPS III the central nervous system (CNS) is always severely affected, resulting in progressive loss of neurocognitive functions.

There is a broad spectrum of disease severity ranging from a severe and rapidly progressing (RP) phenotype, to a more attenuated, slowly progressing (SP) phenotype [12]. In general, the course of disease is characterized by three consecutive phases. After an initial phase of (near) normal development, patients usually present between the age of 1 and 4 years with a developmental delay that mainly affects the domain of speech and language development. After reaching a plateau characterized by halting of cognitive development the second phase of the disease sets in which is accompanied by progressive and often severe behavioral

problems including temper tantrums and hyperactive, fearless and autistic-like behavior [13–16]. Severe dementia and progressive loss of motor functions mark the third and last phase of the disease, progressing into a vegetative state. In patients with an RP phenotype, death usually occurs at the end of the second or beginning of the third decade of life [12,17–21]. SP patients on the other hand, may survive until late adult age. Although disease presentation is comparable to RP patients, especially the second phase is extended and SP MPS III patients can show a stable neurocognitive impairment for many years before further regression sets in. In this group of patients, survival into the seventh decade of life has been reported [22,23]. Although less pronounced than the neurological features, other frequently occurring signs and symptoms in MPS III patients are coarse facial features, recurrent upper airway infections, episodes of diarrhea or constipation, hepatomegaly, and umbilical and/or inguinal hernias.

Birth prevalence

All four MPS III subtypes are inherited in an autosomal recessive manner [24]. With a reported birth prevalence that varies between 1.52 and 1.89 per 100,000 live births, MPS III is one of the most common MPS types worldwide. Nevertheless, a difference in geographic distribution is seen between the MPS III subtypes. For MPS IIIA a higher birth prevalence is found in North-West Europe, whereas type B is most frequently seen in the South-East of Europe [25–27]. Overall, type C and D are significantly less common, with type D being the rarest of the four subtypes.

Pathophysiology

In MPS III the degradation of the GAG HS is impaired. HS is a negatively charged polysaccharide that covalently bound to a core protein forms proteoglycans, which are found on cell surfaces and in the extracellular matrix of all tissues, including the brain.

The lysosomal degradation of HS is a stepwise process that starts with endolytic cleavage mediated by several heparanases. The formed HS fragments and oligosaccharides are subsequently degraded by three exoglycosidases, at least three sulfatases and an acetyltransferase (figure 1). The enzymes SGSH, NAGLU, HGSNAT and GNS are specific for the degradation of HS and the deficiency of one of these enzymes results in the accumulation of non-degraded HS in lysosomes and the extracellular compartments.

The accumulation of HS is generally assumed to be responsible for triggering a pathogenic cascade involving multiple pathways that together result in progressive neuronal damage and dysfunction, leading to CNS deterioration. These mechanisms are reviewed in more detail in chapter 2 of this thesis and include:

1. Formation of storage vesicles in the lysosome affecting the function of other organelles including the Golgi apparatus[28–31] and mitochondria [32–34];
2. HS induced secondary storage of gangliosides GM2 and GM3, and unesterified cholesterol [33,35–42];

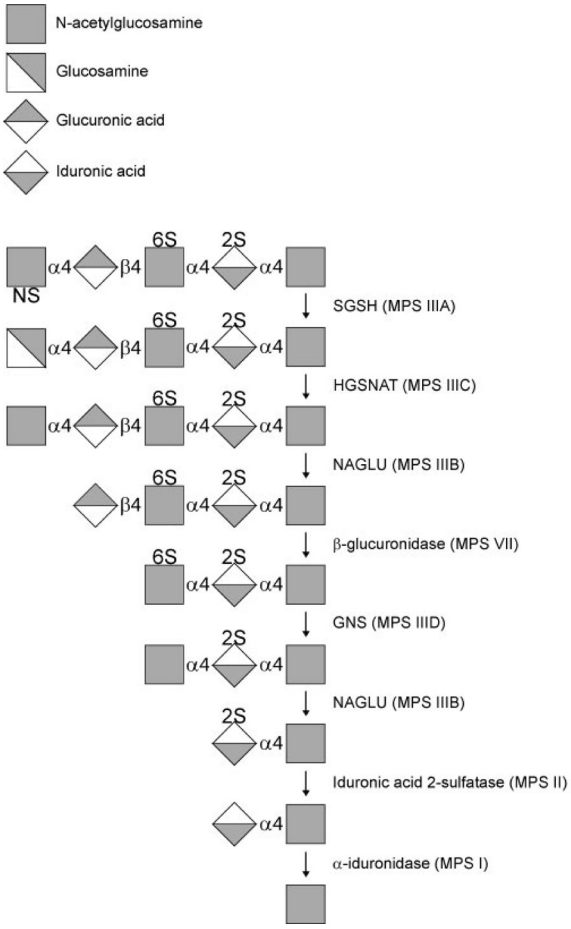


Figure 1. The stepwise degradation of heparan sulfate (HS) and the specific enzymes that are affected in the different MPS subtypes. SGSH: heparan *N*-sulfatase (MPS IIIA); NAGLU: *N*-acetyl-α-glucosaminidase (MPS IIIB); HGSNAT: acetyl CoA:α-glucosaminide *N*-acetyltransferase (MPS IIIC); GNS: *N*-acetylglucosamine 6-sulfatase (MPS IIID).

3. Formation of protein aggregates containing phosphorylated α-synuclein, phosphorylated tau and β-amyloid [43–46];
4. Accumulation of autophagosomes due to involvement of the endosomal-autophagic-lysosomal pathway regulated by transcription factor EB (TFEB) and the CLEAR (coordinated lysosomal expression and regulation) gene network [47–49];
5. Neuroinflammation mediated by toll-like receptors activating both the innate and adaptive immune system, resulting in oxidative stress which further exacerbates neuronal damage and dysfunction [33,40,50–55];
6. Changes in HS structure that may affect signaling pathways and the interaction within and between cells [33,56–61];

7. Changes in synaptic signaling due impaired neurotransmitter release and changes in (post)synaptic proteins [33,62–64].

Diagnosis

A delay in cognitive development and the specific phenotypic features often provide the first clue towards the diagnosis MPS III. Recently, a multiplexed LC-MS/MS assay was introduced as first diagnostic test for quantitative analyses of GAGs in urine and/or plasma [65]. This assay showed to accurately differentiate between the MPS subtypes and, with an improved sensitivity, could replace the traditionally used qualitative approach consisting of the dimethylmethylene blue binding assay and subsequent two-dimensional electrophoresis in most centers. When only elevated levels of HS are found, suggestive for the diagnosis MPS III, measurement of enzyme activity in leukocytes or plasma is performed to determine the underlying enzyme deficiency. Some genotype-phenotype correlations have been established for MPS IIIA, MPS IIIB and MPS IIIC [17,23,66]. However, due to large allelic heterogeneity in all MPS III subtypes and the fact that new disease causing mutations are frequently found, phenotype prediction is overall difficult [23,66–96]. Accurate prediction of disease phenotype will be essential for the assessment of the therapeutic efficacy of upcoming disease- modifying treatments, but can also help current decision making on supportive interventions and (genetic) counseling of parents. Better methods for phenotype prediction are thus needed.

Treatment

There is yet no disease-modifying treatment available for MPS III, although several promising therapies have been developed and are currently under investigation in preclinical and clinical trials. These therapies interfere on different levels of the pathogenic cascade and either focus on restoring the underlying genetic defect by gene therapy, supplementing the deficient enzyme by enzyme replacement therapy (ERT) or hematopoietic stem cell transplantation (HSCT), or the enhancement of residual enzyme activity of mutant enzymes by chaperone therapy. Other strategies focus on the pathogenic consequences of the enzyme deficiency by reducing the accumulating substrate, so called substrate reduction therapy (SRT), or enhancing overall lysosomal function and cellular clearance via the induction of TFEB. Since MPS III is a disorder that mainly affects the CNS, the main challenge in all approaches is that therapies have to be able to reach the brain, either by overcoming or bypassing the blood brain barrier (BBB). These are extensively reviewed in chapter 2 of this thesis and will be further discussed in the general discussion and future perspectives section of chapter 8.

OUTLINE OF THIS THESIS

This thesis addresses the current challenges faced in the field of MPS III and aims for unravelling its pathophysiology, diagnostic process and treatment. Chapter 2 contains a review on the current knowledge about the pathophysiological mechanisms underlying MPS III and the treatment modalities that are under investigation. The first part of this thesis further focuses on the pathophysiology of MPS III. In chapter 3 a model is presented for the prediction of phenotypic severity in MPS IIIB patients, using low culture temperature to enhance residual enzyme activity in patients' fibroblasts. In chapter 4 the underlying processes are elucidated that are responsible for the enhancement of residual enzyme activity in MPS IIIB fibroblasts at low culture temperatures. In an attempt to explain the differences in neuronopathic disease between patients with different MPS subtypes, chapter 5 reports on the differences in sulfation pattern of HS derived disaccharides in plasma of MPS I and MPS III patients. The second part of this thesis addresses the treatment of MPS III. Chapter 6 reports on the results of an high-throughput screen aiming for the identification of compounds that can enhance residual enzyme activity in fibroblasts of MPS IIIB patients. The third part of this thesis, found in chapter 7, concentrates on the diagnosis of MPS III and provides the results of a retrospective study on the delay to diagnosis in MPS I and MPS III patients and whether this delay has changed over time. Finally, in the discussion of this thesis found in chapter 8, suggestions are given for future studies needed to further elucidate the pathophysiology of MPS III, methods to improve the diagnosis as well as an overview of potential therapies for MPS III that are investigated now and need to be investigated the future.

REFERENCES

1. Muenzer J. Overview of the mucopolysaccharidoses. *Rheumatology*. 2011;50 Suppl 5:v4-12.
2. Sanfilippo SJ, Podosin R, Langer L, Good RA. Mental retardation associated with acid mucopolysaccharidurie (heparitin sulfate type). *J Pediatr*. 1963;63(4):837-838.
3. Meyer K, Hoffman P. Hurler's syndrome. *Arthritis Rheum*. 1961;4:552-560.
4. de Duve C, Pressman BC, Gianetto R, Wattiaux R, Appelmans F. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem J*. 1955;60(4):604-617.
5. Appelmans F, Wattiaux R, De Duve C. Tissue fractionation studies. 5. The association of acid phosphatase with a special class of cytoplasmic granules in rat liver. *Biochem J*. 1955;59(3):438-445.
6. De Duve C. The lysosome. *Sci Am*. 1963;208:64-72.
7. Kresse H, Neufeld EF. The Sanfilippo A corrective factor. Purification and mode of action. *J Biol Chem*. 1972;247(7):2164-2170.
8. O'Brien JS. Sanfilippo syndrome: profound deficiency of alpha-acetylglucosaminidase activity in organs and skin fibroblasts from type-B patients. *Proc Natl Acad Sci USA*. 1972;69(7):1720-1722.
9. von Figura K, Kresse H. The sanfilippo B corrective factor: a N-acetyl-alpha-D-glucosamindiase. *Biochem Biophys Res Commun*. 1972;48(2):262-269.
10. Klein U, Kresse H, von Figura K. Sanfilippo syndrome type C: deficiency of acetyl-CoA:alpha-glucosaminide N-acetyltransferase in skin fibroblasts. *Proc Natl Acad Sci USA*. 1978;75(10):5185-5189.
11. Kresse H, Paschke E, von Figura K, Gilberg W, Fuchs W. Sanfilippo disease type D: deficiency of N-acetylglucosamine-6-sulfate sulfatase required for heparan sulfate degradation. *Proc Natl Acad Sci USA*. 1980;77(11):6822-6826.
12. Valstar MJ, Ruijter GJ, Van Diggelen OP, Poorthuis BJ, Wijburg FA. Sanfilippo syndrome: A mini-review. *J Inherit Metab Dis*. 2008;31(2):240-252.
13. Potegal M, Yund B, Rudser K, et al. Mucopolysaccharidosis Type IIIA presents as a variant of Klüver-Bucy syndrome. *J Clin Exp Neuropsychol*. 2013;35(6):608-616.
14. Rumsey RK, Rudser K, Delaney K, Potegal M, Whitley CB, Shapiro E. Acquired autistic behaviors in children with mucopolysaccharidosis type IIIA. *J Pediatr*. 2014;164(5):1147-1151.e1.
15. Shapiro EG, Nestrasil I, Delaney KA, et al. A Prospective Natural History Study of Mucopolysaccharidosis Type IIIA. *J Pediatr*. 2016;170:278-287e4.
16. Shapiro E, King K, Ahmed A, et al. The neurobehavioral phenotype in mucopolysaccharidosis Type IIIB: An exploratory study. *Mol Genet Metab Rep*. 2016;6:41-47.
17. Ruijter GJ, Valstar MJ, van de Kamp JM, et al. Clinical and genetic spectrum of Sanfilippo type C (MPS IIIC) disease in The Netherlands. *Mol Genet Metab*. 2008;93(2):104-111.
18. Meyer A, Kossow K, Gal A, et al. Scoring evaluation of the natural course of mucopolysaccharidosis type IIIA (Sanfilippo syndrome type A). *Pediatrics*. 2007;120(5):e1255-61.
19. Wijburg FA, Węgrzyn G, Burton BK, Tylki-Szymańska A. Mucopolysaccharidosis type III (Sanfilippo syndrome) and misdiagnosis of idiopathic developmental delay, attention deficit/hyperactivity disorder or autism spectrum disorder. *Acta Paediatr*. 2013;102(5):462-470.
20. Buhrman D, Thakkar K, Poe M, Escolar ML. Natural history of Sanfilippo syndrome type A. *J Inherit Metab Dis*. 2014;37(3):431-7.
21. Héron B, Mikaeloff Y, Froissart R, et al. Incidence and natural history of mucopolysaccharidosis type III in France and comparison with United Kingdom and Greece. *Am J Med Genet A*. 2011;155A(1):58-68.
22. Moog U, van Mierlo I, van Schrojenstein Lantman-de Valk HM, Spaapen L, Maaskant MA, Curfs LM. Is Sanfilippo type B in your mind when you see adults with mental retardation and behavioral problems? *Am J Med Genet C Semin Med Genet*. 2007;145C(3):293-301.

23. Valstar MJ, Bruggenwirth HT, Olmer R, et al. Mucopolysaccharidosis type IIIB may predominantly present with an attenuated clinical phenotype. *J Inherit Metab Dis*. 2010;33(6):759-767.
24. Neufeld EF, Muenzer J. The Mucopolysaccharidoses. In: Valle D, Beaudet A, Vogelstein B, et al., eds. *The Metabolic and Molecular Basis of Inherited Disease*. 8th ed. New York, NY, USA: McGraw-Hill; 2001:3421-3452.
25. Poorthuis BJ, Wevers RA, Kleijer WJ, et al. The frequency of lysosomal storage diseases in The Netherlands. *Hum Genet*. 1999;105(1-2):151-156.
26. Meikle PJ, Hopwood JJ, Clague AE, Carey WF. Prevalence of lysosomal storage disorders. *JAMA*. 1999;281(3):249-254.
27. Baehner F, Schmiedeskamp C, Krummenauer F, et al. Cumulative incidence rates of the mucopolysaccharidoses in Germany. *J Inherit Metab Dis*. 2005;28(6):1011-1017.
28. Vitry S, Bruyère J, Hocquemiller M, et al. Storage vesicles in neurons are related to Golgi complex alterations in mucopolysaccharidosis IIIB. *Am J Pathol*. 2010;177(6):2984-2999.
29. Lemonnier T, Blanchard S, Toli D, et al. Modeling neuronal defects associated with a lysosomal disorder using patient-derived induced pluripotent stem cells. *Hum Mol Genet*. 2011;20(18):3653-3666.
30. Roy E, Bruyère J, Flamant P, et al. GM130 gain-of-function induces cell pathology in a model of lysosomal storage disease. *Hum Mol Genet*. 2012;21(7):1481-1495.
31. Nakamura N. Emerging new roles of GM130, a cis-Golgi matrix protein, in higher order cell functions. *J Pharmacol Sci*. 2010;112(3):255-264.
32. Settembre C, Fraldi A, Jahreiss L, et al. A block of autophagy in lysosomal storage disorders. *Hum Mol Genet*. 2008;17(1):119-129.
33. Wilkinson FL, Holley RJ, Langford-Smith KJ, et al. Neuropathology in mouse models of mucopolysaccharidosis type I, IIIA and IIIB. *PLoS One*. 2012;7(4):e35787.
34. Archer LD, Langford-Smith KJ, Bigger BW, Fildes JE. Mucopolysaccharide diseases: a complex interplay between neuroinflammation, microglial activation and adaptive immunity. *J Inherit Metab Dis*. 2014;37(1):1-12.
35. Constantopoulos G, Eiben RM, Schafer IA. Neurochemistry of the mucopolysaccharidoses: brain glycosaminoglycans, lipids and lysosomal enzymes in mucopolysaccharidosis type III B (alpha-N-acetylglucosaminidase deficiency). *J Neurochem*. 1978;31(5):1215-1222.
36. Constantopoulos G, Dekaban AS. Neurochemistry of the mucopolysaccharidoses: brain lipids and lysosomal enzymes in patients with four types of mucopolysaccharidosis and in normal controls. *J Neurochem*. 1978;30(5):965-973.
37. Constantopoulos G, Iqbal K, Dekaban AS. Mucopolysaccharidosis types IH, IS, II, and IIIA: glycosaminoglycans and lipids of isolated brain cells and other fractions from autopsied tissues. *J Neurochem*. 1980;34(6):1399-1411.
38. Jones MZ, Alroy J, Rutledge JC, et al. Human mucopolysaccharidosis IIID: clinical, biochemical, morphological and immunohistochemical characteristics. *J Neuropathol Exp Neurol*. 1997;56(10):1158-1167.
39. Crawley AC, Gliddon BL, Auclair D, et al. Characterization of a C57BL/6 congenic mouse strain of mucopolysaccharidosis type IIIA. *Brain Res*. 2006;1104(1):1-17.
40. Ausseil J, Desmaris N, Bigou S, et al. Early neurodegeneration progresses independently of microglial activation by heparan sulfate in the brain of mucopolysaccharidosis IIIB mice. *PLoS One*. 2008;3(5):e2296.
41. McGlynn R, Dobrenis K, Walkley SU. Differential subcellular localization of cholesterol, gangliosides, and glycosaminoglycans in murine models of mucopolysaccharide storage disorders. *J Comp Neurol*. 2004;480(4):415-426.
42. Fraldi A, Annunziata F, Lombardi A, et al. Lysosomal fusion and SNARE function are impaired by cholesterol accumulation in lysosomal storage disorders. *EMBO J*. 2010;29(21):3607-3620.
43. Ohmi K, Kudo LC, Ryazantsev S, Zhao H-Z, Karsten SL, Neufeld EF. Sanfilippo syndrome type B, a lysosomal storage disease, is also a tauopathy. *Proc Natl Acad Sci USA*. 2009;106(20):8332-8337.
44. Ohmi K, Zhao H-Z, Neufeld EF. Defects in the medial entorhinal cortex and dentate gyrus in the mouse model of Sanfilippo syndrome type B. *PLoS One*. 2011;6(11):e27461.

45. Ginsberg SD, Galvin JE, Lee VM, et al. Accumulation of intracellular amyloid-beta peptide (A beta 1-40) in mucopolysaccharidosis brains. *J Neuropathol Exp Neurol*. 1999;58(8):815-824.
46. Hamano K, Hayashi M, Shioda K, Fukatsu R, Mizutani S. Mechanisms of neurodegeneration in mucopolysaccharidoses II and IIIB: analysis of human brain tissue. *Acta Neuropathol*. 2008;115(5):547-559.
47. Platt FM, Boland B, van der Spoel AC. The cell biology of disease: lysosomal storage disorders: the cellular impact of lysosomal dysfunction. *J Cell Biol*. 2012;199(5):723-734.
48. Sardiello M, Palmieri M, di Ronza A, et al. A gene network regulating lysosomal biogenesis and function. *Science*. 2009;325(5939):473-477.
49. Palmieri M, Impey S, Kang H, et al. Characterization of the CLEAR network reveals an integrated control of cellular clearance pathways. *Hum Mol Genet*. 2011;20(19):3852-3866.
50. Ohmi K, Greenberg DS, Rajavel KS, Ryazantsev S, Li HH, Neufeld EF. Activated microglia in cortex of mouse models of mucopolysaccharidoses I and IIIB. *Proc Natl Acad Sci USA*. 2003;100(4):1902-1907.
51. Kodaira Y, Nair SK, Wrenshall LE, Gilboa E, Platt JL. Phenotypic and functional maturation of dendritic cells mediated by heparan sulfate. *J Immunol*. 2000;165(3):1599-1604.
52. Johnson GB, Brunn GJ, Kodaira Y, Platt JL. Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4. *J Immunol*. 2002;168(10):5233-5239.
53. DiRosario J, Divers E, Wang C, et al. Innate and adaptive immune activation in the brain of MPS IIIB mouse model. *J Neurosci Res*. 2009;87(4):978-990.
54. Villani GR, Gargiulo N, Faraonio R, Castaldo S, Gonzalez Y Reyero E, Di Natale P. Cytokines, neurotrophins, and oxidative stress in brain disease from mucopolysaccharidosis IIIB. *J Neurosci Res*. 2007;85(3):612-622.
55. Villani GR, Di Domenico C, Musella A, Cecere F, Di Napoli D, Di Natale P. Mucopolysaccharidosis IIIB: oxidative damage and cytotoxic cell involvement in the neuronal pathogenesis. *Brain Res*. 2009;1279:99-108.
56. King B, Savas P, Fuller M, Hopwood J, Hemsley K. Validation of a heparan sulfate-derived disaccharide as a marker of accumulation in murine mucopolysaccharidosis type IIIA. *Mol Genet Metab*. 2006;87(2):107-112.
57. Holley RJ, Deligny A, Wei W, et al. Mucopolysaccharidosis type I, unique structure of accumulated heparan sulfate and increased N-sulfotransferase activity in mice lacking α-l-iduronidase. *J Biol Chem*. 2011;286(43):37515-37524.
58. Pan C, Nelson MS, Reyes M, et al. Functional abnormalities of heparan sulfate in mucopolysaccharidosis-I are associated with defective biologic activity of FGF-2 on human multipotent progenitor cells. *Blood*. 2005;106(6):1956-1964.
59. Khan SA, Nelson MS, Pan C, Gaffney PM, Gupta P. Endogenous heparan sulfate and heparin modulate bone morphogenetic protein-4 signaling and activity. *Am J Physiol Cell Physiol*. 2008;294(6):C1387-97.
60. Bishop JR, Schuksz M, Esko JD. Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature*. 2007;446(7139):1030-1037.
61. Lindahl U, Kjellén L. Pathophysiology of heparan sulphate: many diseases, few drugs. *J Intern Med*. 2013;273(6):555-571.
62. Keating DJ, Winter MA, Hemsley KM, et al. Exocytosis is impaired in mucopolysaccharidosis IIIA mouse chromaffin cells. *Neuroscience*. 2012;227:110-118.
63. Vitry S, Ausseil J, Hocquemiller M, Bigou S, Dos Santos Coura R, Heard JM. Enhanced degradation of synaptophysin by the proteasome in mucopolysaccharidosis type IIIB. *Mol Cell Neurosci*. 2009;41(1):8-18.
64. Dwyer CA, Scudder SL, Lin Y, et al. Neurodevelopmental Changes in Excitatory Synaptic Structure and Function in the Cerebral Cortex of Sanfilippo Syndrome IIIA Mice. *Sci Rep*. 2017;7:46576.
65. Langereis EJ, Wagemans T, Kulik W, et al. A multiplex assay for the diagnosis of mucopolysaccharidoses and mucolipidoses. *PLoS One*. 2015;10(9):1-13.
66. Valstar MJ, Neijs S, Bruggenwirth HT, et al. Mucopolysaccharidosis type IIIA: clinical spectrum and genotype-phenotype correlations. *Ann Neurol*. 2010;68(6):876-887.

67. Blanch L, Weber B, Guo XH, Scott HS, Hopwood JJ. Molecular defects in Sanfilippo syndrome type A. *Hum Mol Genet*. 1997;6(5):787-791.
68. Bunge S, Ince H, Steglich C, et al. Identification of 16 sulfamidase gene mutations including the common R74C in patients with mucopolysaccharidosis type IIIA (Sanfilippo A). *Hum Mutat*. 1997;10(6):479-485.
69. Weber B, Guo XH, Wraith JE, et al. Novel mutations in Sanfilippo A syndrome: Implications for enzyme function. *Hum Mol Genet*. 1997;6(9):1573-1579.
70. Di Natale P, Balzano N, Esposito S, Villani GRD. Identification of molecular defects in Italian Sanfilippo a patients including 13 novel mutations. *Hum Mutat*. 1998;11(4):313-320.
71. Muschol N, Storch S, Ballhausen D, et al. Transport, enzymatic activity, and stability of mutant sulfamidase (SGSH) identified in patients with mucopolysaccharidosis type III A. *Hum Mutat*. 2004;23(6):559-566.
72. Muschol N, Pohl S, Meyer A, Gal A, Ullrich K, Braulke T. Residual activity and proteasomal degradation of p.Ser298Pro sulfamidase identified in patients with a mild clinical phenotype of Sanfilippo A syndrome. *Am J Med Genet A*. 2011;155A(7):1634-1639.
73. Beesley CE, Young EP, Vellodi A, Winchester BG. Identification of 12 novel mutations in the α-N-acetylglucosaminidase gene in 14 patients with Sanfilippo syndrome type B (mucopolysaccharidosis type IIIB). *J Med Genet*. 1998;35(11):910-914.
74. Beesley C, Moraitou M, Winchester B, Schulpis K, Dimitriou E, Michelakakis H. Sanfilippo B syndrome: Molecular defects in Greek patients. *Clin Genet*. 2004;65(2):143-149.
75. Beesley CE, Jackson M, Young EP, Vellodi A, Winchester BG. Molecular defects in Sanfilippo syndrome type B (mucopolysaccharidosis IIIB). *J Inherit Metab Dis*. 2005;28(5):759-767.
76. Chinen Y, Tohma T, Izumikawa Y, Uehara H, Ohta T. Sanfilippo type B syndrome: five patients with an R565P homozygous mutation in the α-N-acetylglucosaminidase gene from the Okinawa islands in Japan. *J Hum Genet*. 2005;50(7):357-359.
77. Emre S, Terzioglu M, Tokatli A, et al. Sanfilippo syndrome in Turkey: Identification of novel mutations in subtypes A and B. *Hum Mutat*. 2002;19(2):184-185.
78. Esposito S, Balzano N, Daniele A, et al. Heparan N-sulfatase gene: Two novel mutations and transient expression of 15 defects. *Biochim Biophys Acta*. 2000;1501(1):1-11.
79. van de Kamp JJ, van Pelt JF, Liem KO, Giesberts MA, Niepoth LT, Staalman CR. Clinical variability in Sanfilippo B disease: a report on six patients in two related sibships. *Clin Genet*. 1976;10(5):279-284.
80. Ouesleti S, Brunel V, Turkia H Ben, et al. Molecular characterization of MPS IIIA, MPS IIIB and MPS IIIC in Tunisian patients. *Clin Chim Acta*. 2011;412(23-24):2326-2331.
81. Schmidtchen a, Greenberg D, Zhao HG, et al. NAGLU mutations underlying Sanfilippo syndrome type B. *Am J Hum Genet*. 1998;62(1):64-69.
82. Selmer KK, Gilfillan GD, Strømme P, et al. A mild form of Mucopolysaccharidosis IIIB diagnosed with targeted next-generation sequencing of linked genomic regions. *Eur J Hum Genet*. 2012;20(1):58-63.
83. Tang J, Pan J, Guo Y, et al. Mucopolysaccharidosis type IIIB mutations in Chinese patients: Identification of two novel NAGLU mutations and analysis of two cases involving prenatal diagnosis. *Clin Chim Acta*. 2013;419:33-38.
84. Tessitore A, Villani GR, Di Domenico C, Filocamo M, Gatti R, Di Natale P. Molecular defects in the α-N-acetylglucosaminidase gene in Italian Sanfilippo type B patients. *Hum Genet*. 2000;107(6):568-576.
85. Weber B, Guo XH, Kleijer WJ, van de Kamp JJ, Poorthuis BJ, Hopwood JJ. Sanfilippo type B syndrome (mucopolysaccharidosis III B): allelic heterogeneity corresponds to the wide spectrum of clinical phenotypes. *Eur J Hum Genet*. 1999;7(1):34-44.
86. Zhao HG, Aronovich EL, Whitley CB. Genotype-phenotype correspondence in Sanfilippo syndrome type B. *Am J Hum Genet*. 1998;62(1):53-63.
87. Bunge S, Knigge A, Steglich C, et al. Mucopolysaccharidosis type IIIB (Sanfilippo B): identification of 18 novel α-N-acetylglucosaminidase gene mutations. *J Med Genet*. 1999;36(1):28-31.

88. Champion KJ, Basehore MJ, Wood T, Destrée A, Vannuffel P, Maystadt I. Identification and characterization of a novel homozygous deletion in the α-N-acetylglucosaminidase gene in a patient with Sanfilippo type B syndrome (mucopolysaccharidosis IIIB). *Mol Genet Metab*. 2010;100(1):51-56.

89. Fan X, Zhang H, Zhang S, et al. Identification of the gene encoding the enzyme deficient in mucopolysaccharidosis IIIC (Sanfilippo disease type C). *Am J Hum Genet*. 2006;79(4):738-44.

90. Hrebícek M, Mrázová L, Seyrantepe V, et al. Mutations in TMEM76* cause mucopolysaccharidosis IIIC (Sanfilippo C syndrome). *Am J Hum Genet*. 2006;79(5):807-19.

91. Fedele AO, Filocamo M, Di Rocco M, et al. Mutational analysis of the HGSNAT gene in Italian patients with mucopolysaccharidosis IIIC (Sanfilippo C syndrome). *Hum Mutat*. 2007;28(5):523.

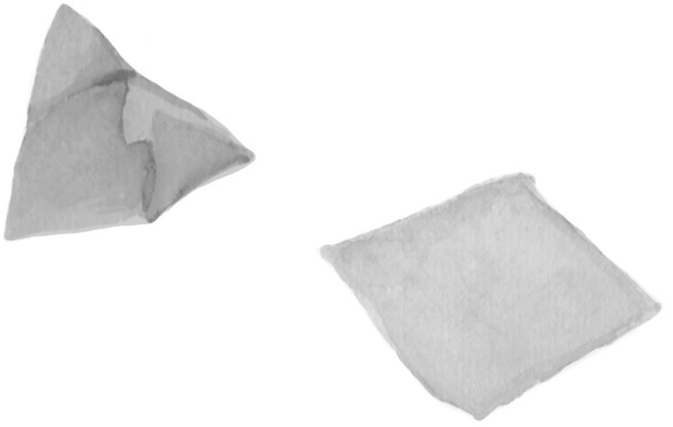
92. Beesley CE, Burke D, Jackson M, Vellodi A, Winchester BG, Young EP. Sanfilippo syndrome type D: identification of the first mutation in the N-acetylglucosamine-6-sulphatase gene. *J Med Genet*. 2003;40(3):192-4.

93. Mok A, Cao H, Hegele RA.Genomic basis of mucopolysaccharidosis type IIID (MIM 252940) revealed bysequencing of GNS encoding N-acetylglucosamine-6-sulfatase. *Genomics*. 2003;81(1):1-5.

94. Beesley CE, Concolino D, Filocamo M, Winchester BG, Strisciuglio P. Identification and characterisation of an 8.7 kb deletion and a novel nonsense mutation in two Italian families with Sanfilippo syndrome type D (mucopolysaccharidosis IIID). *Mol Genet Metab*. 2007;90(1):77-80.

95. Jansen AC, Cao H, Kaplan P, et al.Sanfilippo syndrome type D: natural history and identification of 3 novel mutations in the GNS Gene. *Arch Neurol*. 2007;64(11):1629-34

96. Valstar MJ, Bertoli-Avella AM, Wessels MW, et al. Mucopolysaccharidosis type IIID: 12 new patients and 15 novel mutations. *Hum Mutat*. 2010;31(5):E1348-60



Chapter 2

Treatment of mucopolysaccharidosis type III (Sanfilippo syndrome)

O.L.M. Meijer, N. van Vlies and F.A. Wijburg

Expert Opin Orphan Drug. 2013;1(9):717-730

ABSTRACT

Introduction

Mucopolysaccharidosis type III (MPS III or Sanfilippo syndrome or Sanfilippo disease) is caused by a deficiency in one of four enzymes involved in heparan sulfate (HS) degradation, leading to lysosomal HS accumulation. MPS III is characterized by severe central nervous system (CNS) disease, leading to progressive neurocognitive deterioration. Patients show a broad spectrum of disease progression and severity. To date, no disease-modifying treatment is available for MPS III.

Areas covered

An overview of the different pathophysiological mechanisms involved in MPS III is presented. We discuss the different therapies for MPS III which are currently being investigated, either in animal models or in clinical trials and the advantages and limitations of each therapeutic approach.

Expert opinion

The blood-brain barrier (BBB) is the bottleneck in the development of drugs for MPS III and a number of very promising approaches to target the BBB are currently studied. To assess the full therapeutic potentials of any drug or combination of drugs, trials including very early diagnosed or even fully asymptomatic patients in combination with information on the natural history of the disease are necessary. However, almost all MPS III patients are diagnosed because of developmental delay with behavioral difficulties. At that time substantial and irreversible damage to the CNS may already be present. In the best case scenario, disease-modifying therapy may only halt disease progression. Early diagnosis and initiation of treatment are therefore essential. This will only be feasible through family screening and perinatal diagnosis of patients' siblings or, in the general population, via newborn screening.

INTRODUCTION

The mucopolysaccharidoses (MPSs) comprise a group of seven invariably progressive inborn errors of metabolism, characterized by a defect in the catabolism of glycosaminoglycans (GAGs) (Table 1). The MPSs belong to the extended family of lysosomal storage diseases (LSDs). Within the group of MPSs, mucopolysaccharidosis type III (MPS III or Sanfilippo syndrome) is one of the more common types [1]. MPS III is divided into four subtypes based on the functional deficiency of one of the four lysosomal enzymes involved in the degradation of the GAG heparan sulfate (HS): heparan *N*-sulfatase (SGSH, MPS IIIA; OMIM #252900), *N*-acetyl-α-glucosaminidase (NAGLU, MPS IIIB; OMIM #252920), acetyl CoA:α-glucosaminide *N*-acetyltransferase (HGSNAT, MPS IIIC; OMIM #252930) and *N*-acetylglucosamine 6-sulfatase (GNS, MPS IIID; OMIM #252940). All four subtypes are inherited in an autosomal recessive pattern. The reported incidences vary between 1.52 and 1.89 per 100,000 live births and a difference in geographic distribution is seen between the subtypes. Birth prevalence of MPS IIIA is higher in north-west Europe, whereas type B is most frequently found in south-east Europe [2–4]. Type C and D are both significantly less common.

Clinical presentation

In contrast to the other MPS subtypes, somatic disease in MPS III is relatively mild. MPS III is predominantly characterized by severe central nervous system (CNS) disease, leading to progressive loss of neurocognitive function. There is a broad spectrum of disease severity in all subtypes of MPS III and the course of the disease varies from severe, with death usually within the second decade, to more attenuated with survival well into adulthood [5]. In general, MPS III is characterized by three consecutive phases. After an initial phase of (near) normal development, developmental delay usually becomes apparent between the age of 1 and 4 years. Stagnation of speech development is often one of the first signs. The next phase is characterized by progressive deterioration of cognitive functions, often accompanied by sleep disturbances and behavioral problems characterized by hyperactivity, fearlessness and temper tantrums. With further disease progression the behavioral problems slowly resolve. In the third phase of the disease, the patients show a loss of initiative, severe dementia and progressive loss of motor functions. During this phase patients may develop seizures, dysphagia and pyramidal tract lesions. Patients become fully bedridden and death occurs at the end of the second or beginning of the third decade of life, often due to respiratory complications [5–8].

Patients with a more attenuated phenotype have a similar course of the disease. However, disease progression is more slowly, symptoms are milder and a longer survival is observed. Children present around the age of four with a mild developmental and/or speech delay. Especially the second phase is extended, with moderate cognitive impairment which can

be stable into adolescence or early adulthood, before further regression sets in. Survival into the fourth and even into the seventh decade has been reported [9,10]. Recently, these observations were substantiated by an extensive study into the natural history of disease progression in MPS III patients [11].

Type	Eponym	Enzyme deficiency	Storage product
MPS I	Hurler, Hurler/Scheie, Scheie	α-L-iduronidase	DS, HS
MPS II	Hunter	Iduronate-2-sulfatase	DS, HS
MPS III A	Sanfilippo A	Heparan *N*-sulfatase	HS
MPS III B	Sanfilippo B	*N*-acetyl-α-glucosaminidase	HS
MPS III C	Sanfilippo C	Acetyl CoA:α-glucosaminide *N*-acetyltransferase	HS
MPS III D	Sanfilippo D	*N*-acetylglucosamine 6-sulfatase	HS
MPS IV A	Morquio A	Galactose-6-sulfatase	KS, CS
MPS IV B	Morquio B	β-galactosidase	KS
MPS VI	Maroteaux-Lamy	*N*-acetylgalactosamine 4-sulfatase	DS
MPS VII	Sly	β-glucuronidase	DS, HS, CS
MPS IX		Hyaluronidase	Hyaluronan

Table 1. Overview of the mucopolysaccharidoses. MPS, mucopolysaccharidosis; DS, dermatan sulfate; HS, heparan sulfate; KS, keratan sulfate; CS, chondroitin sulfate.

Pathophysiology

The accumulating GAG in MPS III, HS, is a negatively charged polysaccharide. Covalently attached to a core protein it is part of various proteoglycans found on the cell surfaces and in the extracellular matrix of virtually all tissues. The degradation of HS is a stepwise process that starts with endolytic cleavage by endoglycosidases (heparanases), resulting in the formation of HS fragments and oligosaccharides. Subsequent degradation is facilitated by three exoglycosidases, at least three sulfatases and an acetyltransferase (Figure 1). The four enzymes SGSH, NAGLU, HGSNAT and GNS are specific for the degradation of HS. A deficiency of one of these enzymes results in accumulation of undegraded HS in lysosomes of all cells, including neuronal cells. This accumulation is generally assumed to be the key event leading to neuronal cell dysfunction and subsequent deterioration of CNS functions in MPS III patients. However, the exact pathogenic cascade by which the accumulation of HS leads to neuronal dysfunction is largely unknown. Nevertheless, multiple pathways have been identified to play a role in the pathophysiology of MPS III.

First of all, storage vesicles are abundantly present in MPS III cells. For long these vesicles were identified as dysfunctional lysosomes overloaded with undigested HS. Recent studies in several MPS IIIB cell models have shown that these vesicles contain both the lysosomal-associated membrane protein LAMP1 and GM130 [12–14], a protein essential for

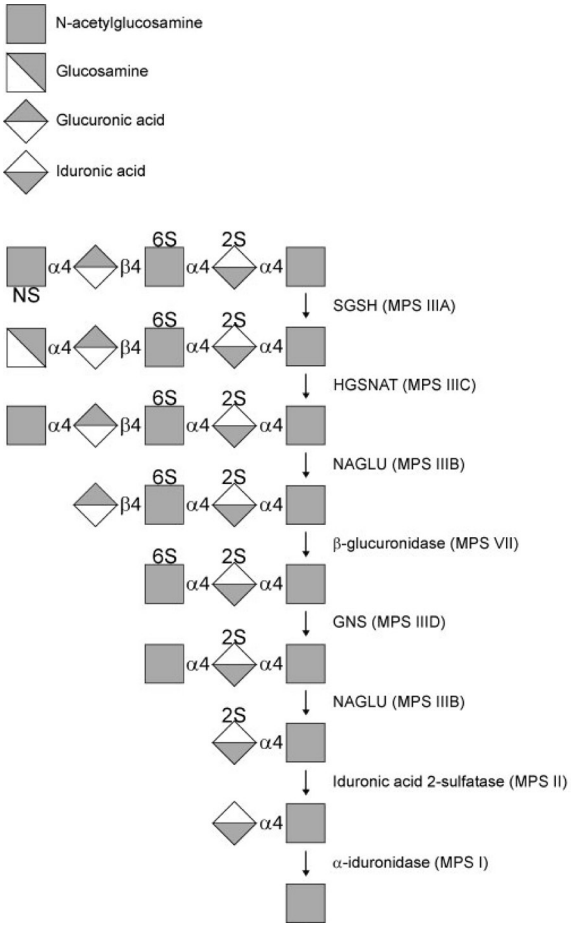


Figure 1. Stepwise degradation of heparan sulfate (HS) and the enzymes that are affected in the different MPS subtypes in which HS is stored. SGSH: heparan *N*-sulfatase (MPS IIIA); NAGLU: *N*-acetyl-α-glucosaminidase (MPS IIIB); HGSNAT: acetyl CoA:α-glucosaminide *N*-acetyltransferase (MPS IIIC); GNS: *N*-acetylglucosamine 6-sulfatase (MPS IIID).

the maintenance of Golgi structure [15]. Also, NAGLU deficient cells show abnormal Golgi architecture which might well participate in the process of vesicle accumulation and neurodegeneration in MPS III.

In addition to the storage of HS, secondary accumulation of the gangliosides GM2 and GM3 is seen in the CNS of MPS III patients and MPS III animal models [16–22]. Secondary to GM2 and GM3 accumulation, storage of unesterified cholesterol in neurons of MPS IIIA and MPS IIIB mice is found, similar to findings in primary ganglioside storage diseases [23]. It was generally assumed that this secondary accumulation resulted from inhibition of ganglioside degrading enzymes by accumulating GAGs. There is, however, evidence that altered synthesis and/or

trafficking of gangliosides from the Golgi compartment might be the underlying mechanism causing ganglioside accumulation. In contrast to what was expected, inhibition of the synthesis of GM2 and other complex gangliosides in MPS IIIA and MPS IIIB mouse models, led to a dramatic decrease in neurological function and survival [24]. The cause of the accumulation of glycosphingolipids is yet unclear, as is their role in neuronal degeneration in MPS III. Also, studies have been performed to evaluate whether HS accumulation results in formation of protein aggregates. Studies in both MPS IIIB mice and MPS III patients have shown divergent results regarding the presence of phosphorylated alpha-synuclein, phosphorylated tau or beta-amyloid-positive lesions [25–27]. Accumulation of only beta-amyloid peptides was shown in post-mortem brain samples of MPS III patients by Ginsberg *et al*. [27], while another group has shown aggregation of α-synuclein [28]. To date, it remains unclear which of these proteins accumulate in MPS III brain tissue and whether aggregation of these proteins contribute to the CNS pathology.

A second mechanism that is probably involved in the cause of neuronal damage in MPS III is impairment of the autophagocytic pathway. Autophagy is needed for the degradation and recycling of cellular components and is therefore essential for cell survival. Double membranes engulf cellular constituents thereby forming an autophagosome. After fusion with a lysosome its content will be degraded. Accumulation of autophagosomes, toxic proteins and dysfunctional mitochondria was detected in brains of MPS IIIA and MPS IIIB mice [22,29]. Dysfunctional mitochondria are well known mediators of apoptotic cell death and impaired autophagy in CNS cells is thought to induce cellular stress and damage, subsequently mobilizing the immune system leading to chronic inflammation and neurodegeneration [30].

Third, apart from components that are released from damaged cells, HS released by exocytosis of HS filled lysosomes, can induce neuroinflammation by interaction with the Toll-like receptor 4 (TLR4) and the MyD88 signaling pathway [21]. In MPS III, this ligation induces the synthesis and activation of inflammatory cytokines and chemokines such as monocyte chemoattractant protein, macrophage inflammatory protein, interleukin-1α, interleukin-1β, cytochrome β-558, interferon-γ, complement components and cathepsin S [21,22,31]. This induces a proinflammatory phenotype in microglia, the resident mononuclear phagocytes, which are abundantly present in the brain [22,31]. Enhanced chemokine expression results in the recruitment and migration of leukocytes from the peripheral circulation into the CNS. Furthermore ligation of TLRs leads to oxidative stress by activation of NADPH oxidase and inducible nitric oxide synthase, which results in the release of superoxide and nitric oxide. This process damages surrounding neurons, further exacerbating disease pathogenesis [32,33].

In addition to the activation of the innate immune system, accumulating GAGs may have the potential to activate the adaptive immune system via both microglia and dendritic cells. Ligation of HS with TLR4 induces dendritic cell maturation and activation by activating the NFκB pathway, leading to an increased expression of co-stimulatory molecules on dendritic

cells such as CD80/86, CD40 and MHCII, resulting in an enhanced antigen presenting potential and T cell proliferation [34–36]. An increase in CD8+ cytotoxic T-cells and elevated MHCI levels has been shown in MPS IIIB mice, which may contribute to neuronal dysfunction in the brain of these animals [36].

The fourth pathophysiological mechanism that is likely to contribute to the neurological symptoms seen in MPS III is altered cell signaling. The HS chains of proteoglycans serve as important carriers for enzymes, lipoproteins, chemokines, interleukins and morphogens, thereby playing an essential role in lipid metabolism, inflammatory processes, angiogenesis and morphogenesis. Acting as coreceptors, HS proteoglycans can directly modulate fibroblast growth factor, Hedgehog, transforming growth factor-β, bone morphogenic protein and Wnt signaling pathways [37–40]. Accumulation of GAGs and changes in the sulfation of HS have been shown to occur in MPS I and MPS III animal models [22,41,42]. Since HS containing GAGs have such wide-ranging functions, these changes may easily affect the signaling pathways and interaction within and between cells.

Finally, impairment of the pre- and postsynaptic signaling function is likely involved in the pathogenesis of MPS III. The endosome-lysosome compartment is crucial for proper neurotransmitter release. In adrenal chromaffin cells of MPS IIIA mice, the presence of an increased number of immature chromaffin granules and impaired catecholamine release has been documented, suggesting a defect in biogenesis and/or cell surface docking and fusion potential of these vesicles [43]. If the same events occur in CNS neurons, this reduced neurotransmitter release could lead to impaired cognitive functioning. Synaptic signaling events may further be affected by changes in synaptic proteins as was observed in MPS I, MPS IIIA and MPS IIIB mice [22,44].

Aim of this review

MPS III is a devastating, progressive inborn error of metabolism that leads to severe neurocognitive deterioration and early death. So far, no disease-modifying therapy exists and treatment focuses on supportive interventions. Unraveling the pathophysiology of MPS III is accompanied by a search for treatment options. This review focuses on the current therapeutic studies and potential future treatment strategies for MPS III.

EMERGING THERAPEUTIC APPROACHES

The different therapeutic approaches that are currently investigated for MPS III will be discussed in this section. An overview is given in table 2.

Enzyme replacement therapy

Since the group of Neufeld *et al.* discovered that co-culturing fibroblasts from patients with distinct MPS subtypes resolved GAG storage by cross-correction of the enzyme deficiency [45], the potentials of intravenously administered exogenous enzyme, enzyme replacement therapy (ERT), for lysosomal storage disorders are continuously investigated. The administered lysosomal hydrolases are transported into the cell and directed towards the lysosomes by the mannose-6-phosphate (M6P) receptor-mediated pathway [46]. ERT was first shown to be highly successful in the treatment of the visceral and somatic signs and symptoms of Gaucher disease [47,48]. ERT has subsequently been registered for other LSDs including Fabry disease, Pompe disease [49] and for the treatment of somatic symptoms in MPS I, MPS II and MPS VI [50–52].

However, intravenously administered enzymes appear not able to cross the blood-brain barrier (BBB), at least not in sufficient quantities [53]. Since the CNS is the most prominently affected organ system in MPS III, intravenous ERT will therefore probably not ameliorate the course of the disease. To overcome this problem, several ERT-based approaches that cross or circumvent the BBB are currently under investigation.

First, receptor-mediated transcytosis might be used to transport macromolecules, including lysosomal hydrolases, across the BBB [54]. For MPS I, the endogenous insulin receptor was used to ferry the missing enzyme, α-L-iduronidase (IDUA), over the BBB, by fusing the enzyme to a monoclonal antibody specific to the human insulin receptor. The fusion protein was taken up by MPS I fibroblasts in a M6P-independent manner, leading to an increase in IDUA enzyme activity and a decrease in GAG levels. After infusion of the fusion protein in primates, 1% of the administered enzyme dose was rapidly taken up in the brain [55]. Similar results were obtained in MPS I mice using the transferrin receptor [56]. Another promising approach is the fusion of therapeutic protein with a receptor-binding peptide such as apolipoprotein E (ApoE) that interferes with low density lipoprotein receptor superfamily proteins which are abundantly present on the BBB and in the brain parenchyma. In a recent study, this approach led to increased IDUA levels and less GAG storage in the brains of MPS I mice [57]. No studies have been reported on transcytosis of fusion proteins in MPS III. In an MPS VII mouse model, removing the M6P residue from recombinant β-glucuronidase in combination with high dose therapy improved enzyme distribution in the brain and cleared neuronal storage, suggesting a different delivery system for crossing the BBB [58,59]. However, removing the M6P residues from SGSH did not improve enzyme delivery in the brain of MPS IIIA mice [60].

Second, enzymes may cross the BBB encapsulated in liposomes or polymeric nanoparticles which are equipped with a target vector to facilitate receptor-mediated transcytosis [61,62]. These nanocarriers have been successfully used for transport of drugs across the BBB in experimental models of several neurodegenerative diseases [63] and have been investigated

in clinical trials to target CNS tumors [64,65] (clinicaltrials.gov, identifier: NCT01386580). In *in vitro* and *in vivo* models of type B Niemann-Pick disease, Fabry disease and Pompe disease, systemic enzyme distribution and uptake was enhanced, when enzyme was coupled to a nanocarrier containing an antibody specific to the intracellular cell adhesion molecule receptor [66–68]. Although this innovative technique is gaining interest, no studies investigating the use of nanoparticles have been conducted so far for drug delivery in LSDs in which the CNS is affected.

A third and currently well studied strategy is the direct delivery of the recombinant enzyme in the CNS. In MPS IIIA mice, enzyme directly delivered to the brain parenchyma via intracerebral injection delayed the onset of neuropathological changes [69]. After frequent injection of recombinant human sulfamidase (rhSGSH) in the cerebrospinal fluid of this MPS IIIA mouse model, lower HS derived mono-sulfated dissacharide levels and less storage vesicles were observed, which was accompanied by improved open-field behavior [70]. Intraventricular delivery of rhSGSH in an MPS IIIA dog model resulted in delivery of enzyme in various brain regions. Furthermore, a dose-dependent decrease in primary as well as secondary storage products and a reduction of neuroinflammation was seen [71,72].

These promising findings have been translated into a phase I/II clinical trial on safety and tolerability of intrathecal delivery of rhSGSH in MPS IIIA patients. The extension of this study is currently ongoing (clinicaltrials.gov, identifier: NCT01299727) and first results are to be expected soon.

Considering the different approaches for the treatment of the MPSs, it is important to bear in mind that any form of ERT is unlikely to be successful in MPS IIIC since HGSNAT is a lysosomal transmembrane protein [73] that is not likely to be replaced by ERT. An important potential drawback of ERT in general is the formation of antibodies directed against the exogenous administered enzyme which diminishes treatment efficacy, as is seen in patients with Pompe disease [74,75], Fabry disease [76], and MPS I Hurler patients who received ERT [77]. So far it is unknown if intrathecal ERT in MPS III patients results in antibody formation. Based on the experiences in other LSDs however, it is essential to monitor the occurrence of a refractory immune response in MPS III patients receiving ERT and to determine the influence on treatment outcome.

Hematopoietic stem cell transplantation

The first successful allogeneic hematopoietic stem cell transplantation (HSCT) in an MPS I patient with the severe Hurler phenotype, paved the way for further research on the use of HSCT in the treatment of the MPSs [78]. The mechanism of HSCT in MPSs is based on the assumption that donor derived HSCs with normal enzyme activity can cross the BBB and transform to microglia cells [79]. These cells may subsequently secrete lysosomal enzymes and thus cross-correct enzyme deficient cells in a way similar to ERT. Several studies have

shown that early HSCT in MPS I Hurler patients can halt or prevent neurocognitive decline and treats several of the somatic symptoms [80–84]. The use of human umbilical cord blood (hUCB) instead of bone marrow as stem cell source, as well as improved chemotherapeutic conditioning regimens, significantly reduced morbidity and mortality and improved engraftment [85,86]. The experience with HSCT in other MPSs is limited and the reported outcomes vary. No clear benefits of HSCT were shown in patients with MPS IIIA and MPS IIIB [87–90]. However, in contrast to MPS I, where patients are generally diagnosed before the age of 2 years on the basis of somatic signs and symptoms, patients with MPS III are diagnosed significantly later in life and almost invariably because of symptoms of CNS disease. It might well be that earlier HSCT, before the onset of clinically detectable CNS involvement, will improve outcome.

The efficacy of HSCT for MPS III has been studied in animal models. While in the MPS IIIA mouse model allogeneic stem cell transplantation did not alter neurological outcome, although a substantial number of donor cells reached the host brain [91], a study in MPS IIIB mice did show behavioral improvement after hUCB cell transplantation [92]. From a group of 19 MPS IIIA and MPS IIIB patients who underwent hUCB transplantation, 9 out of 12 surviving patients showed disease stabilization and probably a more attenuated cognitive impairment [93]. However, the two patients from this cohort that were transplanted before the age of 2 years, still had an overall developmental delay, though modest improvements in their cognitive functioning have been reported. More detailed and prospective studies on the efficacy of HSCT in patients with MPS III, taking into account the variable phenotypic expression of the disease, are urgently needed.

Gene therapy

Similar to the previously discussed therapeutic approaches, gene therapy aims at creating a reservoir of enzyme that is released by cells into the circulation to cross-correct the deficient cells [94]. Since restoration of enzyme activity in affected cells to levels greater than 10% of normal is likely to be therapeutic, a small number of genetically modified cells may allow for sufficient and sustained enzyme production and distribution [94,95]. The cDNA coding for most lysosomal enzymes is relatively small. Therefore, vector systems with limited carrying capacity, such as adeno-associated virus (AAV) are highly effective for gene delivery [96]. There are two different approaches to gene therapy; an *in vivo* and *ex vivo* approach.

In vivo gene therapy

Direct intraventricular or intracerebral vector delivery appears to be a suitable route for targeting the CNS. Several MPS IIIA and MPS IIIB animal studies showed widespread correction of biochemical and histological parameters in the brain along with improvement

of behavioral symptoms after direct administration of a gene therapy vector [97–103]. The use of direct intracerebral gene therapy in MPS IIIA patients is currently under investigation (clinicaltrials.gov, identifier: NCT01474343).

Based on experiences with exogenously administered ERT, it has long been thought that lysosomal enzymes could not cross the BBB. AAV8-mediated liver directed gene transfer resulting in supraphysiological enzyme levels, however, led to less GAG storage in brain and a reduction of CNS pathology in MPS IIIA mice [104]. Similar to modified ERT, it has been shown in MPS IIIA mice that the intravenous administration of a liver-directed modified sulfaminidase, containing an ApoB binding protein, led to successful transcytosis of the enzyme over the BBB and a reduction of neurological pathology [105]. Another method to overcome the BBB is by the use of neurotropic vectors, such as AAV-9 that have the ability to cross the BBB after systemic administration. In MPSIIIA and MPS IIIB mice, correction of neurological disease was seen in studies using AAV-9 as a vector [106,107].

Ex vivo gene therapy

The positive effects of HSCT in some of the LSDs led to studies on *ex vivo* gene therapy. In this approach, autologous bone marrow derived HSCs are transfected *ex vivo*, after which the transfected cells are transfused back into the donor. Using retroviral vectors, this technique was successful in patients with X-linked adrenoleukodystrophy [108], a peroxisomal disorder and in adenosine deaminase-deficient severe combined immune deficiency [109]. A phase I/II study on the safety and efficacy of autologous HSCT transfected with a lentiviral vector in metachromatic leukodystrophy, a LSD, is currently ongoing (clinicalcrials.gov, identifier: NCT01560182).

The disappointing results of allogeneic HSCT in MPS III patients argue against *ex vivo* HSC-directed gene therapy in this disorder. HSC-directed gene therapy in MPS IIIB mice, however, resulted in migration of HSCs into the brain and a reduction of neuropathologic changes [110]. Also, transplantation with wild type cells transfected with a lentiviral vector expressing the SGSH gene, ameliorated neuropathology and increased SGSH activity in brain cells of MPS IIIA mice to a greater extent than transfected MPS IIIA cells, indicating that the level of enzyme expression influences treatment efficacy [111].

The use of autologous cells reduces the morbidity and mortality related to allogeneic HSCT and prevents the onset of graft-versus-host disease. Nevertheless, the risks are not fully known and may be considerable. Potential risks of both *in* and *ex vivo* gene therapy consist of retrovirally induced mutagenesis [112] and transcriptional interference between the vector and flanking endogenous genes that can lead to silencing and transcriptional deregulation [113]. A disadvantage of *in vivo* gene therapy is the immunogenic response to viral vectors and the potential need for immune suppression.

Substrate reduction therapy

The aim of substrate reduction therapy (SRT) is to reduce GAG synthesis and subsequently prevent the accumulation of HS. For this purpose several small molecules have been identified. The best studied compound for MPS III is genistein (4',5,7-trihydroxyisoflavone or 5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one). This isoflavone belongs to the group of flavonoids and is mainly found in soy based foods [114]. It interferes with the epidermal growth factor dependent pathway which regulates expression of genes involved in the biosynthesis of GAGs [115]. There is evidence that small quantities of genistein can cross the blood-brain barrier [116].

In vitro studies have demonstrated that genistein inhibits GAG synthesis in MPS IIIA en MPS IIIB patients' fibroblasts [117]. In an MPS IIIB mouse model it has been shown that highly dosed genistein reduces total GAGs levels and lysosome compartment size in liver [118]. Using the same mouse model it has been demonstrated that continuous, long-term genistein treatment reduces GAG storage in brain and subsequent neuroinflammation. Furthermore, these MPS IIIB mice showed normalized behavior on genistein treatment [119].

Although an earlier open label study in MPS III patients showed promising results, a double blind placebo controlled cross-over trial could not detect any clinical effects and only small, though significant, biochemical effects, of genistein at 10 mg/kg/day in 30 MPS III patients [120–122]. Possibly, a higher dose of genistein increases efficacy as was suggested by recent studies in patients and animal models [118,119,123].

New strategies for the use of SRT in the treatment of MPS III are still exploited. The combined treatment of genistein or analogue isoflavones and flavonoids showed promising synergistic effects *in vitro* [124,125]. A recent study identified synthetic genistein derivatives with the ability to effectively inhibit GAG synthesis in MPS IIIA and MPS IIIB patients' fibroblasts [125]. These approaches will probably be further investigated in the near future in clinical trials in MPS III patients.

Another approach of SRT that aims at the reduction of secondary storage products has also been investigated. Secondary to HS storage, GM2 and GM3 accumulation has been observed in neurons of MPS III patients [16–19]. Miglustat (*N*-butyldeoxynojirimycin, OGT 918) is an iminosugar that is registered for mild to moderate type I Gaucher disease in cases in which ERT is considered not to be suitable [126]. In higher doses it can reach the brain where it has been shown to reduce brain ganglioside levels in mice by reversibly inhibiting glucosylceramide synthase, the enzyme that catalyzes the rate limiting step in glycosphingolipid biosynthesis [127]. A randomized double-blind placebo controlled trial on the use of orally administered miglustat in MPS III patients could not detect any effect on cognitive function or ganglioside levels, although a significant amount of drug did pass the BBB [128].

Substrate optimization therapy

Substrate optimization therapy offers another and relatively new approach for the treatment of several MPSs, including MPS III. By modifying the biosynthesis of GAGs, degradation of GAGs could be completed by alternative enzymes, circumventing the need for the deficient lysosomal proteins [129]. Changing the structure of HS by inhibiting the 2-O sulfation, decreased HS accumulation in MPS I, MPS II and MPS III patients' fibroblasts [130] and these compounds have shown to penetrate the BBB in mice. However it is unclear whether changes in sulfation of HS and HS containing proteoglycans will affect its biological function.

Chaperones

Lysosomal enzymes are synthesized in the endoplasmic reticulum (ER) where endogenous molecular chaperones facilitate proper protein folding. The molecular chaperones are part of the protein quality control system. Chaperones interact with misfolded proteins in the ER thereby promoting folding into their native states [131]. Proteins are then further modified in the Golgi apparatus and transported to the lysosome [132]. In several genetic diseases, including LSDs, certain missense mutations cause protein misfolding, retention of the protein in the ER and subsequent degradation via the ubiquitin-proteasome pathway. It is not surprising that upregulation of the molecular chaperone machinery has been studied as a therapeutic approach in protein conformation-associated diseases such as certain neurodegenerative disorders [133]. To date, this approach has been shown to attenuate lysosomal storage in fibroblasts of patients with two different LSDs, Niemann Pick disease type C and Gaucher disease respectively [134,135].

Proof-of-concept for the use of pharmacological chaperones has been established for several LSDs [136], resulting in clinical trials for Fabry disease, Gaucher disease, GM2 gangliosidosis and Pompe disease. While in a phase II clinical trial in Fabry patients the treatment was well tolerated, increased enzyme activity and lowered storage levels [137], some patients with late-onset GM2-gangliosidosis showed serious neurologic side effects after chaperone therapy [138] and a phase II clinical trial on the safety and tolerability of a pharmacological chaperone in Pompe patients was preliminary terminated because of treatment related side effects (clinicaltrials.gov, identifier: NCT00688597). No results have been published yet for a phase II study in patients with Gaucher disease (clinicaltrials.gov, identifier: NCT00813865) and patients with Pompe disease (clinicaltrials.gov identifier: NCT01380743) [139].

In the group of MPSs, the potentials of chaperone therapy have been studied for MPS IIIB and MPS IIIC. Two *N*-acetylglucosaminidase inhibitors, namely 2-acetamido-1,2-dideoxynojirimycin and 6-acetamido-6-deoxycastanospermine have been identified to act as potential chemical chaperones that can bind and stabilize mutant NAGLU [140]. These compounds however, are rather nonspecific thereby acting as potent inhibitors of several other enzymes as well [141]. In MPS IIIC it has been shown that an overall majority of missense

mutations cause enzyme misfolding, abnormal glycosylation and protein retention in the ER and that the competitive HSGNAT inhibitor glucosamine is able to mediate refolding in patients' fibroblasts [142].

Although *in vitro* data are promising, more studies are needed to confirm the potentials of chaperones for the treatment of LSDs and MPS III in particular. Important to bear in mind are the restrictions of chaperone therapy. First of all, chaperone therapy will be only applicable to a subset of patients, i.e. who have missense mutations which do not prohibit proper folding and have only a mild effect on enzyme activity [136]. Furthermore, pharmacological chaperones bind to the same active site as the substrate, thereby stabilizing the mutant enzyme, but potentially preventing full enzyme activity [143]. Preferably, next generation chaperones stabilize proteins by binding at alternative sites to prevent enzyme inhibition.

Anti-inflammatory and immunosuppressive treatment

It is clear that the accumulation of undegraded HS in cells of MPS III patients triggers multiple pathophysiological cascades. As long as no definite strategy has been identified to prevent HS storage, treatment options are now primarily aimed at halting or slowing down the processes leading to neuronal death. Inflammation has been shown to be an important factor in CNS disease and several approaches that aim at slowing this process have been investigated. Indeed, MPS IIIA and MPS IIIB mice have shown to benefit from immunosuppressive and anti-inflammatory treatment with prednisone and acetylsalicylic acid, respectively [36,144]. Slowing of disease progression by treatment with nonsteroidal anti-inflammatory drugs was also seen in animal models of other LSDs, i.e. Sandhoff disease and Niemann-Pick disease type C [145,146]. In the future, these drugs might play a role in the treatment of MPS III patients as part of a combined treatment regimen together with the currently investigated therapeutic agents.

EXPERT OPINION

A rapid increase of knowledge of the underlying pathophysiological mechanisms marks the field of MPS III and related LSDs, resulting in exciting and encouraging therapeutic developments. Although this has not yet resulted in any approved disease-modifying treatment for MPS III, it seems highly likely that a number of treatment options will be registered within the next decade.

MPS III predominantly affects the CNS, initially leading to a developmental delay and behavioral problems, followed by progressive neurocognitive deterioration and finally pyramidal tract disease. Therefore, targeting the BBB is the biggest challenge in the development of drugs for MPS III. Several promising approaches have been explored in

	Proof of principle in animal studies	Clinical studies
Enzyme replacement therapy		
Receptor-mediated transcytosis of modified ERT	No studies performed yet in MPS III Promising results in MPS I [55–57]	
ERT packaged in nanocarriers	Brain delivery of drugs in several LSD animal models [66–68]	
ERT delivery into the CNS	Disease attenuation in MPS IIIA mice and dogs [69–72]	Phase I/II clinical trial in MPS IIIA patients is ongoing (clinicaltrials.gov identifier: NCT01299727)
Hematopoietic stem cell transplantation		
HSCT	Donor cell engraftment in brain, but no improvement in disease outcome in MPS IIIA mice [91]	Used as treatment for MPS I [80–84] No effect seen in patients with MPS IIIA and MPS IIIB [87–90]
UCBT	Behavioral improvement in MPS IIIB mice [92]	Disease stabilization and attenuation of cognitive impairment in MPS III patients [93]
Gene therapy		
In vivo gene therapy	Amelioration of disease after direct CNS delivery of vector in MPS IIIA and MPS IIIB animals [97–103] Reduction of neuropathology by a liver targeted vector, resulting in supraphysiological SGSH expression [104] Reduction of neuropathology in MPS IIIA mice by modified SGSH allowing for receptor-mediated transcytosis over the BBB [105] Attenuation of disease with a brain targeted vector in MPSIIIA and MPS IIIB mice [106,107]	Phase I/II clinical trial in MPS IIIA patients is ongoing (clinicaltrials.gov identifier: NCT01474343).
Ex vivo gene therapy	Promising results in MPSIIIA and MPS IIIB mice [110,111]	
Small molecules		
Substrate reduction therapy	High dose genistein treatment improved disease outcome in MPS IIIB mice [119]	Mixed results of genistein in MPS III patients [120–123] No effect of miglustat on cognitive function of MPS III patients [128]
Substrate optimization therapy	Compounds which cross the BBB and inhibit HS sulfation reduced storage in MPS III cells [130]	
Chaperones	Established for several LSDs [136], for MPS III only tested in cells [140,142]	

Table 2. Studies performed on different therapies for MPSIII. ERT, enzyme replacement therapy; MPS, mucopolysaccharidosis; LSD, lysosomal storage disorder; HSCT, hematopoietic stem cell transplantation; UCBT, umbilical cord blood transplantation; CNS, central nervous system; SGSH, heparan *N*-sulfatase; BBB, blood-brain barrier; HS, heparan sulfate.

experimental models of MPS III and related LSDs. Receptor-mediated transcytosis by fusion of the enzyme with proteins that are recognized by receptors lining the BBB, has shown promising results in MPS I [55–57]. It is to be expected that this approach will be explored in MPS III in the near future. Already, AAV-mediated gene transfer to the liver *in vivo* resulted in successful production of an endogenous sulfaminidase-ApoB fusion protein, which crossed the BBB [105]. Also, *ex vivo* gene therapy techniques overexpressing the deficient enzyme in autologous HSCs, have been shown to significantly reduce neuropathologic changes [110,111]. This approach offers new possibilities for HSCT in the treatment of MPS III.

In addition to treatment strategies based on replacing the deficient enzyme, much research is directed towards drugs that may enhance residual enzyme activity. Pharmacological chaperones, which stabilize misfolded enzymes, are an attractive therapeutic option, since their small size may allow for transport over the BBB. As many patients with MPS III have missense mutations causing protein misfolding [142], further exploration of the potentials of chaperone therapy is needed. The first clinical trials in other LSDs, however, showed variable tolerability and potential deleterious effects of chaperones need to be controlled.

In view of the complex pathophysiological cascades, all triggered by the accumulation of HS, combination therapy will probably be the most successful approach. Furthermore, optimal treatment strategies may differ between the MPS III subtypes. For instance, MPS IIIC patients are unlikely to benefit from ERT, gene therapy or HSCT because the deficient enzyme HGSNAT is a transmembrane protein and unlikely to be inserted into the proper location when it is produced outside the target cell, in contrast to the soluble enzymes affected in the other MPS III subtypes. MPS IIIC patients however could benefit from chaperone therapy and/or SRT. Finally, patients at different stages of the disease may need different therapeutic regimes for optimal efficacy. For instance, patients initiated on treatment at a very early stage of the disease might be optimally treated by ERT, gene therapy or HSCT as single treatment, while patients with more advanced disease may need addition of anti-inflammatory drugs to stop otherwise ongoing neuroinflammation.

To optimize treatment strategy for different groups of patients, double-blind placebo controlled trials are needed to objectively evaluate treatment efficacy. In these studies, preservation of cognitive function will be the most relevant clinical endpoint. In the current situation, improvement or preservation of cognitive function may be difficult to assess, as many patients already show significant loss of cognition at the time of diagnosis and it may be difficult to stop the progression of neuronal loss on time. Therefore, the use of biomarkers of disease progression, studied in natural history studies, are essential for establishing a reasonably likely effect of drugs in studies in symptomatic patients. Natural history studies for MPS IIIA and MPS IIIB are currently recruiting (clinciltrials.gov identifier: NCT01047306 and NCT01509768 respectively).

Despite all promising developments for treatment of MPS III, efficacy of any kind of treatment will benefit tremendously from early initiation, preferably before the onset of symptoms. Currently, almost all MPS III patients are diagnosed based on a developmental delay and behavioural problems. At that moment severe and irreversible damage to the CNS has already set in. In the best case scenario, a disease-modifying therapy may only halt disease progression. Apart from sibling screening, the only way to identify MPS III patients before the onset of clinical CNS disease is by newborn population screening. Traditionally, newborn screening (NBS) programs aim at diagnosing genetic disorders in a very early stage in order to prevent or reduce clinical symptoms by early initiation of treatment. Based on the classical screening criteria of Wilson and Junger, the availability of an effective treatment is required to allow screening for a particular disease [147]. However, the interpretation of this last criterion is widely discussed. Early diagnosis of a disorder for which there is still no disease-modifying therapy has several important advantages. These include early initiation of disease specific supportive interventions, which will improve quality of life of the patient, as well as providing the opportunity for genetic counseling of affected families. In addition, early diagnosis will allow for trials on the efficacy of treatment in presymptomatic patients, which may be of particular relevance in MPS III. A recent study demonstrated the feasibility of NBS for MPS III [148].

In conclusion, MPS III is a devastating and primarily neurodegenerative and invariably progressive disease that severely affects quality of life of both patients and their families. We strongly believe that the current efforts to unravel the pathophysiology of MPS III and the search for therapies to prevent disease onset and/or modify its course will result in a disease-modifying treatment in the near future. However, presymptomatic diagnosis allowing early treatment is essential for optimal treatment efficacy. This will only be feasible through NBS.

REFERENCES

1. Muenzer J. Overview of the mucopolysaccharidoses. *Rheumatology*. 2011;50 Suppl 5:v4-12.
2. Poorthuis BJ, Wevers RA, Kleijer WJ, et al. The frequency of lysosomal storage diseases in The Netherlands. *Hum Genet*. 1999;105(1-2):151-156.
3. Meikle PJ, Hopwood JJ, Clague AE, Carey WF. Prevalence of lysosomal storage disorders. *JAMA*. 1999;281(3):249-254.
4. Baehner F, Schmiedeskamp C, Krummenauer F, et al. Cumulative incidence rates of the mucopolysaccharidoses in Germany. *J Inherit Metab Dis*. 2005;28(6):1011-1017.
5. Valstar MJ, Ruijter GJ, Van Diggelen OP, Poorthuis BJ, Wijburg FA. Sanfilippo syndrome: A mini-review. *J Inherit Metab Dis*. 2008;31(2):240-252.
6. Ruijter GJ, Valstar MJ, van de Kamp JM, et al. Clinical and genetic spectrum of Sanfilippo type C (MPS IIIC) disease in The Netherlands. *Mol Genet Metab*. 2008;93(2):104-111.
7. Meyer A, Kossow K, Gal A, et al. Scoring evaluation of the natural course of mucopolysaccharidosis type IIIA (Sanfilippo syndrome type A). *Pediatrics*. 2007;120(5):e1255-61.
8. Wijburg FA, Węgrzyn G, Burton BK, Tylki-Szymańska A. Mucopolysaccharidosis type III (Sanfilippo syndrome) and misdiagnosis of idiopathic developmental delay, attention deficit/hyperactivity disorder or autism spectrum disorder. *Acta Paediatr*. 2013;102(5):462-470.
9. Moog U, van Mierlo I, van Schrojenstein Lantman-de Valk HM, Spaapen L, Maaskant MA, Curfs LM. Is Sanfilippo type B in your mind when you see adults with mental retardation and behavioral problems? *Am J Med Genet C Semin Med Genet*. 2007;145C(3):293-301.
10. Valstar MJ, Bruggenwirth HT, Olmer R, et al. Mucopolysaccharidosis type IIIB may predominantly present with an attenuated clinical phenotype. *J Inherit Metab Dis*. 2010;33(6):759-767.
11. Héron B, Mikaeloff Y, Froissart R, et al. Incidence and natural history of mucopolysaccharidosis type III in France and comparison with United Kingdom and Greece. *Am J Med Genet A*. 2011;155A(1):58-68.
12. Vitry S, Bruyère J, Hocquemiller M, et al. Storage vesicles in neurons are related to Golgi complex alterations in mucopolysaccharidosis IIIB. *Am J Pathol*. 2010;177(6):2984-2999.
13. Lemonnier T, Blanchard S, Toli D, et al. Modeling neuronal defects associated with a lysosomal disorder using patient-derived induced pluripotent stem cells. *Hum Mol Genet*. 2011;20(18):3653-3666.
14. Roy E, Bruyère J, Flamant P, et al. GM130 gain-of-function induces cell pathology in a model of lysosomal storage disease. *Hum Mol Genet*. 2012;21(7):1481-1495.
15. Nakamura N. Emerging new roles of GM130, a cis-Golgi matrix protein, in higher order cell functions. *J Pharmacol Sci*. 2010;112(3):255-264.
16. Constantopoulos G, Eiben RM, Schafer IA. Neurochemistry of the mucopolysaccharidoses: brain glycosaminoglycans, lipids and lysosomal enzymes in mucopolysaccharidosis type III B (alpha-N-acetylglucosaminidase deficiency). *J Neurochem*. 1978;31(5):1215-1222.
17. Constantopoulos G, Dekaban AS. Neurochemistry of the mucopolysaccharidoses: brain lipids and lysosomal enzymes in patients with four types of mucopolysaccharidosis and in normal controls. *J Neurochem*. 1978;30(5):965-973.
18. Constantopoulos G, Iqbal K, Dekaban AS. Mucopolysaccharidosis types IH, IS, II, and IIIA: glycosaminoglycans and lipids of isolated brain cells and other fractions from autopsied tissues. *J Neurochem*. 1980;34(6):1399-1411.
19. Jones MZ, Alroy J, Rutledge JC, et al. Human mucopolysaccharidosis IIID: clinical, biochemical, morphological and immunohistochemical characteristics. *J Neuropathol Exp Neurol*. 1997;56(10):1158-1167.

20. Crawley AC, Gliddon BL, Auclair D, et al. Characterization of a C57BL/6 congenic mouse strain of mucopolysaccharidosis type IIIA. *Brain Res*. 2006;1104(1):1-17.
21. Ausseil J, Desmaris N, Bigou S, et al. Early neurodegeneration progresses independently of microglial activation by heparan sulfate in the brain of mucopolysaccharidosis IIIB mice. *PLoS One*. 2008;3(5):e2296.
22. Wilkinson FL, Holley RJ, Langford-Smith KJ, et al. Neuropathology in mouse models of mucopolysaccharidosis type I, IIIA and IIIB. *PLoS One*. 2012;7(4):e35787.
23. McGlynn R, Dobrenis K, Walkley SU. Differential subcellular localization of cholesterol, gangliosides, and glycosaminoglycans in murine models of mucopolysaccharide storage disorders. *J Comp Neurol*. 2004;480(4):415-426.
24. Mohammed EE, Snella EM, Rutz-Mendicino MM, et al. Accelerated clinical disease and pathology in mucopolysaccharidosis type IIIB and GalNAc transferase double knockout mice. *Mol Genet Metab*. 2012;107(1-2):129-135.
25. Ohmi K, Kudo LC, Ryazantsev S, Zhao H-Z, Karsten SL, Neufeld EF. Sanfilippo syndrome type B, a lysosomal storage disease, is also a tauopathy. *Proc Natl Acad Sci USA*. 2009;106(20):8332-8337.
26. Ohmi K, Zhao H-Z, Neufeld EF. Defects in the medial entorhinal cortex and dentate gyrus in the mouse model of Sanfilippo syndrome type B. *PLoS One*. 2011;6(11):e27461.
27. Ginsberg SD, Galvin JE, Lee VM, et al. Accumulation of intracellular amyloid-beta peptide (A beta 1-40) in mucopolysaccharidosis brains. *J Neuropathol Exp Neurol*. 1999;58(8):815-824.
28. Hamano K, Hayashi M, Shioda K, Fukatsu R, Mizutani S. Mechanisms of neurodegeneration in mucopolysaccharidoses II and IIIB: analysis of human brain tissue. *Acta Neuropathol*. 2008;115(5):547-559.
29. Settembre C, Fraldi A, Jahreiss L, et al. A block of autophagy in lysosomal storage disorders. *Hum Mol Genet*. 2008;17(1):119-129.
30. Archer LD, Langford-Smith KJ, Bigger BW, Fildes JE. Mucopolysaccharide diseases: a complex interplay between neuroinflammation, microglial activation and adaptive immunity. *J Inherit Metab Dis*. 2014;37(1):1-12.
31. Ohmi K, Greenberg DS, Rajavel KS, Ryazantsev S, Li HH, Neufeld EF. Activated microglia in cortex of mouse models of mucopolysaccharidoses I and IIIB. *Proc Natl Acad Sci USA*. 2003;100(4):1902-1907.
32. Villani GR, Gargiulo N, Faraonio R, Castaldo S, Gonzalez Y Reyero E, Di Natale P. Cytokines, neurotrophins, and oxidative stress in brain disease from mucopolysaccharidosis IIIB. *J Neurosci Res*. 2007;85(3):612-622.
33. Villani GR, Di Domenico C, Musella A, Cecere F, Di Napoli D, Di Natale P. Mucopolysaccharidosis IIIB: oxidative damage and cytotoxic cell involvement in the neuronal pathogenesis. *Brain Res*. 2009;1279:99-108.
34. Kodaira Y, Nair SK, Wrenshall LE, Gilboa E, Platt JL. Phenotypic and functional maturation of dendritic cells mediated by heparan sulfate. *J Immunol*. 2000;165(3):1599-1604.
35. Johnson GB, Brunn GJ, Kodaira Y, Platt JL. Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4. *J Immunol*. 2002;168(10):5233-5239.
36. DiRosario J, Divers E, Wang C, et al. Innate and adaptive immune activation in the brain of MPS IIIB mouse model. *J Neurosci Res*. 2009;87(4):978-990.
37. Pan C, Nelson MS, Reyes M, et al. Functional abnormalities of heparan sulfate in mucopolysaccharidosis-I are associated with defective biologic activity of FGF-2 on human multipotent progenitor cells. *Blood*. 2005;106(6):1956-1964.
38. Khan SA, Nelson MS, Pan C, Gaffney PM, Gupta P. Endogenous heparan sulfate and heparin modulate bone morphogenetic protein-4 signaling and activity. *Am J Physiol Cell Physiol*. 2008;294(6):C1387-97.
39. Bishop JR, Schuksz M, Esko JD. Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature*. 2007;446(7139):1030-1037.
40. Lindahl U, Kjellén L. Pathophysiology of heparan sulphate: many diseases, few drugs. *J Intern Med*. 2013;273(6):555-571.
41. King B, Savas P, Fuller M, Hopwood J, Hemsley K. Validation of a heparan sulfate-derived disaccharide as a marker of accumulation in murine mucopolysaccharidosis type IIIA. *Mol Genet Metab*. 2006;87(2):107-112.

42. Holley RJ, Deligny A, Wei W, et al. Mucopolysaccharidosis type I, unique structure of accumulated heparan sulfate and increased N-sulfotransferase activity in mice lacking α-l-iduronidase. *J Biol Chem*. 2011;286(43):37515-37524.

43. Keating DJ, Winter MA, Hemsley KM, et al. Exocytosis is impaired in mucopolysaccharidosis IIIA mouse chromaffin cells. *Neuroscience*. 2012;227:110-118.

44. Vitry S, Ausseil J, Hocquemiller M, Bigou S, Dos Santos Coura R, Heard JM. Enhanced degradation of synaptophysin by the proteasome in mucopolysaccharidosis type IIIB. *Mol Cell Neurosci*. 2009;41(1):8-18.

45. Fratantoni JC, Hall CW, Neufeld EF. Hurler and Hunter syndromes: mutual correction of the defect in cultured fibroblasts. *Science*. 1968;162(3853):570-572.

46. Braulke T, Bonifacino JS. Sorting of lysosomal proteins. *Biochim Biophys Acta*. 2009;1793(4):605-614.

47. Weinreb NJ, Charrow J, Andersson HC, et al. Effectiveness of enzyme replacement therapy in 1028 patients with type 1 Gaucher disease after 2 to 5 years of treatment: a report from the Gaucher Registry. *Am J Med*. 2002;113(2):112-119.

48. Andersson H, Kaplan P, Kacena K, Yee J. Eight-year clinical outcomes of long-term enzyme replacement therapy for 884 children with Gaucher disease type 1. *Pediatrics*. 2008;122(6):1182-1190.

49. Lachmann RH. Enzyme replacement therapy for lysosomal storage diseases. *Curr Opin Pediatr*. 2011;23(6):588-593.

50. Wraith JE, Clarke LA, Beck M, et al. Enzyme replacement therapy for mucopolysaccharidosis I: a randomized, double-blinded, placebo-controlled, multinational study of recombinant human alpha-L-iduronidase (laronidase). *J Pediatr*. 2004;144(5):581-588.

51. Muenzer J, Wraith JE, Beck M, et al. A phase II/III clinical study of enzyme replacement therapy with idursulfase in mucopolysaccharidosis II (Hunter syndrome). *Genet Med*. 2006;8(8):465-473.

52. Harmatz P, Giugliani R, Schwartz I, et al. Enzyme replacement therapy for mucopolysaccharidosis VI: a phase 3, randomized, double-blind, placebo-controlled, multinational study of recombinant human N-acetylgalactosamine 4-sulfatase (recombinant human arylsulfatase B or rhASB) and follow-on, open-label extension study. *J Pediatr*. 2006;148(4):533-539.

53. Kakkis ED, McEntee MF, Schmidtchen A, et al. Long-term and high-dose trials of enzyme replacement therapy in the canine model of mucopolysaccharidosis I. *Biochem Mol Med*. 1996;58(2):156-167.

54. Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ. Structure and function of the blood-brain barrier. *Neurobiol Dis*. 2010;37(1):13-25.

55. Boado RJ, Zhang Y, Zhang Y, Xia C, Wang Y, Pardridge WM. Genetic engineering of a lysosomal enzyme fusion protein for targeted delivery across the human blood-brain barrier. *Biotechnol Bioeng*. 2008;99(2):475-484.

56. Boado RJ, Hui EK, Lu JZ, Zhou QH, Pardridge WM. Reversal of lysosomal storage in brain of adult MPS-I mice with intravenous Trojan horse-iduronidase fusion protein. *Mol Pharm*. 2011;8(4):1342-1350.

57. Wang D, El-Amouri SS, Dai M, et al. Engineering a lysosomal enzyme with a derivative of receptor-binding domain of apoE enables delivery across the blood-brain barrier. *Proc Natl Acad Sci USA*. 2013;110(8):2999-3004.

58. Grubb JH, Vogler C, Levy B, Galvin N, Tan Y, Sly WS. Chemically modified beta-glucuronidase crosses blood-brain barrier and clears neuronal storage in murine mucopolysaccharidosis VII. *Proc Natl Acad Sci USA*. 2008;105(7):2616-2621.

59. Huynh HT, Grubb JH, Vogler C, Sly WS. Biochemical evidence for superior correction of neuronal storage by chemically modified enzyme in murine mucopolysaccharidosis VII. *Proc Natl Acad Sci USA*. 2012;109(42):17022-17027.

60. Rozaklis T, Beard H, Hassiotis S, et al. Impact of high-dose, chemically modified sulfamidase on pathology in a murine model of MPS IIIA. *Exp Neurol*. 2011;230(1):123-130.

61. Chen Y, Liu L. Modern methods for delivery of drugs across the blood-brain barrier. *Adv Drug Deliv Rev*. 2012;64(7):640-665.

62. Kanwar JR, Sun X, Punj V, et al. Nanoparticles in the treatment and diagnosis of neurological disorders: untamed dragon with fire power to heal. *Nanomedicine*. 2012;8(4):399-414.
63. Re F, Gregori M, Masserini M. Nanotechnology for neurodegenerative disorders. *Nanomedicine*. 2012;8 Suppl 1:S51-8.
64. Beier CP, Schmid C, Gorlia T, et al. RNOP-09: pegylated liposomal doxorubicine and prolonged temozolomide in addition to radiotherapy in newly diagnosed glioblastoma - a phase II study. *BMC Cancer*. 2009;9:308.
65. Ananda S, Nowak AK, Cher L, et al. Phase 2 trial of temozolomide and pegylated liposomal doxorubicin in the treatment of patients with glioblastoma multiforme following concurrent radiotherapy and chemotherapy. *J Clin Neurosci*. 2011;18(11):1444-1448.
66. Garnacho C, Dhami R, Simone E, et al. Delivery of acid sphingomyelinase in normal and niemann-pick disease mice using intercellular adhesion molecule-1-targeted polymer nanocarriers. *J Pharmacol Exp Ther*. 2008;325(2):400-408.
67. Hsu J, Serrano D, Bhowmick T, et al. Enhanced endothelial delivery and biochemical effects of α-galactosidase by ICAM-1-targeted nanocarriers for Fabry disease. *J Control Release*. 2011;149(3):323-331.
68. Hsu J, Northrup L, Bhowmick T, Muro S. Enhanced delivery of α-glucosidase for Pompe disease by ICAM-1-targeted nanocarriers: comparative performance of a strategy for three distinct lysosomal storage disorders. *Nanomedicine*. 2012;8(5):731-739.
69. Savas PS, Hemsley KM, Hopwood JJ. Intracerebral injection of sulfamidase delays neuropathology in murine MPS-IIIA. *Mol Genet Metab*. 2004;82(4):273-285.
70. Hemsley KM, King B, Hopwood JJ. Injection of recombinant human sulfamidase into the CSF via the cerebellomedullary cistern in MPS IIIA mice. *Mol Genet Metab*. 2007;90(3):313-328.
71. Hemsley KM, Norman EJ, Crawley AC, et al. Effect of cisternal sulfamidase delivery in MPS IIIA Huntaway dogs - a proof of principle study. *Mol Genet Metab*. 2009;98(4):383-392.
72. Crawley AC, Marshall N, Beard H, et al. Enzyme replacement reduces neuropathology in MPS IIIA dogs. *Neurobiol Dis*. 2011;43(2):422-434.
73. Meikle PJ, Whittle AM, Hopwood JJ. Human acetyl-coenzyme A:alpha-glucosaminide N-acetyltransferase. Kinetic characterization and mechanistic interpretation. *Biochem J*. 1995;308 (Pt 1):327-333.
74. Kishnani PS, Goldenberg PC, DeArmey SL, et al. Cross-reactive immunologic material status affects treatment outcomes in Pompe disease infants. *Mol Genet Metab*. 2010;99(1):26-33.
75. Bali DS, Goldstein JL, Banugaria S, et al. Predicting cross-reactive immunological material (CRIM) status in Pompe disease using GAA mutations: lessons learned from 10 years of clinical laboratory testing experience. *Am J Med Genet C Semin Med Genet*. 2012;160(1):40-49.
76. Rombach SM, Aerts JM, Poorthuis BJ, et al. Long-term effect of antibodies against infused alpha-galactosidase A in Fabry disease on plasma and urinary (lyso)Gb3 reduction and treatment outcome. *PLoS One*. 2012;7(10):e47805.
77. Saif MA, Bigger BW, Brookes KE, et al. Hematopoietic stem cell transplantation improves the high incidence of neutralizing allo-antibodies observed in Hurler's syndrome after pharmacological enzyme replacement therapy. *Haematologica*. 2012;97(9):1320-1328.
78. Hobbs JR, Hugh-Jones K, Barrett AJ, et al. Reversal of clinical features of Hurler's disease and biochemical improvement after treatment by bone-marrow transplantation. *Lancet*. 1981;2(8249):709-712.
79. Krivit W, Sung JH, Shapiro EG, Lockman LA. Microglia: the effector cell for reconstitution of the central nervous system following bone marrow transplantation for lysosomal and peroxisomal storage diseases. *Cell Transpl*. 1995;4(4):385-392.
80. Peters C, Balthazor M, Shapiro EG, et al. Outcome of unrelated donor bone marrow transplantation in 40 children with Hurler syndrome. *Blood*. 1996;87(11):4894-4902.
81. Peters C, Shapiro EG, Anderson J, et al. Hurler syndrome: II. Outcome of HLA-genotypically identical sibling and HLA-haploidentical related donor bone marrow transplantation in fifty-four children. The Storage Disease Collaborative Study Group. *Blood*. 1998;91(7):2601-2608.

82. Souillet G, Guffon N, Maire I, et al. Outcome of 27 patients with Hurler's syndrome transplanted from either related or unrelated haematopoietic stem cell sources. *Bone Marrow Transpl*. 2003;31(12):1105-1117.
83. Guffon N, Souillet G, Maire I, Straczek J, Guibaud P. Follow-up of nine patients with Hurler syndrome after bone marrow transplantation. *J Pediatr*. 1998;133(1):119-125.
84. Malm G, Gustafsson B, Berglund G, et al. Outcome in six children with mucopolysaccharidosis type IH, Hurler syndrome, after haematopoietic stem cell transplantation (HSCT). *Acta Paediatr*. 2008;97(8):1108-1112.
85. Boelens JJ, Aldenhoven M, Purtill D, et al. Outcomes of transplantation using various hematopoietic cell sources in children with Hurler syndrome after myeloablative conditioning. *Blood*. 2013;121(19):3981-3987.
86. Staba SL, Escolar ML, Poe M, et al. Cord-blood transplants from unrelated donors in patients with Hurler's syndrome. *N Engl J Med*. 2004;350(19):1960-1969.
87. Vellodi A, Young E, New M, Pot-Mees C, Hugh-Jones K. Bone marrow transplantation for Sanfilippo disease type B. *J Inherit Metab Dis*. 1992;15(6):911-918.
88. Güngör N, Tunçbilek E. Sanfilippo disease type B. A case report and review of the literature on recent advances in bone marrow transplantation. *Turk J Pediatr*. 1995;37(2):157-163.
89. Shapiro EG, Lockman LA, Balthazor M, Krivit W. Neuropsychological outcomes of several storage diseases with and without bone marrow transplantation. *J Inherit Metab Dis*. 1995;18(4):413-429.
90. Sivakumur P, Wraith JE. Bone marrow transplantation in mucopolysaccharidosis type IIIA: a comparison of an early treated patient with his untreated sibling. *J Inherit Metab Dis*. 1999;22(7):849-850.
91. Lau AA, Hannouche H, Rozaklis T, Hassiotis S, Hopwood JJ, Hemsley KM. Allogeneic stem cell transplantation does not improve neurological deficits in mucopolysaccharidosis type IIIA mice. *Exp Neurol*. 2010;225(2):445-454.
92. Garbuzova-Davis S, Klasko SK, Sanberg PR. Intravenous administration of human umbilical cord blood cells in an animal model of MPS III B. *J Comp Neurol*. 2009;515(1):93-101.
93. Prasad VK, Mendizabal A, Parikh SH, et al. Unrelated donor umbilical cord blood transplantation for inherited metabolic disorders in 159 pediatric patients from a single center: influence of cellular composition of the graft on transplantation outcomes. *Blood*. 2008;112(7):2979-2989.
94. Sands MS, Davidson BL. Gene therapy for lysosomal storage diseases. *Mol Ther*. 2006;13(5):839-849.
95. Schueler UH, Kolter T, Kaneski CR, Zirzow GC, Sandhoff K, Brady RO. Correlation between enzyme activity and substrate storage in a cell culture model system for Gaucher disease. *J Inherit Metab Dis*. 2004;27(5):649-658.
96. Daly TM, Sands MS. Gene therapy for lysosomal storage diseases. *Expert Opin Investig Drugs*. 1998;7(10):1673-1682.
97. Fu H, Samulski RJ, McCown TJ, Picornell YJ, Fletcher D, Muenzer J. Neurological correction of lysosomal storage in a mucopolysaccharidosis IIIB mouse model by adeno-associated virus-mediated gene delivery. *Mol Ther*. 2002;5(1):42-49.
98. Cressant A, Desmaris N, Verot L, et al. Improved behavior and neuropathology in the mouse model of Sanfilippo type IIIB disease after adeno-associated virus-mediated gene transfer in the striatum. *J Neurosci*. 2004;24(45):10229-10239.
99. Fraldi A, Hemsley K, Crawley A, et al. Functional correction of CNS lesions in an MPS-IIIA mouse model by intracerebral AAV-mediated delivery of sulfamidase and SUMF1 genes. *Hum Mol Genet*. 2007;16(22):2693-2702.
100. Fu H, Kang L, Jennings JS, et al. Significantly increased lifespan and improved behavioral performances by rAAV gene delivery in adult mucopolysaccharidosis IIIB mice. *Gene Ther*. 2007;14(14):1065-1077.
101. Fu H, DiRosario J, Kang L, Muenzer J, McCarty DM. Restoration of central nervous system alpha-N-acetylglucosaminidase activity and therapeutic benefits in mucopolysaccharidosis IIIB mice by a single intracisternal recombinant adeno-associated viral type 2 vector delivery. *J Gene Med*. 2010;12(7):624-633.

102. McIntyre C, Byers S, Anson DS. Correction of mucopolysaccharidosis type IIIA somatic and central nervous system pathology by lentiviral-mediated gene transfer. *J Gene Med*. 2010;12(9):717-728.

103. Ellinwood NM, Ausseil J, Desmaris N, et al. Safe, efficient, and reproducible gene therapy of the brain in the dog models of Sanfilippo and Hurler syndromes. *Mol Ther*. 2011;19(2):251-259.

104. Ruzo A, Garcia M, Ribera A, et al. Liver production of sulfamidase reverses peripheral and ameliorates CNS pathology in mucopolysaccharidosis IIIA mice. *Mol Ther*. 2012;20(2):254-266.

105. Sorrentino NC, D'Orsi L, Sambri I, et al. A highly secreted sulphamidase engineered to cross the blood-brain barrier corrects brain lesions of mice with mucopolysaccharidoses type IIIA. *EMBO Mol Med*. 2013;5(5):675-690.

106. Ruzo A, Marcó S, García M, et al. Correction of pathological accumulation of glycosaminoglycans in central nervous system and peripheral tissues of MPSIIIA mice through systemic AAV9 gene transfer. *Hum Gene Ther*. 2012;23(12):1237-1246.

107. Fu H, DiRosario J, Killedar S, Zaraspe K, McCarty DM. Correction of neurological disease of mucopolysaccharidosis IIIB in adult mice by rAAV9 trans-blood-brain barrier gene delivery. *Mol Ther*. 2011;19(6):1025-1033.

108. Cartier N, Hacein-Bey-Abina S, Bartholomae CC, et al. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science*. 2009;326(5954):818-823.

109. Candotti F, Shaw KL, Muul L, et al. Gene therapy for adenosine deaminase-deficient severe combined immune deficiency: clinical comparison of retroviral vectors and treatment plans. *Blood*. 2012;120(18):3635-3646.

110. Zheng Y, Ryazantsev S, Ohmi K, et al. Retrovirally transduced bone marrow has a therapeutic effect on brain in the mouse model of mucopolysaccharidosis IIIB. *Mol Genet Metab*. 2004;82(4):286-295.

111. Langford-Smith A, Wilkinson FL, Langford-Smith KJ, et al. Hematopoietic stem cell and gene therapy corrects primary neuropathology and behavior in mucopolysaccharidosis IIIA mice. *Mol Ther*. 2012;20(8):1610-1621.

112. Hacein-Bey-Abina S, von Kalle C, Schmidt M, et al. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med*. 2003;348(3):255-256.

113. De Palma M, Montini E, Santoni de Sio FR, et al. Promoter trapping reveals significant differences in integration site selection between MLV and HIV vectors in primary hematopoietic cells. *Blood*. 2005;105(6):2307-2315.

114. Wegrzyn G, Jakóbkiewicz-Banecka J, Gabig-Cimińska M, et al. Genistein: a natural isoflavone with a potential for treatment of genetic diseases. *Biochem Soc Trans*. 2010;38(2):695-701.

115. Jakóbkiewicz-Banecka J, Piotrowska E, Narajczyk M, Barańska S, Wegrzyn G. Genistein-mediated inhibition of glycosaminoglycan synthesis, which corrects storage in cells of patients suffering from mucopolysaccharidoses, acts by influencing an epidermal growth factor-dependent pathway. *J Biomed Sci*. 2009;16:26.

116. Tsai TH. Concurrent measurement of unbound genistein in the blood, brain and bile of anesthetized rats using microdialysis and its pharmacokinetic application. *J Chromatogr A*. 2005;1073(1-2):317-322.

117. Piotrowska E, Jakóbkiewicz-Banecka J, Barańska S, et al. Genistein-mediated inhibition of glycosaminoglycan synthesis as a basis for gene expression-targeted isoflavone therapy for mucopolysaccharidoses. *Eur J Hum Genet*. 2006;14(7):846-852.

118. Malinowska M, Wilkinson FL, Bennett W, et al. Genistein reduces lysosomal storage in peripheral tissues of mucopolysaccharide IIIB mice. *Mol Genet Metab*. 2009;98(3):235-242.

119. Malinowska M, Wilkinson FL, Langford-Smith KJ, et al. Genistein improves neuropathology and corrects behaviour in a mouse model of neurodegenerative metabolic disease. *PLoS One*. 2010;5(12):e14192.

120. Piotrowska E, Jakóbkiewicz-Banecka J, Tylki-Szymanska A, et al. Genistin-rich soy isoflavone extract in substrate reduction therapy for Sanfilippo syndrome: an open-label, pilot study in 10 pediatric patients. *Curr Ther Res Clin Exp*. 2008;69(2):166-179.

121. Piotrowska E, Jakobkiewicz-Banecka J, Maryniak A, et al. Two-year follow-up of Sanfilippo Disease patients treated with a genistein-rich isoflavone extract: assessment of effects on cognitive functions and general status of patients. *Med Sci Monit*. 2011;17(4):CR196-202.

122. de Ruijter J, Valstar MJ, Narajczyk M, et al. Genistein in Sanfilippo disease: a randomized controlled crossover trial. *Ann Neurol*. 2012;71(1):110-120.

123. Malinová V, Węgrzyn G, Narajczyk M. The use of elevated doses of genistein-rich soy extract in the gene expression-targeted isoflavone therapy for Sanfilippo disease patients. *JIMD Rep*. 2012;5:21-25.

124. Arfi A, Richard M, Gandolphe C, Scherman D. Storage correction in cells of patients suffering from mucopolysaccharidoses types IIIA and VII after treatment with genistein and other isoflavones. *J Inherit Metab Dis*. 2010;33(1):61-67.

125. Kloska A, Jakóbkiewicz-Banecka J, Narajczyk M, Banecka-Majkutewicz Z, Węgrzyn G. Effects of flavonoids on glycosaminoglycan synthesis: implications for substrate reduction therapy in Sanfilippo disease and other mucopolysaccharidoses. *Metab Brain Dis*. 2011;26(1):1-8.

126. Elstein D, Hollak C, Aerts JM, et al. Sustained therapeutic effects of oral miglustat (Zavesca, N-butyldeoxynojirimycin, OGT 918) in type I Gaucher disease. *J Inherit Metab Dis*. 2004;27(6):757-766.

127. Platt FM, Neises GR, Dwek RA, Butters TD. N-butyldeoxynojirimycin is a novel inhibitor of glycolipid biosynthesis. *J Biol Chem*. 1994;269(11):8362-8365.

128. Guffon N, Bin-Dorel S, Decullier E, Paillet C, Guitton J, Fouilhoux A. Evaluation of miglustat treatment in patients with type III mucopolysaccharidosis: a randomized, double-blind, placebo-controlled study. *J Pediatr*. 2011;159(5):838-844.e1.

129. Brown JR, Crawford BE, Esko JD. Glycan antagonists and inhibitors: a fount for drug discovery. *Crit Rev Biochem Mol Biol*. 2007;42(6):481-515.

130. Crawford B, Brown J, Tolmie K, et al. Small molecule inhibitors of Glycosaminoglycan Biosynthesis as substrate optimization therapy for the Mucopolysaccharidoses. *Mol Genet Metab*. 2011;102(2):S12-S13.

131. Hartl FU, Bracher A, Hayer-Hartl M. Molecular chaperones in protein folding and proteostasis. *Nature*. 2011;475(7356):324-332.

132. Desnick RJ, Schuchman EH. Enzyme replacement and enhancement therapies: lessons from lysosomal disorders. *Nat Rev Genet*. 2002;3(12):954-966.

133. Cattaneo M, Dominici R, Cardano M, Diaferia G, Rovida E, Biunno I. Molecular chaperones as therapeutic targets to counteract proteostasis defects. *J Cell Physiol*. 2012;227(3):1226-1234.

134. Pipalia NH, Cosner CC, Huang A, et al. Histone deacetylase inhibitor treatment dramatically reduces cholesterol accumulation in Niemann-Pick type C1 mutant human fibroblasts. *Proc Natl Acad Sci USA*. 2011;108(14):5620-5625.

135. Lu J, Yang C, Chen M, et al. Histone deacetylase inhibitors prevent the degradation and restore the activity of glucocerebrosidase in Gaucher disease. *Proc Natl Acad Sci USA*. 2011;108(52):21200-21205.

136. Valenzano KJ, Khanna R, Powe AC, et al. Identification and characterization of pharmacological chaperones to correct enzyme deficiencies in lysosomal storage disorders. *Assay Drug Dev Technol*. 2011;9(3):213-235.

137. Germain DP, Giugliani R, Hughes DA, et al. Safety and pharmacodynamic effects of a pharmacological chaperone on α-galactosidase A activity and globotriaosylceramide clearance in Fabry disease: report from two phase 2 clinical studies. *Orphanet J Rare Dis*. 2012;7(1):91.

138. Clarke JT, Mahuran DJ, Sathe S, et al. An open-label Phase I/II clinical trial of pyrimethamine for the treatment of patients affected with chronic GM2 gangliosidosis (Tay-Sachs or Sandhoff variants). *Mol Genet Metab*. 2011;102(1):6-12.

139. Kishnani P, Tarnopolsky M, Sivakumar K, et al. T.P.45 An ongoing phase 2a study to investigate drug–drug interactions between escalating doses of AT2220 (duvoglustat hydrochloride) and acid alpha glucosidase in subjects with Pompe disease – Preliminary results. *Neuromusc Dis*. 2012;22(9-10):852.

140. Ficko-Blean E, Stubbs KA, Nemirovsky O, Vocadlo DJ, Boraston AB. Structural and mechanistic insight into the basis of mucopolysaccharidosis IIIB. *Proc Natl Acad Sci USA*. 2008;105(18):6560-6565.

141. Macauley MS, Whitworth GE, Debowski AW, Chin D, Vocadlo DJ. O-GlcNAcase uses substrate-assisted catalysis: kinetic analysis and development of highly selective mechanism-inspired inhibitors. *J Biol Chem*. 2005;280(27):25313-25322.

142. Feldhammer M, Durand S, Pshezhetsky A V. Protein misfolding as an underlying molecular defect in mucopolysaccharidosis III type C. *PLoS One*. 2009;4(10):e7434.

143. Lieberman RL, D'aquino JA, Ringe D, Petsko GA. Effects of pH and iminosugar pharmacological chaperones on lysosomal glycosidase structure and stability. *Biochemistry*. 2009;48(22):4816-4827.

144. Arfi A, Richard M, Gandolphe C, Bonnefont-Rousselot D, Thérond P, Scherman D. Neuroinflammatory and oxidative stress phenomena in MPS IIIA mouse model: the positive effect of long-term aspirin treatment. *Mol Genet Metab*. 2011;103(1):18-25.

145. Jeyakumar M, Smith DA, Williams IM, et al. NSAIDs increase survival in the Sandhoff disease mouse: synergy with N-butyldeoxynojirimycin. *Ann Neurol*. 2004;56(5):642-649.

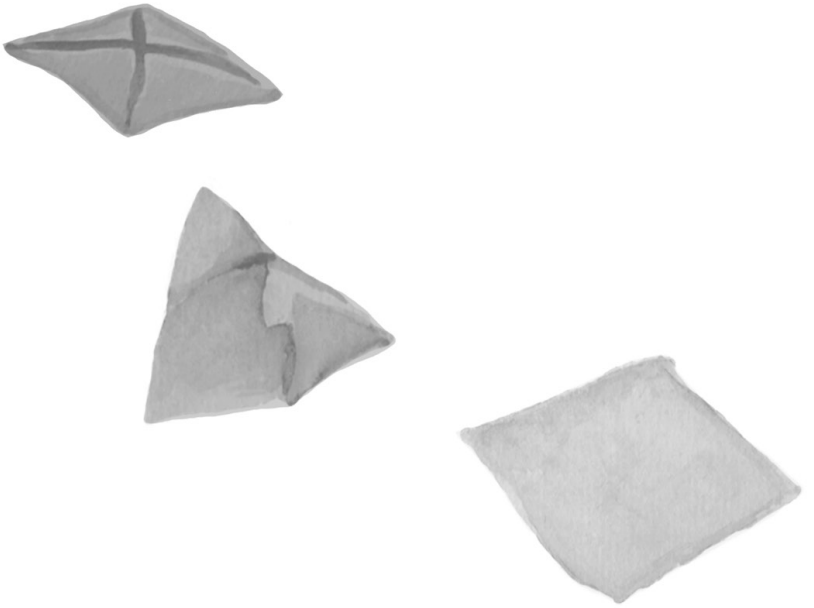
146. Smith D, Wallom KL, Williams IM, Jeyakumar M, Platt FM. Beneficial effects of anti-inflammatory therapy in a mouse model of Niemann-Pick disease type C1. *Neurobiol Dis*. 2009;36(2):242-251.

147. Wilson J, Jungner G. Principles and practice of screening for disease. *Public Health Pap*. 1968;(34). http://whqlibdoc.who.int/php/WHO_PHP_34.pdf.

148. de Ruijter J, de Ru MH, Wagemans T, et al. Heparan sulfate and dermatan sulfate derived disaccharides are sensitive markers for newborn screening for mucopolysaccharidoses types I, II and III. *Mol Genet Metab*. 2012;107(4):705-710.

Part I

Pathophysiology



Chapter 3

Residual *N*-acetyl-α-glucosaminidase activity in fibroblasts correlates with disease severity in patients with mucopolysaccharidosis type IIIB

O.L.M. Meijer, L. Welling, M.J. Valstar, L.H. Hoefsloot, H.T. Brüggenwirth, A.T. van der Ploeg, G.J. Ruijter, T. Wagemans, F.A. Wijburg and N. van Vlies

J Inherit Metab Dis. 2016;39(3):437-45

ABSTRACT

Background

Mucopolysaccharidosis type IIIB (MPS IIIB) is a rare genetic disorder in which deficiency of the lysosomal enzyme *N*-acetyl-α-glucosaminidase (NAGLU) results in the accumulation of heparan sulfate (HS), leading to progressive neurocognitive deterioration. In MPS IIIB a wide spectrum of disease severity is seen. Due to a large allelic heterogeneity, establishing genotype-phenotype correlations is difficult. However, reliable prediction of the natural course of the disease is needed, for instance for the assessment of the efficacy of potential therapies.

Methods

To identify markers that correlate with disease severity, all Dutch patients diagnosed with MPS IIIB were characterised as either rapid (RP; classical, severe phenotype) or slow progressors (SP; non-classical, less severe phenotype), based on clinical data. NAGLU activity and HS levels were measured in patients' fibroblasts after culturing at different temperatures.

Results

A small, though significant difference in NAGLU activity was measured between RP and SP patients after culturing at 37°C ($p < 0.01$). Culturing at 30°C resulted in more pronounced and significantly higher NAGLU activity levels in SP patients ($p < 0.001$) with a NAGLU activity of 0.58 $nmol.mg^{-1}.hr^{-1}$ calculated to be the optimal cut-off value to distinguish between the groups (sensitivity and specificity 100%). A lower capacity of patients' fibroblasts to increase NAGLU activity at 30°C could significantly predict for the loss of several disease specific functions.

Conclusions

NAGLU activity in fibroblasts cultured at 30°C can be used to discriminate between RP and SP MPS IIIB patients and the capacity of cells to increase NAGLU activity at lower temperatures correlates with disease symptoms.

INTRODUCTION

Mucopolysaccharidosis type III (MPS III or Sanfilippo disease) is a rare autosomal recessive lysosomal storage disorder in which the deficiency of one of four enzymes involved in the degradation of heparan sulfate (HS) leads to the accumulation of this specific glycosaminoglycan (GAG) [1]. The reported birth incidence for MPS III ranges from 1.52 to 1.89 per 100,000 newborns [2,3]. Based on the deficient enzyme, four different MPS III subtypes are distinguished referred to as MPS IIIA (OMIM #252900), B (OMIM #252920), C (OMIM #252930) and D (OMIM #252940) [1]. Clinically these subtypes are indistinguishable.

The clinical course of the disease is generally divided into three stages. After a symptom-free interval, patients normally present between the age of 1 and 4 years with a delay in cognitive development, especially in the development of speech and language skills. The second phase of the disease starts at the age of 3 to 4 years and is marked by a progressive intellectual decline which is accompanied by behavioral and sleeping problems. Eventually, the third phase sets in, characterized by the regression of motor functions. Patients lose the ability to walk independently, become wheelchair bound and fully care dependent. Patients with this rapidly progressing classical phenotype (rapid progressors, RP) usually die at the end of the second or in the beginning of the third decade of life [4]. However, in recent years it has been recognized that MPS III is characterized by a much broader spectrum of disease progression and severity. Survival well into adulthood has been reported and patients may show a stable developmental impairment for many years [5–7]. In the Netherlands, most patients with MPS IIIB display this slowly progressing attenuated phenotype (slow progressors, SP) [6].

MPS IIIB is caused by a deficiency of the enzyme *N*-acetyl-α-glucosaminidase (EC 3.2.1.50) which is responsible for the hydrolysis of the α, 1→4 linkage between *N*-acetylglucosamine and the adjacent glucuronic or iduronic acid residue [8]. The gene encoding for NAGLU is localized on chromosome 17q21.1 [9] and over 100 mutations in the NAGLU gene (HGNC: 7632) have been identified [6,10–25]. Due to this large allelic heterogeneity, establishing a genotype-phenotype correlation is difficult.

Although there is no disease-modifying treatment available for MPS IIIB, several promising therapies are currently under investigation. A trial on intravenous enzyme replacement therapy (ERT) has recently been initiated (clinicaltrials.gov identifier: NCT02324049) and a study knon intrathecal ERT is in a preclinical phase for MPS IIIB (http://www.bmrn.com/pipeline/index.php). The latter approach is already in a phase 2b study for MPS IIIA (clinicaltrials.gov identifiers: NCT01299727 and NCT02060526). Intracerebral gene therapy has been developed for MPS IIIB and is now in a phase 1/2 study (ISRCTN identifier: ISRCTN19853672). Finally, the efficacy of high dose oral genistein, which reduces the accumulation of HS in MPS III mice [26,27] is being investigated in patients with all MPS III subtypes (EudraCT number: 2013-001479-18).

In order to properly target a disease-modifying therapy, and to allow accurate evaluation of potential clinical benefits, it is essential to reliably predict the natural course of the disease for each individual patient at an early stage. We therefore aimed to identify markers that correlate with disease severity in MPS IIIB patients. We analyzed NAGLU activity and levels of stored HS in fibroblasts of MPS IIIB patients after culturing cells at different temperatures and correlated this to their clinical phenotype.

MATERIAL AND METHODS

Patients

All Dutch patients diagnosed with MPS IIIB of whom cultured skin fibroblasts were available and essential clinical data could be retrieved from medical records, were included in this study. Data was collected on survival and the age at which the ability to speak (defined as: to verbally communicate intentionally with meaningful words) and to walk (defined as: to walk independently without any assistance) was lost. Based on the essential clinical data set, patients were divided into two groups by a clinician experienced in the diagnosis and treatment of lysosomal storage disorders, including MPS IIIB (FAW). The rapid progressor group (RP) consisted of patients with a classical, severe form of the disease and the slow progressor group (SP) of patients with a non-classical, less severe phenotype.
Informed consent for the use of patient data and fibroblasts was obtained from parents or legal representatives for all patients. This study was submitted to the Medical Ethical Review Board of the Academic Medical Center in Amsterdam who declared that ethical approval was not necessary because of the observational nature of the study.

Mutation analysis

Mutation analysis had been performed in most patients within the scope of the diagnostic workup. If not, mutation analysis of the NAGLU gene was performed in fibroblasts by standard Sanger sequencing methods. Mutation nomenclature is according the HGVS (http://www.hgvs.org/mutnomen/). Sequence reference for the NAGLU gene is NM 000263.3.

Cell culture

Fibroblast cell lines were tested for mycoplasma contamination and were subsequently cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% inactivated Fetal Bovine Serum (FBS) and 100 U/mL penicillin, 100 µg/mL streptomycin and 250 µg/mL amphotericin in a humidified atmosphere containing 5% CO_2. To remove bovine NAGLU in the FBS, NAGLU was inactivated by incubation of FBS at 65°C for 35 minutes.

Fibroblasts were plated at 70% confluence and cultured at a temperature of either 30°C or 37°C for 7 days. After a week the medium was removed and cell layers were washed twice with phosphate buffered saline (PBS). After harvesting, cell pellets were washed once with PBS and twice with 0.9% NaCl and stored at -20°C until further analysis.

Enzymatic activity of NAGLU in fibroblasts

NAGLU activity in fibroblasts was measured using the fluorogenic substrate 4-methylumbelliferyl-2-acetamido-2-deoxy-α-D-glucopyranoside(4MU-α-GlcNAc) (Moscerdam, Oegstgeest, The Netherlands)using a modified version of the method described by Marsh *et al.* [28]. The 4MU-α-GlcNAc substrate was dissolved to a concentration of 6 mg/mL in 0.1 M citrate 0.2 M phosphate buffer pH 4.3. Cell pellets were resuspended in milliQ (Synergy® Water Purification System, Millipore, Billerica, MA, USA) supplemented with one cOmplete™ protease inhibitor cocktail tablet per 10 mL milliQ (Roche, Mannheim, Germany). Cells were disrupted by sonification uing a Vibra Cell sonicator (Sonics & Materials Inc., Newtown, CT, USA). Subsequently, protein concentration was measured in whole cell lysates as described by Lowry *et al.* [29]. For the determination of NAGLU activity, cell lysates were diluted to a concentration of 1 mg/mL and 30 µL of cell lysate was added to 15 µL 4MU-α-GlcNAc solution and 15 µL of a 0.1 M citrate 0.2 M phosphate buffer with pH 3.85. After 4 hours incubation at 37°C, the reaction was stopped with 1440 µL 0.2 M sodium carbonate 0.5 M glycine buffer, pH 10.5. Released 4-methylumbelliferone was measured fluorometrically with an excitation wavelength of 360 nm and emission wavelength of 450 nm using a Perkin Elmer LS45 fluorescence spectrometer (Perkin Elmer, Waltham, MA, USA). NAGLU activity in each sample was calculated using a calibration curve of 4-methylumbelliferone (Glycosynth Ltd., Warrington, Cheshire, UK). All enzyme activity assays were performed in duplicate and repeated at least once in independent fibroblast cultures.

HS analysis in fibroblasts

HS levels were determined as described previously [30]. HS in 25 µg of fibroblast lysate was enzymatically digested into disaccharides. As a final deproteination step samples were loaded on an Amicon Ultra 10 kD centrifugal filter (EMD Millipore, Billerica, MA, USA) and centrifuged at 14.000 g for 30 minutes at a temperature of 25°C.

Statistical analysis

All data analyses were performed using SPSS software for Windows (version 21.0, SPSS Inc., Chicago, IL, USA). Non-parametric statistical tests were used to analyze the relationship between MPS IIIB phenotype and NAGLU activity levels after cell culturing at 37°C and 30°C and NAGLU activity ratio at 30°C over 37°C, respectively. Linear regression was performed to determine the correlation between the ratio of NAGLU activity after cell culturing at 30°C

over 37°C and clinical signs of disease progression. Cut-off values of NAGLU activity that could discriminate between MPS III phenotypes, were identified using receiver operating characteristic (ROC) curve analysis. True positive rates (sensitivity) were plotted against false positive rates (1-specificity) for all classification points and p-values were calculated for the area under the curve. A p-value of < 0.05 was considered statistically significant.

RESULTS

Patients

Twenty-eight patients from 17 different families were included in this study (table 1). Six patients were classified as RP patients and 22 as SP patients. At the time of this study 15 out of 28 patients were still alive. In the RP patients death occurred at a younger age than in the SP patients (median age 14 years, range 13-20 years vs. median age 51 years, range 28-69 years, respectively. $p < 0.001$). RP patients lost their ability to communicate in a meaningful way at a median age of 6 years (range 5-10 years). In patients with the SP phenotype this was 24 years (range 8-69 years) ($p < 0.001$). The median age at loss of the ability to walk independently was 14 years (range 8-18 years) in RP patients and 50 years (range 18-68 years) in the SP patients ($p < 0.001$).

Mutations

For all patients mutations are presented in table 1. All mutations in our cohort have been reported previously, except the missense mutation c.509G>A; p.(Gly170Asp) found in patient 15 [6,9,10,18,22].

NAGLU activity in fibroblasts after culturing at 37°C and 30°C

To investigate whether residual enzyme activity in cultured skin fibroblasts differentiates between RP and SP patients, NAGLU activity in fibroblasts was measured. In figure 1A, NAGLU activity levels in fibroblasts of all individual MPS IIIB patients are depicted after culturing at 37°C and 30°C. Very low levels of NAGLU activity were observed for all patients after culturing at 37°C. Under these conditions fibroblasts from RP patients showed a median enzymatic activity of 0.17 $nmol.mg^{-1}.hr^{-1}$ (range 0.14-0.23 $nmol.mg^{-1}.hr^{-1}$) compared to a median enzymatic activity of 0.27 $nmol.mg^{-1}.hr^{-1}$ (range 0.16-3.84 $nmol.mg^{-1}.hr^{-1}$) in fibroblasts from SP patients. As is shown in figure 1B, a small, though significant difference in NAGLU activity between the RP and SP group was seen after culturing at 37°C ($p < 0.01$). However, when looking at individual values of enzymatic activity there is still considerable overlap between the two groups. Figure 1C shows that culturing fibroblasts at 30°C for 1 week resulted in a more pronounced difference in NAGLU activity between the two groups.

General information					Genetic characteristics [1]				Clinical characteristics		
Patient	Family	M / F	Year of birth	Phenotype	cDNA change 1.	cDNA change 2.	Protein change 1.	Protein change 2.	Age (of death)	Loss of speech	Loss of walking
Patient 1	1.1	F	1988	RP	c.214_237dup24	c.214_237dup24	p.(Ala72_Gly79dup8)	p.(Ala72_Gly79dup8)	26	5	18
Patient 2	1.2	M	1991	RP	c.214_237dup24	c.214_237dup24	p.(Ala72_Gly79dup8)	p.(Ala72_Gly79dup8)	23	8	16
Patient 3	2.1	M	1972	RP	Not identified	Not identified	Not identified	Not identified	20†	10	17
Patient 4	3.1	M	1969	RP	c.889C>T	c.217_221dup5	p.(Arg297*)	p.(Val75fs)	14†	7	11
Patient 5	3.2	F	1971	RP	c.889C>T	c.217_221dup5	p.(Arg297*)	p.(Val75fs)	13†	5	8
Patient 6	4.1	M	1963	RP	c.889C>T	c.889C>T	p.(Arg297*)	p.(Arg297*)	14†	5	9
Patient 7	5.1	M	1961	SP	c.889C>T	c.1834A>G	p.(Arg297*)	p.(Ser612Gly)	53	51	
Patient 8*	6.1	F	1979	SP	c.1834A>G	c.1927C>T	p.(Ser612Gly)	p.(Arg643Cys)	36		
Patient 9*	6.2	F	1984	SP	c.1834A>G	c.1927C>T	p.(Ser612Gly)	p.(Arg643Cys)	30		
Patient 10	7.1	F	1988	SP	c1693C>T	c.1900G>A	p.(Arg565Trp)	p.(Glu634Lys)	27	24	
Patient 11	7.2	F	1989	SP	c1693C>T	c.1900G>A	p.(Arg565Trp)	p.(Glu634Lys)	25	24	
Patient 12	8.1	M	1983	SP	c.187G>A	Not identified	p.(Asp63Asn)	Not identified	31	30	
Patient 13**	9.1	M	1988	SP	c.419A>G	c.1489C>G	p.(Tyr140Cys)	p.(Leu497Val)	26		
Patient 14	10.1	M	1997	SP	c.1834A>G	c.1834A>G	p.(Ser612Gly)	p.(Ser612Gly)	17		
Patient 15**	11.1	F	1952	SP	c.509G>A	c.743A>G	p.(Gly170Asp)	p.(His248Arg)	63	39	53
Patient 16	12.1	M	1971	SP	c.237ins24	c.1694G>A	p.(Ala72_Gly79dup8)	p.(Arg565Gln)	29†	16	19
Patient 17	12.2	M	1973	SP	c.237ins24	c.1694G>A	p.(Ala72_Gly79dup8)	p.(Arg565Gln)	42	18	18
Patient 18	13.1	F	1932	SP	c.1834A>G	c.1834A>G	p.(Ser612Gly)	p.(Ser612Gly)	69†	69	68
Patient 19	14.1	F	1947	SP	c.1927C>T	c.1927C>T	p.(Arg643Cys)	p.(Arg643Cys)	62†	24	46
Patient 20	14.2	F	1948	SP	c.1927C>T	c.1927C>T	p.(Arg643Cys)	p.(Arg643Cys)	28†	23	28
Patient 21	14.3	M	1951	SP	c.1927C>T	c.1927C>T	p.(Arg643Cys)	p.(Arg643Cys)	51†	47	49
Patient 22	14.4	F	1953	SP	c.1927C>T	c.1927C>T	p.(Arg643Cys)	p.(Arg643Cys)	58†	18	36
Patient 23	14.5	F	1954	SP	c.1927C>T	c.1927C>T	p.(Arg643Cys)	p.(Arg643Cys)	60	36	47
Patient 24	14.6	F	1956	SP	c.1927C>T	c.1927C>T	p.(Arg643Cys)	p.(Arg643Cys)	52†	19	36
Patient 25	15.1	M	1984	SP	c.845C>T	c.1172A>G	p.(Ala282Val)	p.(Tyr391Cys)	31		
Patient 26	16.1	F	1937	SP	c.1927C>T	c.281G>C + c.283G>C	p.(Arg643Cys)	p.(Arg94_Asp95delins2)	47†	8	34
Patient 27	16.2	M	1942	SP	c.1927C>T	c.281G>C + c.283G>C	p.(Arg643Cys)	p.(Arg94_Asp95delins2)	50†	8	18
Patient 28	17.1	M	1955	SP	c.1562C>T	c.1489C>G	p.(Pro521Leu)	p.(Leu497Val)	60	45	50

Table 1. Clinical and genetic characteristics of the MPS IIIB patients. All patients for whom no data are given are still able to speak or walk. Siblings 14.2, 14.3, 14.5 and 14.6 are cousins of the siblings 14.1 and 14.4. Most patients were previously reported by Valstar *et al.* [6]. * Patients not previously reported. ** Patients previously reported, but without mutations. [1] Sequence reference NM 000263.3.

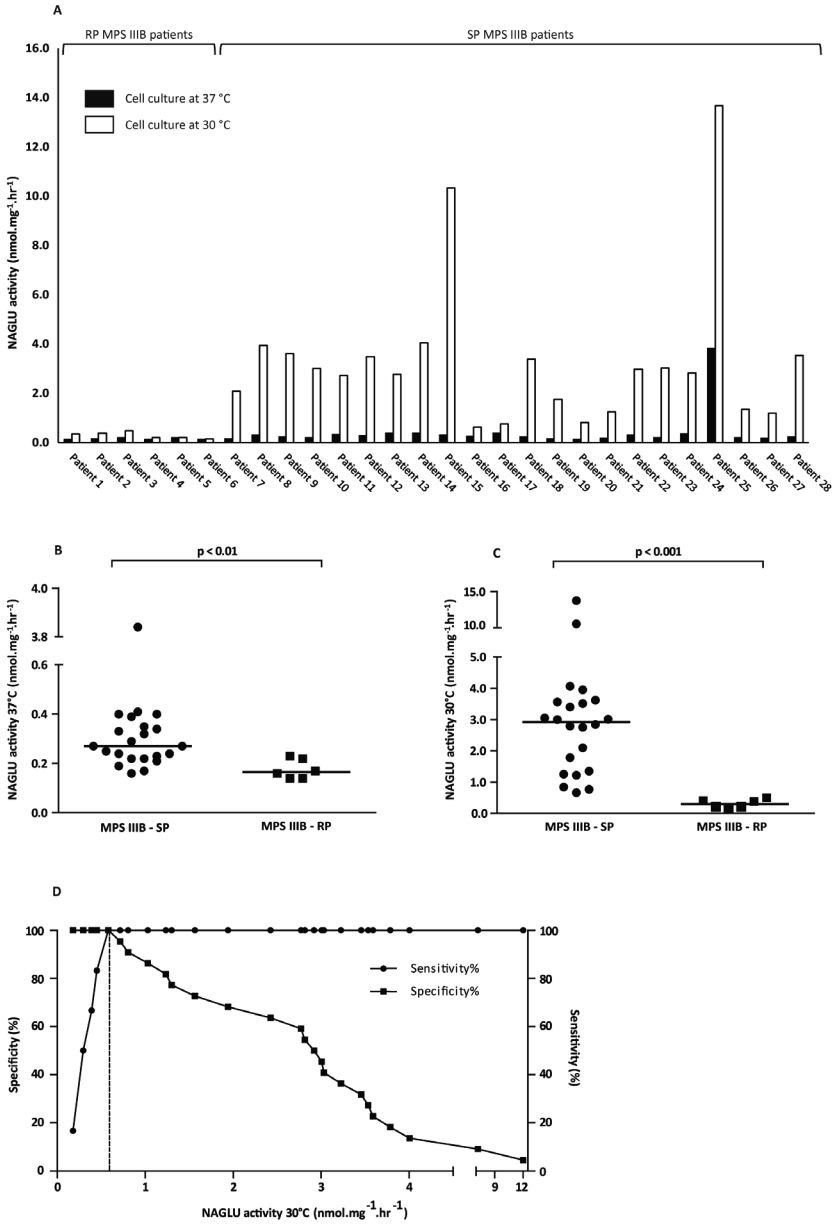


Figure 1. A. Mean NAGLU activity (nmol.mg^{-1}.hr^{-1}) of duplicate measurement in fibroblasts of the individual MPS IIIB patients after culturing cells at 37°C and 30°C for 1 week. Patient numbers correspond with the numbers in table 1. Data of one representative experiment are shown. B and C. NAGLU activity (nmol.mg^{-1}.hr^{-1}) measured in fibroblasts of SP and RP MPS IIIB patients after culturing cells at 37°C and 30°C for 1 week, respectively. Medians are given. Data of one representative experiment are shown. D. Sensitivity and specificity for cut-off levels of NAGLU activity in fibroblasts after culturing at 30°C to distinguish between RP and SP MPS IIIB patients. The dashed line indicates the calculated optimal cut-off level of 0.58 nmol.mg^{-1}.hr^{-1}.

After culturing fibroblasts at 30°C significantly higher NAGLU activity levels were found in SP patients (median NAGLU activity 2.92 nmol.mg^{-1}.hr^{-1} (range 0.66-13.70 nmol.mg^{-1}.hr^{-1})) compared to RP patients (median NAGLU activity after culturing at 30°C 0.30 nmol.mg^{-1}.hr^{-1} (range 0.15-0.50 nmol.mg^{-1}.hr^{-1})) ($p < 0.001$). ROC analysis showed an area under the curve of 1.0, indicating that NAGLU activity after culturing at 30°C is a very accurate tool to discriminate between RP and SP patients ($p < 0.001$). A NAGLU activity of 0.58 nmol.mg^{-1}.hr^{-1} was calculated to be the optimal cut-off value to distinguish RP patients from SP patients with a sensitivity and specificity of 100% (figure 1D).

HS levels in fibroblasts

HS levels in RP and SP MPS IIIB patients' fibroblasts were analyzed to assess whether storage levels are influenced by the different NAGLU activities observed between the two groups. No significant difference was found in HS level between the groups when fibroblasts were cultured at 37°C (figure 2). However, in fibroblasts cultured at 30°C HS levels were significantly lower in fibroblasts from patients with an SP phenotype compared to fibroblasts from patients with an RP phenotype ($p < 0.05$). Culturing fibroblasts of RP patients at either 37°C or 30°C, did not result in any significant differences in HS levels, while significantly lower HS levels after culturing at 30°C were found in patients within the SP group, compared to levels at 37°C ($p < 0.01$). This indicates that the increased NAGLU activity, measured *in vitro* after culturing cells at 30°C, indeed corresponds to higher levels of functional NAGLU in fibroblasts of SP patients.

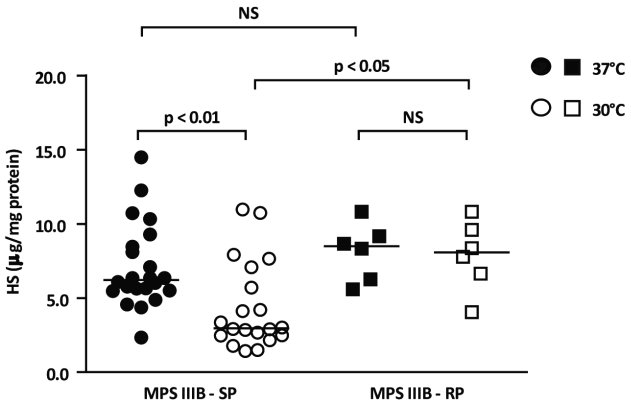


Figure 2. HS levels (µg/mg protein) in fibroblasts of SP and RP MPS IIIB patients after culturing cells at 37°C and 30°C. Medians are given. Data of one representative experiment are shown.

NAGLU activity ratio at 30°C over 37°C and disease symptoms

The capacity of patients' fibroblasts to increase residual enzyme activity was further assessed by calculating the ratio of NAGLU activity after culturing at 30°C to the activity after culturing at 37°C. The ratio of NAGLU activity at 30°C over 37°C was 9.36 (range 1.93-31.49) in SP patients, which was significantly higher compared to patients in the RP group, who had a median ratio of NAGLU activity of 1.78 (range 0.96-2.39) ($p < 0.001$) (figure 3A). Because MPS IIIB comprises a continuous spectrum of disease severity, we assessed whether this ratio correlated more specifically with the age at which patients lost specific functions. A lower capacity of patients' fibroblasts to increase NAGLU activity at 30°C could significantly predict for the loss of the ability to communicate verbally in a meaningful manner and walk independently at a younger age (figure 3B and 3C). Also, the age of demise correlated with the NAGLU activity ratio at 30°C over 37°C (figure 3D).

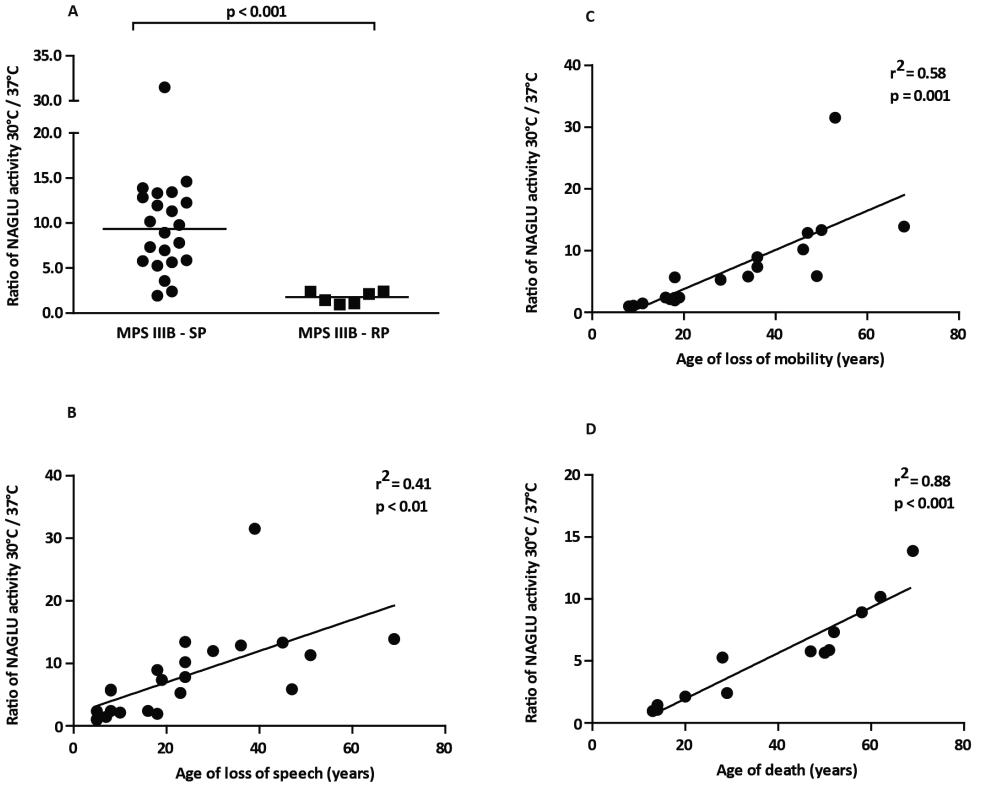


Figure 3. A. Ratio of NAGLU activity after culturing at 30°C over NAGLU activity after culturing at 37°C measured in fibroblasts of SP and RP MPS IIIB patients. Medians are given. Data of one representative experiment are shown. B-D. Ratio of NAGLU activity after culturing at 30°C over NAGLU activity after culturing at 37°C in fibroblasts, correlated to the age of speech loss (23 out of 28 patients), to the age of loss of mobility (19 out of 28 patients) and correlated to the age of demise in (13 out of 28 patients who had died at time of this study). Data of one representative experiment are shown.

DISCUSSION

Over recent years, a number of studies on potential disease-modifying treatment options for MPS IIIB have been initiated. For evaluating clinical efficacy as well as for assessing which patients may benefit the most from a specific treatment, it will be essential to predict the natural course of the disease for each individual patient at an early stage. Here, we investigate an approach to discriminate between MPS IIIB patients with a rapidly progressing (RP, classical or severe) phenotype and patients with a slowly progressing (SP, attenuated) phenotype, using cultured skin fibroblasts.

We observed a significant difference in residual activity of NAGLU between fibroblasts from RP and SP patients when fibroblasts were cultured at 37°C. However, NAGLU activity did not completely discriminate between the two groups. Culturing fibroblasts at 30°C, however, allowed for complete separation between RP and SP patients. Accordingly, HS levels after culturing at 30°C were significantly lower in fibroblasts from the SP group than in fibroblasts from the RP group. This indicates that the increased NAGLU activity measured in protein lysates after culture at 30°C comprises a form of the enzyme that is biochemically active in living cells and exerts its catalytic function in the lysosome. Nevertheless, it should be taken into consideration that other factors, such as alteration of GAG synthesis, are responsible for lower HS levels. However, it is unlikely that this only affects the SP group.

MPS IIIB is characterized by a large genetic heterogeneity which is probably the most important cause of the phenotypic variability. Although some genotype-phenotype correlations have been established in MPS IIIB, previously unrecognized mutations are frequently reported. In our limited series of patients, the RP patients all have two nonsense or frameshift mutations, whereas all the SP patients have at least one missense mutation. Irrespectively of genotype, NAGLU activity in fibroblasts after culturing at 30°C appears to reliably predict for an RP or SP phenotype.

MPS IIIB comprises a continuous spectrum of disease severity, and a division in only an RP and an SP group does no justice to the clinical variability observed in patients. Valstar *et al.* showed that the loss to communicate in a meaningful manner and the ability to walk independently are key symptoms in the assessment of disease progression [6]. We therefore correlated the loss of these functions to the ratio of NAGLU activity at 30°C over 37°C. The capacity of fibroblasts to enhance residual enzyme activity at 30°C correlated with the course of the disease. However, since not all patients had already reached these stages of disease, patient numbers are small and more patients would be needed to validate these correlations.

The increased levels of residual enzyme measured in SP patients after culturing cells at 30°C, might be due to more efficient protein folding at this lower temperature, thereby stimulating

the activity of mutant enzymes [31,32]. The positive effect of culturing human fibroblasts at 30°C on protein quantity and enzymatic activity has already been demonstrated for a distinct metabolic disorder by another group in our lab [33]. Cells of patients with a mild mevalonate kinase deficiency showed higher enzymatic activity after culturing at 30°C which correlated with higher protein levels on Western blot. Culture temperature had no effect on cells of patients with a severe mevalonate kinase deficiency. We hypothesize that the differences between SP patients might be caused by the presence of molecular chaperones or differences in the regulation of the endoplasmic-reticulum-associated protein degradation machinery which only become apparent at 30°C culture conditions, at least in the *in vitro* setting.

In most SP fibroblasts, culturing at 30°C resulted in levels of NAGLU activity that exceeded 10% of control enzyme activity (reference values for NAGLU activity in fibroblasts used in our diagnostic laboratory are 9-17 nmol.mg^{-1}.hr^{-1}). Ten percent of control NAGLU activity is often associated with the amelioration of symptoms. Our findings indicate that residual NAGLU activity can potentially be enhanced and that MPS IIIB patients with an SP phenotype might benefit from therapies that interfere with protein folding, such as chemical and pharmacological chaperones[34]. However, more research is needed to elucidate the underlying processes and the effects of therapies that interfere with these mechanisms.

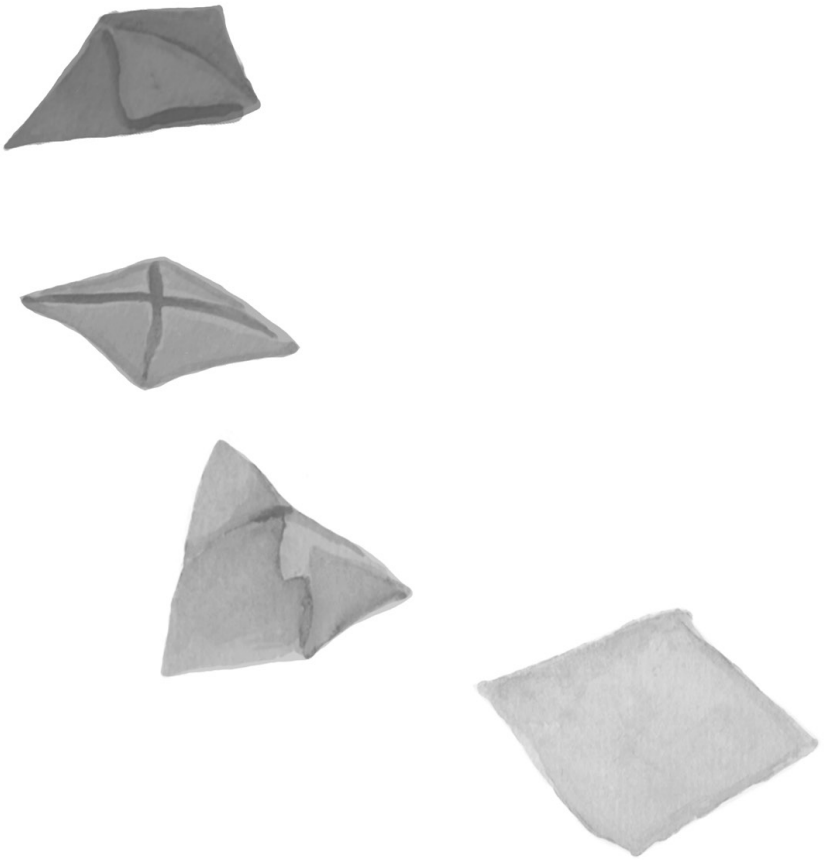
To be able to measure low levels of NAGLU activity and to detect minor changes in enzyme activity at different conditions in all patient cell lines, we optimized the assay normally used in our diagnostic department. Using this optimized assay, in patient 25 remarkably high levels of NAGLU activity and low HS levels were found at all culture conditions, despite previous genetic and biochemical confirmation of the diagnosis MPS IIIB. The use of high concentrations of 4MU-α-G1cNAc substrate (1.5 mg/mL final concentration) in combination with an optimized pH and incubation time, might have favored the particular kinetic properties of the mutant enzyme in this patient. Furthermore, NAGLU with this particular mutation might have an increased affinity to the 4MU-substrate, as compared to natural substrates present *in vivo*. This is currently further investigated.

In conclusion, we show that NAGLU activity in fibroblasts cultured at 30°C can be used to discriminate between RP and SP MPS IIIB patients and that the capacity of cells to increase NAGLU activity at lower temperatures correlates with disease severity and progression. Prediction of the phenotype of an individual patient may become of high relevance in the near future for assessment of the efficacy of disease-modifying treatments for MPS IIIB.

REFERENCES

1. Muenzer J. Overview of the mucopolysaccharidoses. *Rheumatology*. 2011;50 Suppl 5:v4-12.
2. Poorthuis BJ, Wevers RA, Kleijer WJ, et al. The frequency of lysosomal storage diseases in The Netherlands. *Hum Genet*. 1999;105(1-2):151-156.
3. Baehner F, Schmiedeskamp C, Krummenauer F, et al. Cumulative incidence rates of the mucopolysaccharidoses in Germany. *J Inherit Metab Dis*. 2005;28(6):1011-1017.
4. Valstar MJ, Ruijter GJ, Van Diggelen OP, Poorthuis BJ, Wijburg FA. Sanfilippo syndrome: A mini-review. *J Inherit Metab Dis*. 2008;31(2):240-252.
5. Valstar MJ, Neijs S, Bruggenwirth HT, et al. Mucopolysaccharidosis type IIIA: clinical spectrum and genotype-phenotype correlations. *Ann Neurol*. 2010;68(6):876-887.
6. Valstar MJ, Bruggenwirth HT, Olmer R, et al. Mucopolysaccharidosis type IIIB may predominantly present with an attenuated clinical phenotype. *J Inherit Metab Dis*. 2010;33(6):759-767.
7. Moog U, van Mierlo I, van Schrojenstein Lantman-de Valk HM, Spaapen L, Maaskant MA, Curfs LM. Is Sanfilippo type B in your mind when you see adults with mental retardation and behavioral problems? *Am J Med Genet C Semin Med Genet*. 2007;145C(3):293-301.
8. Esko JD, Kimata K, Lindahl U. Chapter 16 Proteoglycans and Sulfated Glycosaminoglycans. In: Varki A, Cummings RD, Esko JD, eds. *Essentials of Glycobiology*. 2nd ed. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press; 2009.
9. Zhao HG, Li HH, Bach G, Schmidtchen A, Neufeld EF. The molecular basis of Sanfilippo syndrome type B. *Proc Natl Acad Sci USA*. 1996;93(12):6101-6105.
10. Beesley CE, Young EP, Vellodi A, Winchester BG. Identification of 12 novel mutations in the α-N-acetylglucosaminidase gene in 14 patients with Sanfilippo syndrome type B (mucopolysaccharidosis type IIIB). *J Med Genet*. 1998;35(11):910-914.
11. Beesley C, Moraitou M, Winchester B, Schulpis K, Dimitriou E, Michelakakis H. Sanfilippo B syndrome: Molecular defects in Greek patients. *Clin Genet*. 2004;65(2):143-149.
12. Beesley CE, Jackson M, Young EP, Vellodi A, Winchester BG. Molecular defects in Sanfilippo syndrome type B (mucopolysaccharidosis IIIB). *J Inherit Metab Dis*. 2005;28(5):759-767.
13. Chinen Y, Tohma T, Izumikawa Y, Uehara H, Ohta T. Sanfilippo type B syndrome: five patients with an R565P homozygous mutation in the α-N-acetylglucosaminidase gene from the Okinawa islands in Japan. *J Hum Genet*. 2005;50(7):357-359.
14. Emre S, Terzioglu M, Tokatli A, et al. Sanfilippo syndrome in Turkey: Identification of novel mutations in subtypes A and B. *Hum Mutat*. 2002;19(2):184-185.
15. Esposito S, Balzano N, Daniele A, et al. Heparan N-sulfatase gene: Two novel mutations and transient expression of 15 defects. *Biochim Biophys Acta*. 2000;1501(1):1-11.
16. van de Kamp JJ, van Pelt JF, Liem KO, Giesberts MA, Niepoth LT, Staalman CR. Clinical variability in Sanfilippo B disease: a report on six patients in two related sibships. *Clin Genet*. 1976;10(5):279-284.
17. Ouesleti S, Brunel V, Turkia H Ben, et al. Molecular characterization of MPS IIIA, MPS IIIB and MPS IIIC in Tunisian patients. *Clin Chim Acta*. 2011;412(23-24):2326-2331.
18. Schmidtchen a, Greenberg D, Zhao HG, et al. NAGLU mutations underlying Sanfilippo syndrome type B. *Am J Hum Genet*. 1998;62(1):64-69.
19. Selmer KK, Gilfillan GD, Strømme P, et al. A mild form of Mucopolysaccharidosis IIIB diagnosed with targeted next-generation sequencing of linked genomic regions. *Eur J Hum Genet*. 2012;20(1):58-63.
20. Tang J, Pan J, Guo Y, et al. Mucopolysaccharidosis type IIIB mutations in Chinese patients: Identification of two novel NAGLU mutations and analysis of two cases involving prenatal diagnosis. *Clin Chim Acta*. 2013;419:33-38.

21. Tessitore A, Villani GR, Di Domenico C, Filocamo M, Gatti R, Di Natale P. Molecular defects in the α-N-acetylglucosaminidase gene in Italian Sanfilippo type B patients. *Hum Genet*. 2000;107(6):568-576.
22. Weber B, Guo XH, Kleijer WJ, van de Kamp JJ, Poorthuis BJ, Hopwood JJ. Sanfilippo type B syndrome (mucopolysaccharidosis III B): allelic heterogeneity corresponds to the wide spectrum of clinical phenotypes. *Eur J Hum Genet*. 1999;7(1):34-44.
23. Zhao HG, Aronovich EL, Whitley CB. Genotype-phenotype correspondence in Sanfilippo syndrome type B. *Am J Hum Genet*. 1998;62(1):53-63.
24. Bunge S, Knigge A, Steglich C, et al. Mucopolysaccharidosis type IIIB (Sanfilippo B): identification of 18 novel α-N-acetylglucosaminidase gene mutations. *J Med Genet*. 1999;36(1):28-31.
25. Champion KJ, Basehore MJ, Wood T, Destrée A, Vannuffel P, Maystadt I. Identification and characterization of a novel homozygous deletion in the α-N-acetylglucosaminidase gene in a patient with Sanfilippo type B syndrome (mucopolysaccharidosis IIIB). *Mol Genet Metab*. 2010;100(1):51-56.
26. Malinowska M, Wilkinson FL, Bennett W, et al. Genistein reduces lysosomal storage in peripheral tissues of mucopolysaccharide IIIB mice. *Mol Genet Metab*. 2009;98(3):235-242.
27. Malinowska M, Wilkinson FL, Langford-Smith KJ, et al. Genistein improves neuropathology and corrects behaviour in a mouse model of neurodegenerative metabolic disease. *PLoS One*. 2010;5(12):e14192.
28. Marsh J, Fensom AH. 4-Methylumbelliferyl α-N-acetylglucosaminidase activity for diagnosis of Sanfilippo B disease. *Clin Genet*. 1985;27(3):258-262.
29. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193(1):265-275.
30. Kingma SDK, Langereis EJ, de Klerk CM, et al. An algorithm to predict phenotypic severity in mucopolysaccharidosis type I in the first month of life. *Orphanet J Rare Dis*. 2013;8(1):99.
31. Gootjes J, Schmohl F, Mooijer PA, et al. Identification of the molecular defect in patients with peroxisomal mosaicism using a novel method involving culturing of cells at 40°C: Implications for other inborn errors of metabolism. *Hum Mutat*. 2004;24(2):130-139.
32. Diekman EF, Ferdinandusse S, van der Pol L, et al. Fatty acid oxidation flux predicts the clinical severity of VLCAD deficiency. *Genet Med*. 2015;17(12):989-94.
33. Houten SM, Frenkel J, Rijkers GT, Wanders RJ a, Kuis W, Waterham HR. Temperature dependence of mutant mevalonate kinase activity as a pathogenic factor in hyper-IgD and periodic fever syndrome. *Hum Mol Genet*. 2002;11(25):3115-3124.
34. Meijer OLM, van Vlies N, Wijburg FA. Treatment of mucopolysaccharidosis type III (Sanfilippo syndrome). *Expert Opin Orphan Drugs*. 2013;1(9):717-730.



Chapter 4

Processing of mutant *N*-acetyl-α-glucosaminidase in mucopolysaccharidosis type IIIB fibroblasts cultured at low temperature

O.L.M. Meijer, H. te Brinke, R. Ofman, L. IJlst,
F.A. Wijburg and N. van Vlies

Mol Genet Metab. 2017;122(1-2):100-106

ABSTRACT

Background

The autosomal recessive, neurodegenerative disorder mucopolysaccharidosis type IIIB (MPSIIIB) is caused by a deficiency of the lysosomal enzyme *N*-acetyl-α-glucosaminidase (NAGLU), resulting in accumulation of heparan sulfate. The disease spectrum comprises a severe, rapidly progressing (RP) phenotype and a more attenuated, slowly progressing (SP) phenotype. Previous studies showed significantly higher NAGLU activity in skin fibroblasts of SP patients when cultured at 30°C which may be relevant for development of novel therapeutic strategies. Here we report on the processes involved in this phenomenon.

Methods

Fibroblasts from controls, one RP patient (homozygous for the p.R297* mutation) and three SP MPSIIIB patients (homozygous for the mutation p.S612G or p.R643C, or compound heterozygous for the mutations p.A72_G79dup8 and p.R565Q) were cultured at temperatures ranging from 37°C to 27°C and harvested at different time points to assess NAGLU activity, mRNA and protein levels, and NAGLU glycosylation. Intracellular localization of wild-type and mutant mCherry-tagged NAGLU was analyzed by immunofluorescence.

Results

In control fibroblasts NAGLU was present as a 85 kDa precursor and a 82 kDa mature form. In SP patients' fibroblasts cultured at 37°C, only the 85 kDa form was detected. Culturing at lower temperatures resulted in higher NAGLU mRNA levels, increased levels of both precursor and mature NAGLU protein and improved processing. The formation of mature NAGLU corresponded with higher NAGLU activity levels.

Conclusions

We show that the NAGLU protein consists of a precursor and a mature form and that in SP MPSIIIB patients' fibroblasts only the precursor protein is present at 37°C. Culturing at lower temperatures resulted in the formation of the mature, enzymatically active form, due to higher mRNA levels and improved processing.

INTRODUCTION

Mucopolysaccharidosis type IIIB (MPS IIIB or Sanfilippo disease type B) is an autosomal recessive lysosomal storage disorder (LSD) caused by mutations in the gene encoding for the lysosomal enzyme *N*-acetyl-α-glucosaminidase (NAGLU; EC: 3.2.1.50), leading to impaired degradation and subsequent accumulation of the glycosaminoglycan heparan sulfate (HS) [1]. MPS IIIB is a multisystem disease characterized by progressive brain disease which initially predominantly affects speech and language skills and is accompanied by, often severe, behavioral problems. Neurocognitive decline is followed by loss of motor functions and life expectancy is limited. Somatic disease is relatively mild in MPS IIIB. There is a wide spectrum of disease severity, ranging from a severe, rapidly progressing phenotype (RP) to a more attenuated, slowly progressing phenotype (SP) which is reflected by considerable differences in disease survival [2]. There is still no approved disease-modifying treatment for MPS III(B).
Von Figura *et al.* showed that NAGLU is synthesized as a precursor peptide and is later processed to its mature form [3]. The processing of soluble lysosomal enzymes, including NAGLU, takes place in the ER and Golgi apparatus. In the late Golgi-compartments the majority of proteins destined for the lysosome is provided with mannose 6-phosphate (M6P) moieties, which serve as recognition signal for the M6P receptor in the trans-Golgi-network, where they enter the endosomal lysosomal pathway and are transported to the lysosome [4,5].
In both RP and SP MPS IIIB patients basal NAGLU activity levels are very low [6,7]. A recent study has demonstrated that residual NAGLU activity in fibroblasts of SP MPS IIIB patients can be significantly increased by culturing at 30°C, resulting in significantly lower HS levels in these cells [7]. The exact mechanism by which culturing at lower temperatures results in higher activity of mutant enzymes, has, however, not been unraveled. This study aims to elucidate the processes involved in processing of normal and mutant NAGLU under different temperature conditions. A better understanding of these processes may provide clues to novel therapeutic approaches in MPSIIIB patients with an SP phenotype.

MATERIAL AND METHODS

Cell culture

Fibroblast cell lines were derived from healthy controls and MPS IIIB patients with either an RP or SP phenotype. The RP MPS IIIB fibroblast cell line used in this study was homozygous for the nonsense mutation p.R297* [8,9]. The SP MPS IIIB cell lines used, were homozygous for the mutation p.S612G or p.R643C, or compound heterozygous for the mutations p.A72_G79dup8 and p.R565Q, all known to convey a more attenuated, slowly progressing phenotype [2,7].

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 100 U/mL penicillin, 100 µg/mL streptomycin and 250 µg/mL amphotericin at temperatures ranging from 37°C to 27°C in a humidified atmosphere containing 5% CO_2 . In addition to their build-in temperature sensor, all incubators had an extra externally applied temperature sensor. Before supplementing the culture medium with FBS, bovine NAGLU in FBS was inactivated by incubation at 65°C for 35 minutes.

Chemicals

Bortezomib was obtained from Bio-Connect, Huissen, The Netherlands. Stock solutions were prepared in DMSO and diluted in culture medium to a final DMSO concentration of 0.1% (v/v). Peptide-*N*-glycosidase F (PNGase F) was from New England Biolabs, Ipswich, Massachusetts, USA and was used following the manufacturer's instructions.

NAGLU activity assay

Fibroblasts of control subjects and MPS IIIB patients were harvested and stored at -20°C until further analysis. Cell pellets were dissolved in milliQ supplemented with cOmplete™ protease inhibitor cocktail (Roche, Mannheim, Germany) and disrupted by sonification. Protein concentration in whole cell lysates was determined with the BCA protein assay according to the manufacturer's instructions (ThermoFisher Scientific, Waltham, Massachusetts, USA). Subsequently, NAGLU activity in protein homogenates was measured as described earlier [7]. All samples were measured in duplicate.

Western blot analysis

For western blot analysis 50 µg protein was loaded on a NuPAGE Novex 4-12% Bis-Tris pre-cast polyacrylamide gel (Invitrogen, Carlsbad, California, USA) and after electrophoresis, transferred onto an Amersham Protran Nitrocellulose Blotting Membrane by semidry blotting (GE Healthcare Life Sciences, Little Chalfont, UK). Membranes were blocked in 30 g/L bovine serum albumin (Sigma-Aldrich, St. Louis, Missouri, USA) in 0.1% Tween-20 in PBS (TPBS).

Rabbit anti-NAGLU antibody 1:800 (ab169874; Abcam, Cambridge, UK) and mouse anti-β-actin antibody 1:10,000 (Sigma-Aldrich, St. Louis, Missouri, USA), dissolved in TPBS, were used as primary antibodies, and goat anti-rabbit antibody 1:10,000 and donkey anti-mouse antibody 1:10,000 (IRDye 800CW and IRDye 680RD, respectively; LI-COR Biosciences, Lincoln, Nebraska, USA), dissolved in TPBS with Odyssey® blocking buffer and 0.01% SDS (w/v), as secondary antibodies. Between antibody incubations membranes were washed five times with TPBS. Blots were analyzed using the Odyssey® CLx Infrared Imaging System (LI-COR Biosciences, Nebraska, USA).

Quantitative real-time PCR

For total RNA isolation from human fibroblasts TRI Reagent® (Sigma-Aldrich, St. Louis, Missouri, USA) was used and cDNA was produced using Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany). Real-time PCR analysis for NAGLU was performed using LightCycler® 480 SYBR Green I Master and cDNA samples were run on the LightCycler480 Instrument (Roche, Mannheim, Germany). Data were analyzed using linear regression calculations as earlier described [10]. All analyses were performed in triplicate. NAGLU mRNA was normalized using the geometric mean derived from the reference genes Cyclophillin B (*PPIB*) and H3 Histone Family Member 3A (*H3F3A*). Primer sequences are given in table S1.

Cloning of human wild-type and mutant NAGLU

An EST-clone containing full-length human NAGLU cDNA was obtained from Source BioScience (Nottingham, UK; IMAGE clone 6213852, IRAUp969F07104D) and used to clone the open reading frame (ORF) into the pmCherry-N1 vector (Clonetech, Mountain View, California, USA; PT3974-5). Using the EST-clone as template, the 3' prime end of NAGLU ORF (c.866_2229 without the stopcodon) was amplified by PCR, using primers Fw866NAGLU and Rv2229NAGLUr*Age*I (table S1). The amplicon was ligated into a pGEMT plasmid. Subsequently, pNAGLU-mCherry was created as follows: first, a 1552 bp fragment consisting of 24 bp of the 5' prime UTR, followed by the first 1528 bp of the NAGLU coding sequence, was excised from the EST clone using restriction enzymes *Eco*RI and *Sbf*I. Then, the 3 'prime end of NAGLU was cut from the pGEMT vector using *Sbf*I and *Age*I. Both fragments were ligated into the *Eco*RI and *Age*I sites of the pmCherry-N1 vector.

To introduce the mutations c.1834A>G (p.S612G) and c.1927C>T (p.R643C) into NAGLU-mCherry, a 1758 bp fragment of the NAGLU ORF was cut from pNAGLU-mCherry by digestion with *Kpn*I, and ligated into pcDNA3.1 to serve as template for site directed mutagenesis (Site Directed Mutagenesis Kit, Agilent, Amstelveen, The Netherlands) using primers FwNAGLU_c.1834A>G and RvNAGLU_ c.1834A>G, and FwNAGLU_c.1927C>T and RvNAGLU_c.1927C>T, respectively (table S1). After validation of the nucleotide sequence, the *Kpn*I restriction fragment was ligated back into pNAGLU-mCherry. To generate the c.214_237dup24 (p.A72_G79dup8) variant, a pUC19 plasmid was obtained (GenScript, Jiangning, Nanjing, Jiangsu Province, China) containing a synthesized fragment of human NAGLU(c.-23_1187)whichincludedthe24bpduplication(GCGGCGCGCGTGCGGGTGCGCGGC). The *Eco*RI and *Acc*I fragment was ligated into pNAGLU-mCherry. To generate the c.1694G>A (p.R565Q) mutation, cDNA (c.227_870) was amplified by PCR from cDNA of a patient cell line compound heterozygous for this mutation using primers FwNAGLU_c.1694G>A and RvNAGLU_c.1694G>A (table S1) and ligated into the pGEMT vector. A pGEMT vector containing the NAGLU fragment with the mutation was selected and used for subcloning into wild-type pNAGLU-mCherry using the restriction enzymes *Acc*I and *Xmn*I. The complete

nucleotide sequences of all NAGLU constructs were verified by BDT sequence analysis using the sequence primers listed in (table S1).

Confocal microscopy

Transfection of fibroblasts was performed using the AMAXA NHDF Nucleofector Kit (Lonza, Basel, Switzerland) according to the manufacturer's instructions (program U23). For co-localization studies, 2 µg of plasmid (empty pmCherry, pmCherry containing full-length NAGLU cDNA and its variants p.S612G, p.R643C, p.R565C, p.A72_G79dup8) was transfected into 500,000 p.R297* RP MPS IIIB patient fibroblasts, which were subsequently cultured at 37°C. Forty-eight hour post transfection, cells were incubated with 0.075 µM LysoTracker®Green DND-26 and Hoechst 33342 reagent (Enzo Life Science, Farmingdale, New York, USA) for 1 hour. Cells were imaged with a Leica TCS SP8 filter-free spectral confocal microscope. Acquisition settings were adjusted to achieve a red and green signal of approximately equal intensity, and subsequently the same settings were used throughout all experiments.

Statistical analysis

Statistical analyses were performed using SPSS software for Windows (version 23.0, SPSS Inc., Chicago, Illinois, USA). Independent-samples T-tests were used to analyze the differences in NAGLU mRNA levels after cell culturing at 37°C and 30°C in control, RP and SP MPS IIIB fibroblast cell lines and the ratio of NAGLU mRNA levels at 30°C over 37°C. A p-value of < 0.05 was considered statistically significant.

RESULTS

Time dependence of NAGLU protein and activity at 30°C culture conditions

In line with previous reports, we found that in control fibroblasts NAGLU consists of a precursor and a mature form, with an apparent molecular weight of 85 kDa and 82 kDA, respectively (figure 1A) [3,11]. In SP MPS IIIB cell lines cultured at 37°C, NAGLU was predominantly present in the precursor form. Culturing at 30°C for one to seven days resulted in increased levels of NAGLU precursor. In addition, the levels of the mature form of NAGLU increased over time, which corresponded with a gradual increase in NAGLU activity (figure 1B-D). Quantification of the levels of both the precursor and mature form of NAGLU revealed a progressive increase in the ratio of mature over precursor NAGLU in the SP cell lines carrying the p.S612G and p.R643C mutation, but remained unchanged in the SP cell line with the NAGLU mutation p.R565Q/p.A72_G79dup8 (figure 1C). As expected, in the p.R297* RP cell line, NAGLU protein was completely absent under normal culture conditions. Culturing at

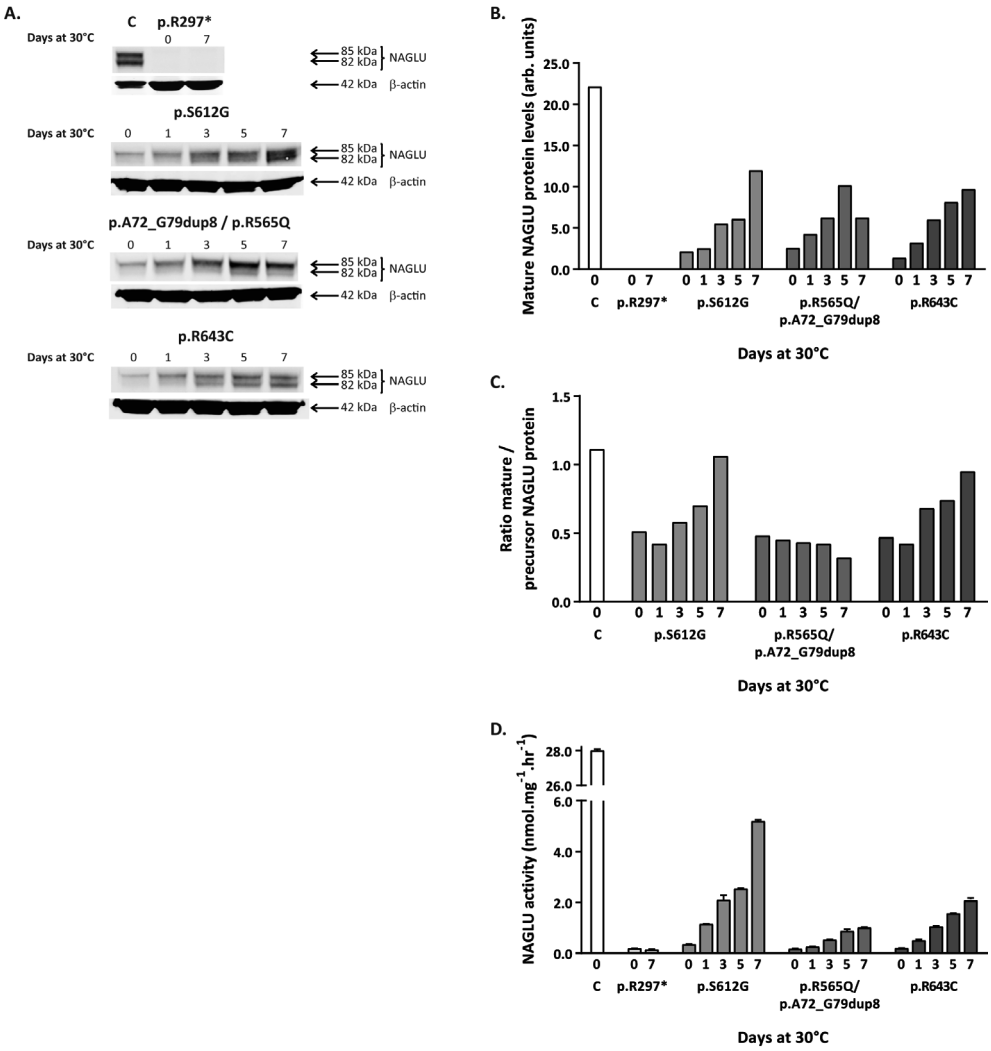


Figure 1. Time dependence of NAGLU protein and activity at 30°C culture conditions. A. Western blot analysis of NAGLU protein levels in control, p.R297* RP MPS IIIB and p.S612G, A72_G79dup8 / p.R565Q and p.R643C SP MPS IIIB fibroblasts after culturing at 30°C for different periods of time. B. Quantification of mature NAGLU protein levels on western blot (arb. units). C. Ratio of mature over precursor NAGLU protein as quantified on western blot. D. Corresponding NAGLU activity levels (nmol.mg^{-1}.hr^{-1}). Mean and SDs are given (n=2). Data of one representative experiment is shown, performed in duplicate.

30°C did not result in the formation of precursor or mature NAGLU and no enzyme activity could be detected.

Temperature dependence of NAGLU protein and activity

To study the effect of different temperatures on the synthesis and maturation of mutant NAGLU, SP cell lines were cultured for seven days at different temperatures ranging from 37°C to 27°C. An increase in total NAGLU protein levels as well as the formation of the mature form of NAGLU was already noticeable at 35°C, and reached its maximum at 29°C (figures 2A and 2B). In all cases the increase in mature NAGLU protein correlated with an increase in NAGLU activity (figure 2C).

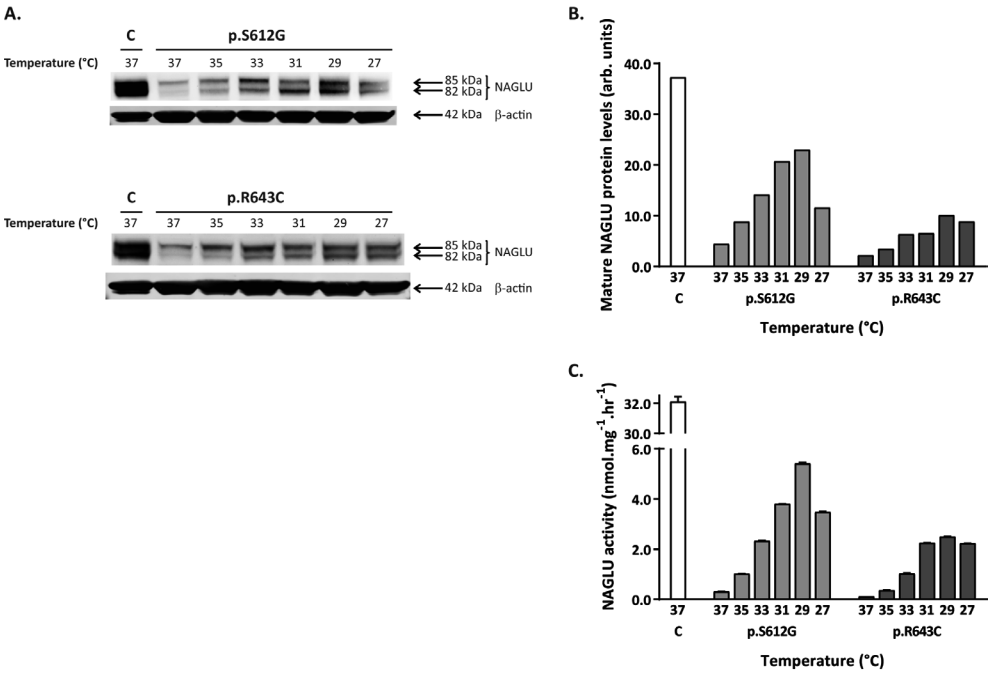


Figure 2. Temperature dependence of NAGLU protein and activity. A. Western blot analysis of NAGLU protein levels in control, p.S612G and p.R643C SP MPS IIIB fibroblasts after culturing at temperatures ranging from 27°C to 37°C for seven days. B. Quantification of mature NAGLU protein levels on western blot (arb. units). C. Corresponding NAGLU activity levels (nmol.mg^{-1}.hr^{-1}). Mean and SDs are given (n=2).

Effect of temperature on NAGLU mRNA levels

To investigate whether the increase in NAGLU protein resulted from an increase in NAGLU mRNA levels, fibroblast cell lines from controls, RP and SP patients were cultured at 37°C and 30°C for seven days. Next, mRNA levels of NAGLU and the reference genes *PPIB* and *H3F3A* were measured. In all SP fibroblast cell lines, NAGLU mRNA levels increased significantly when cultured at 30°C compared to levels at 37°C ($p < 0.01$) (figure 3A). In only

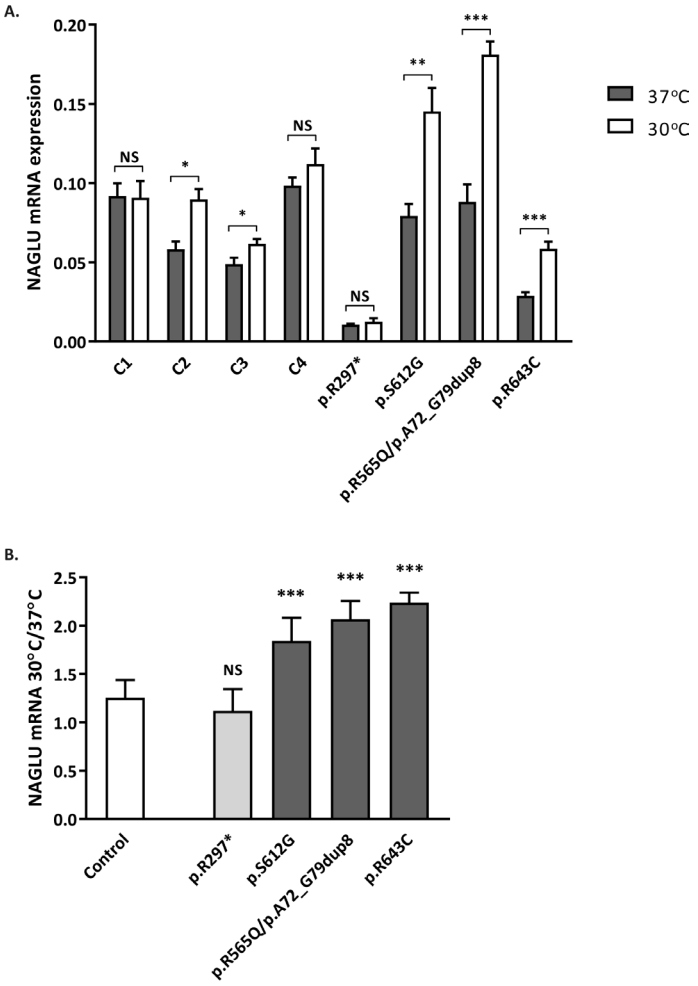


Figure 3. Effect of temperature on NAGLU mRNA levels. A. NAGLU mRNA expression after culturing four control fibroblast cell lines (C1-C4), p.R297* RP MPS IIIB and p.S612G, A72_G79dup8/p.R565Q and p.R643C SP MPS IIIB fibroblasts at 30°C for seven days. B. Ratio of NAGLU mRNA levels at 30°C over NAGLU mRNA levels at 37°C in p.R297* RP MPS IIIB and p.S612G, A72_G79dup8/p.R565Q and p.R643C SP MPS IIIB fibroblasts, compared to the mean ratio in control fibroblasts (C1-C4)**.** In all cases mean and SDs are given (n = 3). Data of one representative experiment is shown, performed in duplicate. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

two control cell lines a small but significant increase in NAGLU mRNA levels after culturing at 30°C was seen ($p < 0.05$). Compared to control fibroblasts the increase in NAGLU mRNA levels measured in SP cell lines was significantly more pronounced ($p < 0.001$) (figure 3B). In the RP fibroblasts no detectable effect of culturing at 30°C on NAGLU mRNA levels was seen.

Proteasome inhibition and the effect on mutant NAGLU precursor levels

Mutant and misfolded proteins are generally considered to be broken down by the proteasome complex. To assess if mutant NAGLU in SP fibroblasts is destined for proteasomal degradation, fibroblasts were cultured under normal conditions in the presence of bortezomib, a well-known proteasome inhibitor [12]. Indeed, treatment of p.R643C SP fibroblasts with different concentrations bortezomib for 48 hours at 37°C resulted in higher levels of the precursor form of NAGLU (figure 4). Quantification of the protein levels found on western blot revealed that treatment with 300 nM bortezomib resulted in 3.46 times higher NAGLU precursor levels, compared to levels found in non-treated p.R643C SP fibroblasts. This suggests that under normal conditions, mutant precursor NAGLU is degraded by the proteasome. However, the increase in the precursor form of mutant NAGLU did not result in higher levels of the mature form or an increase in NAGLU enzyme activity (data not shown).

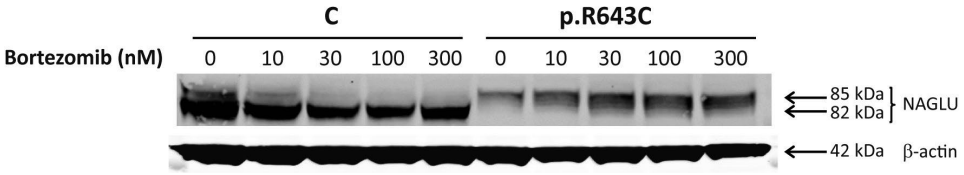


Figure 4. Proteasome inhibition and the effect on mutant NAGLU precursor levels. Western blot analysis of NAGLU protein levels after culturing control and p.R643C SP MPS IIIB fibroblasts in the presence of different concentrations bortezomib for 48 hours. Data of one representative experiment is shown, performed in duplicate.

Protein glycosylation of mutant NAGLU at 37°C versus 30°C

To determine the effect of low temperature on glycosylation of mutant NAGLU protein, control and p.S612G SP fibroblasts were cultured at 37°C or 30°C for seven days. Subsequently, cell lysates were incubated in the presence or absence of PNGase F, followed by western blot analysis of NAGLU protein. In control fibroblasts cultured at 37°C, NAGLU was mainly present in its mature 82 kDa form and to a lesser extend in its 85 kDa precursor form (figure 5). Culturing control fibroblasts at 30°C resulted in a decrease in the level of mature NAGLU. After treatment with PNGase F, only a complete deglycosylated form of NAGLU was seen, with an apparent molecular weight of 75 kDa. In the p.S612G SP cells cultured at 37°C, NAGLU protein levels were low and mainly present in the glycosylated 85 kDa precursor form. However, also a small band was visible that corresponded with the 75

kDa deglycosylated form of NAGLU that was also seen in control fibroblasts after treatment with PNGase F. Upon culturing the SP fibroblasts at 30°C, an increase in NAGLU precursor was observed and also the mature form of NAGLU appeared, as was shown in previous experiments. Treatment of mutant NAGLU with PNGase F resulted in a single protein band with the same molecular weight as completely deglycosylated NAGLU in the control fibroblasts. Furthermore, in control and SP MPS IIIB fibroblasts, precursor and mature NAGLU gave the same product after treatment with PNGase F, indicating that both forms of NAGLU only differ in their glycosylation status.

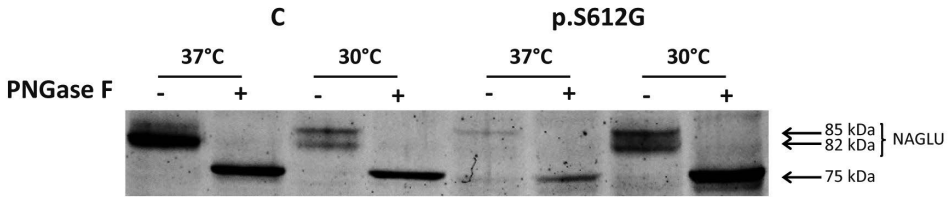
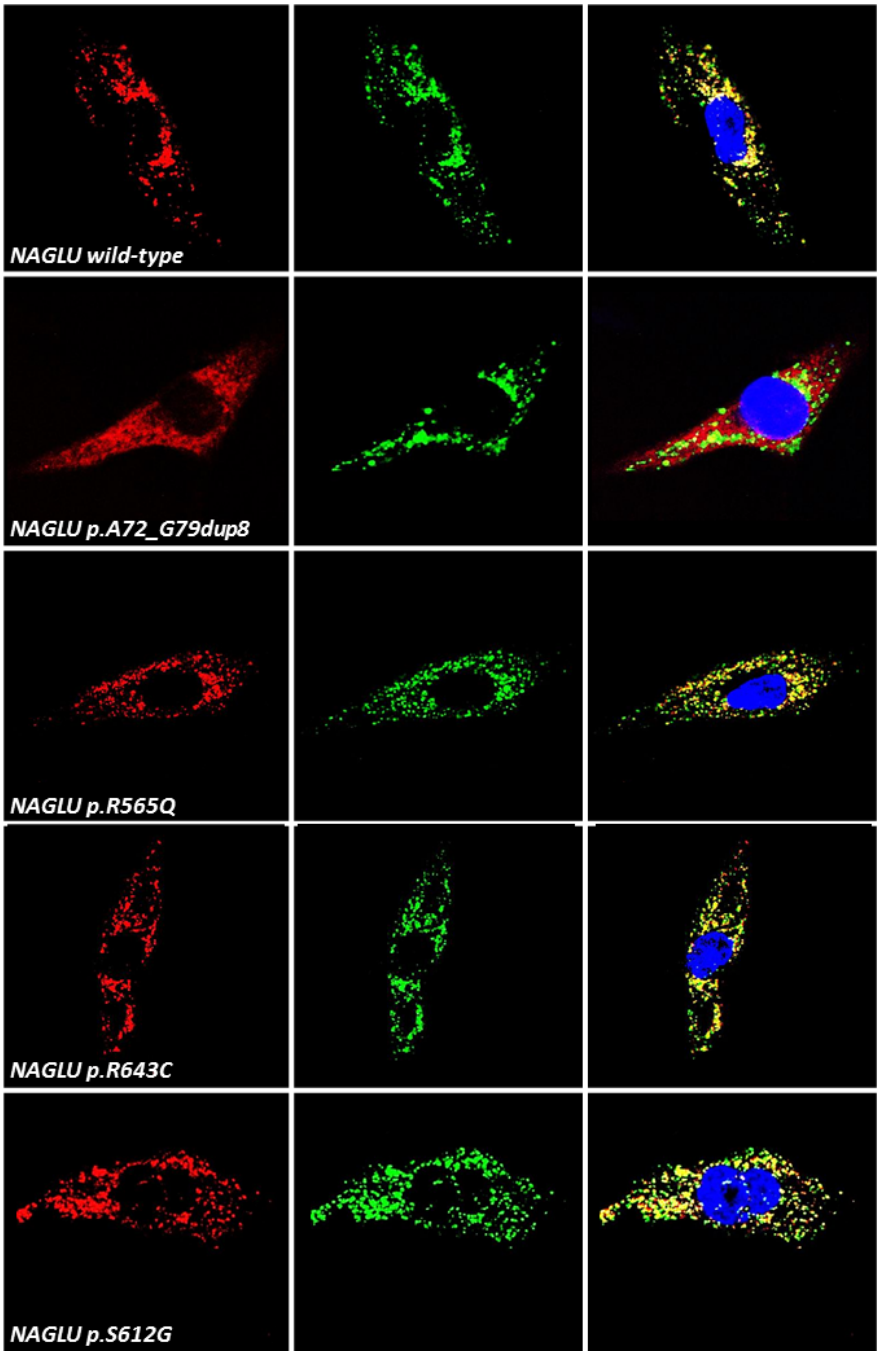


Figure 5. Protein glycosylation of mutant NAGLU at 37°C versus 30°C. Western blot analysis of NAGLU protein levels in control and p.S612G SP MPS IIIB fibroblasts cultured at 37°C or 30°C for seven days and treated with or without peptide-*N*-glycosidase F (PNGase F). Data of one representative experiment is shown, performed in duplicate.

Intracellular localization of NAGLU

To evaluate the subcellular localization of mutant NAGLU, plasmids for the expression of normal and mutant NAGLU tagged with mCherry at the carboxy-terminus were constructed. The plasmids were transfected into RP p.R297* fibroblasts and cultured at 37°C for 48 hours before further analysis. As expected, normal NAGLU-mCherry colocalized with lysotracker green (figure 6A) and, with an enzyme activity of 5.09 nmol.mg^{-1} .hr^{-1}, this construct was biochemically active (figure 6B). Also, NAGLU-mCherry carrying the missense mutations p.R565Q, p.S612G and p.R643C was localized predominantly in lysosomes after culturing at 37°C, but NAGLU activity in these cell lines did not significantly differ from activity levels in the mock transfected p.R297* RP fibroblasts (0.11 nmol.mg^{-1} .hr^{-1}), although protein levels of mutant NAGLU-mCherry were comparable to wild-type NAGLU-mCherry (figure 6C). Only in NAGLU-mCherry carrying the p.A72_G79dup8 mutation no colocalisation with lysosomes could be observed.

A.


NAGLU wild-type
NAGLU p.A72_G79dup8
NAGLU p.R565Q
NAGLU p.R643C
NAGLU p.S612G

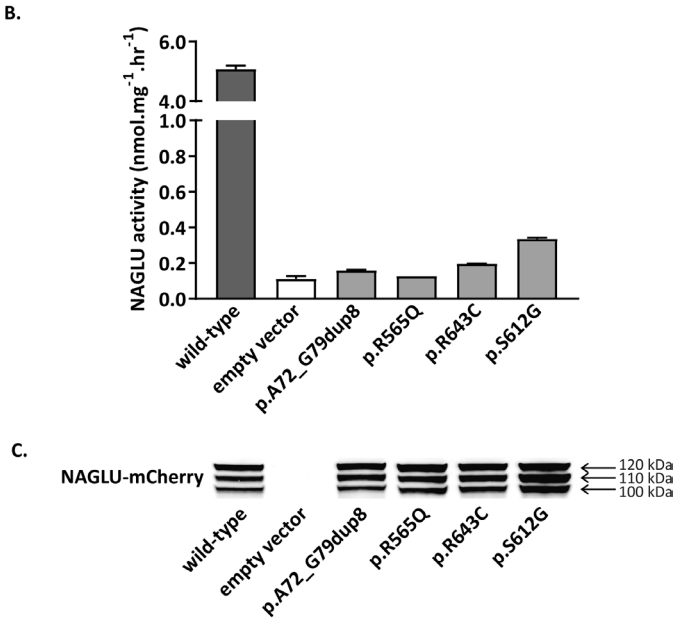


Figure 6. Intracellular localization of NAGLU. A. Colocalization of NAGLU-mCherry (left column) with lysotracker green (middle column), of wild-type NAGLU-mCherry and NAGLU-mCherry carrying the mutations p.R565Q, p.S612G, p.R643C and p.A72_G79dup8, transfected into p.R297* RP MPS IIIB fibroblasts and cultured at 37°C for 48 hours after transfection. In the right column the merged pictures are shown. B. Corresponding NAGLU activity levels ($nmol.mg^{-1}.hr^{-1}$). Mean and SDs are given (n=2). C. Western blot analysis of NAGLU-mCherry in the corresponding cell lysates. Data of one representative experiment is shown, performed in duplicate.

DISCUSSION

Previous work revealed that residual NAGLU activity in SP MPS IIIB patients' fibroblasts increased significantly upon culturing at 30°C, which was not seen in fibroblasts from RP MPS IIIB patients [7]. The strong correlation between the capacity to increase residual NAGLU activity and the MPS IIIB phenotype implies that this is a major contributor to the clinical course of the disease in these patients. Here, we show that culturing fibroblasts of SP patients under hypothermic conditions not only results in higher residual NAGLU activity, but also in increased levels of both precursor and mature NAGLU protein. Moreover, we observed an improvement in the processing of precursor protein into the mature and enzymatically active form. Unexpectedly, culturing at lower temperatures resulted in a significant increase in NAGLU mRNA levels in SP patients' fibroblasts.

The finding that in all tested SP cell lines, NAGLU mRNA levels increased significantly upon culturing at lower temperatures is remarkable, especially because this effect was seen to a much lesser extent in control fibroblast cell lines. In other mammalian cell systems it has been shown that mild cold stress (27-32˚C) influences the expression of a wide variety of genes and results in the induction of a number of cold-shock proteins, of which the best characterized proteins are cold-inducible RNA binding protein and RNA binding motif protein 3 [13,14]. Although their exact working mechanism is still unknown, these proteins are thought to modulate transcription and translation, and to function as RNA chaperones [15,16]. This could well explain the higher mRNA levels measured in SP patients' fibroblasts.

Increasing the amount of NAGLU precursor by blocking proteasomal breakdown of mutant NAGLU precursor protein, did not result in higher levels of mature protein or NAGLU activity. This suggests that the increase of mature NAGLU at low temperatures is not only due to increased synthesis of precursor NAGLU but that improved folding and/or processing under these conditions also plays a crucial role. This was further supported by the fact that at 37°C mutant NAGLU-mCherry was found to be predominantly localized in lysosomes but was not enzymatically active, suggesting improper NAGLU folding and/or processing. However, the lysosomal localization of mutant NAGLU-mCherry could be due to the fact that an overexpression system was used. Nevertheless, these results show that increasing NAGLU protein levels alone is not sufficient to increase the levels of active enzyme and that culturing at low temperatures also initiates other processes which ultimately result in the formation of mature and biochemically active NAGLU in SP MPS IIIB fibroblast cell lines.

In fibroblasts compound heterozygous for the *NAGLU* mutations p.R565Q and p.A72_G79dup8, higher enzyme activity was measured at hypothermic culture conditions, and both precursor and mature protein levels were increased under these conditions. However, in contrast to the other SP MPS IIIB cell lines, there was no additional improvement in the ratio of mature over precursor NAGLU, a measure for NAGLU processing, which thus appears to be, a least partially, mutation dependent. Hypothermia might lead to higher precursors levels of both NAGLU mutants, but since fluorescence microscopy revealed that p.A72_G79dup8 NAGLU did not reach the lysosomes, it might well be possible that only p.R565Q precursor NAGLU could be processed into its mature form. Despite the induction of several temperature sensitive proteins, mild cold stress seems to result in an overall decrease of transcription and translation, which might also allow more time for protein folding and processing [15]. This further suggests that the temperature induced increase in residual NAGLU activity and higher levels of mature, processed protein, depend on multiple factors that affect gene expression, including transcription start site and splicing decisions, mRNA levels and stability, and overall protein synthesis [16–19].

The positive effect of low culture temperature on the activity of mutant enzymes in MPS IIIB fibroblasts of patients with an SP phenotype is convincing and robust. In addition to

this study, a recent study has shown similar results in fibroblasts of MPS IIIA patients with different disease severity [20]. In order to mimic these effects by the use of pharmacological agents, more knowledge is needed about the mechanisms underlying this multifactorial process.

SUPPLEMENTARY DATA

Primer sequences for qPCR	
Fw960NAGLU	TTCCTCAGAGCCCTCCTACC
Rv1151NAGLU	AACAGGTCCAGAACCAGGAG

Primer sequences for cloning NAGLU '3 prime end	
Fw866NAGLU	CCATCATCGGGAGCCTCTTC
Rv2229NAGLU*Age*I	ATATACCGGTTCGCCTCCGGAGCCCCAAGAGCCGGCCACCCAG

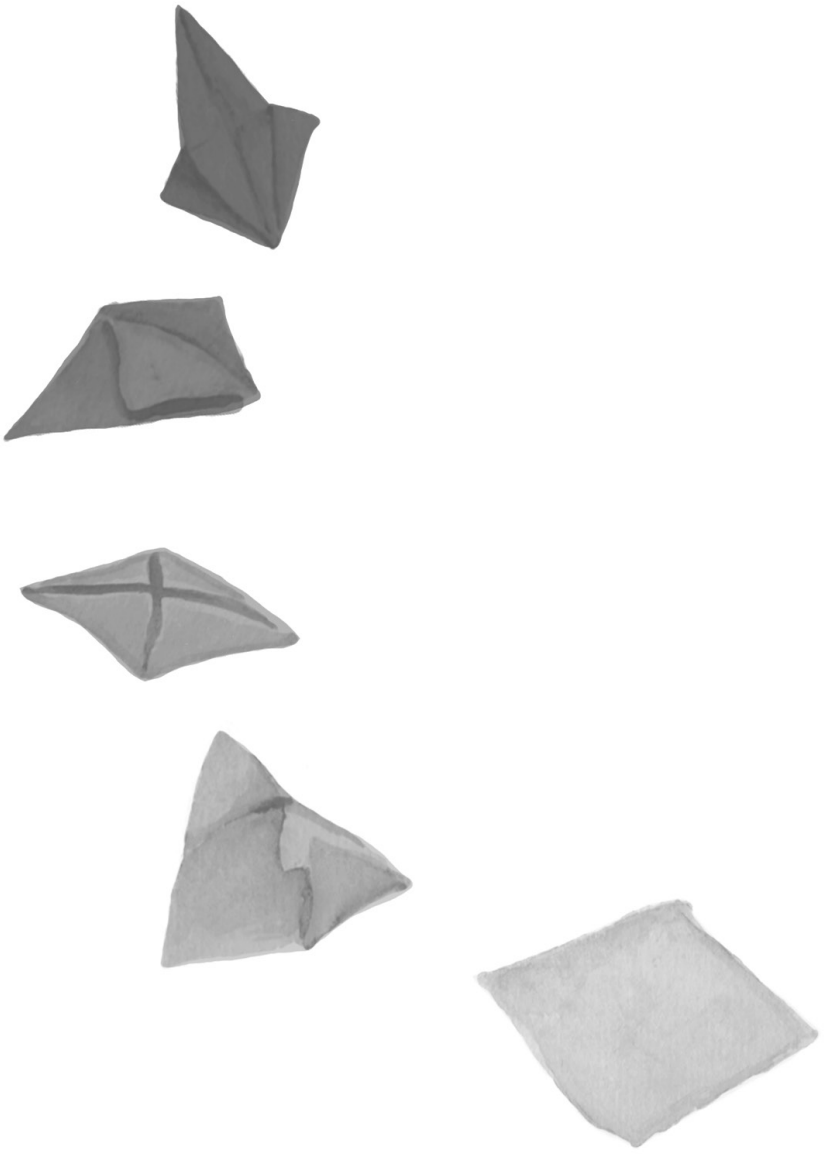
Primer sequences for mutagenesis	
FwNAGLU_c.1834A>G	CGAGGTGCTGGCTGGTGACAGCCGCT
RvNAGLU_c.1834A>G	AAGCGGCTGTCACCAGCCAGCACCTCG
FwNAGLU_c.1927C>T	ACGAGCAGAACAGCTGCTACCAGCTGACC
RvNAGLU_c.1927C>T	GGTCAGCTGGTAGCAGCTGTTCTGCTCGT
FwNAGLU_c.1694G>A	CATCGGGAGCCTCTTCCTGCGA
RvNAGLU_c.1694G>A	CACCCAGCGGGGGTAATATTTG

Primer sequences for NAGLU sequencing	
Fw448NAGLU	GGGAGCGAGAGATAGACTGG
Fw1900NAGLU	AGGCCGATTTCTACGAGCAG
Rv1819NAGLU	CACTAGCCAGCACCTCGT
Rv1392NAGLU	AAATCTGGCACTGGGTCCTT
Rv924NAGLU	TTGAAAGTGTCGGCCCCATA
Rv419NAGLU	AGTCCCACCACACGAAAGAG

Table S1. Primer sequences used for quantitative RT-PCR and cloning of human wild-type and mutant NAGLU. Restriction sites are underscored.

REFERENCES

1. Muenzer J. Overview of the mucopolysaccharidoses. *Rheumatology*. 2011;50 Suppl 5:v4-12.
2. Valstar MJ, Bruggenwirth HT, Olmer R, et al. Mucopolysaccharidosis type IIIB may predominantly present with an attenuated clinical phenotype. *J Inherit Metab Dis*. 2010;33(6):759-767.
3. von Figura K, Hasilik A, Steckel F, van de Kamp J. Biosynthesis and maturation of alpha-N-acetylglucosaminidase in normal and Sanfilippo B-fibroblasts. *Am J Hum Genet*. 1984;36:93-100.
4. Hasilik A. The early and late processing of lysosomal enzymes: proteolysis and compartmentation. *Experientia*. 1992;48(2):130-151.
5. Braulke T, Bonifacino JS. Sorting of lysosomal proteins. *Biochim Biophys Acta*. 2009;1793(4):605-614.
6. O'Brien JS. Sanfilippo syndrome: profound deficiency of alpha-acetylglucosaminidase activity in organs and skin fibroblasts from type-B patients. *Proc Natl Acad Sci USA*. 1972;69(7):1720-1722.
7. Meijer O, Welling L, Valstar M, et al. Residual N-acetyl-α-glucosaminidase activity in fibroblasts correlates with disease severity in patients with mucopolysaccharidosis type IIIB. *J Inherit Metab Dis*. 2016;39(3):437-445.
8. Zhao HG, Aronovich EL, Whitley CB. Genotype-phenotype correspondence in Sanfilippo syndrome type B. *Am J Hum Genet*. 1998;62(1):53-63.
9. Weber B, Guo XH, Kleijer WJ, van de Kamp JJ, Poorthuis BJ, Hopwood JJ. Sanfilippo type B syndrome (mucopolysaccharidosis III B): allelic heterogeneity corresponds to the wide spectrum of clinical phenotypes. *Eur J Hum Genet*. 1999;7(1):34-44. d
10. Ramakers C, Ruijter JM, Lekanne Deprez RH, Moorman AFM. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett*. 2003;339(1):62-66.
11. Weber B, Blanch L, Clements PR, Scott HS, Hopwood JJ. Cloning and expression of the gene involved in Sanfilippo B syndrome (mucopolysaccharidosis III B). *Hum Mol Genet*. 1996;5(6):771-777.
12. Adams J, Palombella VJ, Sausville EA, et al. Proteasome inhibitors: a novel class of potent and effective antitumor agents. *Cancer Res*. 1999;59(11):2615-2622.
13. Al-Fageeh MB, Marchant RJ, Carden MJ, Smales CM. The cold-shock response in cultured mammalian cells: harnessing the response for the improvement of recombinant protein production. *Biotechnol Bioeng*. 2006;93(5):829-835.
14. Roobol A, Carden MJ, Newsam RJ, Smales CM. Biochemical insights into the mechanisms central to the response of mammalian cells to cold stress and subsequent rewarming. *FEBS J*. 2009;276(1):286-302.
15. Fujita J. Cold shock response in mammalian cells. *J Mol Microbiol Biotechnol*. 1999;1(2):243-255.
16. Dresios J, Aschrafi A, Owens GC, Vanderklish PW, Edelman GM, Mauro VP. Cold stress-induced protein Rbm3 binds 60S ribosomal subunits, alters microRNA levels, and enhances global protein synthesis. *Proc Natl Acad Sci USA*. 2005;102(6):1865-1870.
17. Al-Fageeh MB, Smales CM. Control and regulation of the cellular responses to cold shock: the responses in yeast and mammalian systems. *Biochem J*. 2006;397(2):247-259.
18. Ars E, Serra E, de la Luna S, Estivill X, Lázaro C. Cold shock induces the insertion of a cryptic exon in the neurofibromatosis type 1 (NF1) mRNA. *Nucleic Acids Res*. 2000;28(6):1307-1312.
19. Neutelings T, Lambert CA, Nusgens B V., Colige AC. Effects of mild cold shock (25°C) followed by warming up at 37°C on the cellular stress response. *PLoS One*. 2013;8(7):e69687.
20. Nijmeijer S, Knottnerus S, Wijburg F, van Vlies N. Phenotypic severity in MPS IIIA strongly correlates with increase in residual SGSH activity in skin fibroblasts cultured at 30°C. *Mol Genet Metab*. 2017;120(1-2):S103.



Chapter 5

Quantity and structure of stored heparan sulfate may affect the nature and course of neuronopathic disease in mucopolysaccharidosis type I and type III

O.L.M Meijer. T. Wagemans, H. van Lenthe,
F.A. Wijburg and Naomi van Vlies

Manuscript in preparation

ABSTRACT

Introduction

Heparan sulfate (HS) is one of the accumulating glycosaminoglycans in several mucopolysaccharidoses (MPSs), including MPS I (Hurler, Hurler-Scheie and Scheie syndromes) and MPS III (Sanfilippo disease). Both MPS types are characterized by severe central nervous system (CNS) disease, although striking differences in CNS symptoms are observed between the two MPSs and between MPS I and MPS III patients with different disease severity. Here, the relation between total HS (tHS) levels and sulfation pattern of HS derived disaccharides and the type and severity of CNS disease is studied in plasma of MPS I and MPS III patients.

Methods

To study the relation of total tHS levels and sulfation pattern with type and severity of CNS disease, we analyzed tHS concentrations and sulfation of HS derived disaccharides in plasma of patients with MPS I (N = 24) and MPS III (N = 44).

Results

tHS levels and HS derived disaccharide fractions differed significantly between MPS III patients with different disease severity. A correlation between tHS and disease severity was also detected in MPS I. Fractions of HS derived disaccharides in plasma differed between MPS I and MPS III patients, with a trend towards higher levels of sulfated HS derived disaccharides in MPS III.

Conclusions

Both tHS levels and HS derived disaccharide fractions differ significantly between MPS III patients with different disease severity. The same correlation between tHS and disease severity was detected in MPS I, although no differences in HS derived disaccharide fractions were found between the MPS I phenotypes.

Moreover, HS derived disaccharide fractions in plasma differed between MPS I and MPS III patients, which may relate to differences in CNS phenotype between MPS I and MPS III patients.

INTRODUCTION

The glycosaminoglycan heparan sulfate (HS) is a linear polysaccharide that consists of the repeating disaccharide subunits *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA). Covalently bound to a core protein, HS forms HS proteoglycans (HSPG) that are found in all cell types. During its biosynthesis, HS undergoes extensive modification and epimerization in the Golgi apparatus. Epimerization of a subset of the GlcA subunits results in the generation of iduronic acid (IdoA). In addition, sulfate groups can be added to *N*-, 2-O and 6-O atoms of the different disaccharide residues. A mature HS chain consists of modified HS segments which are interspersed by sections of unmodified disaccharides. It is the degree and pattern of HS modification, and especially of sulfation, that defines the extensive and highly specific ability of HSPGs to bind various protein ligands [1].

As component of the plasma membrane and extracellular matrix (ECM), HSPGs are abundantly present throughout the body, including in the central nervous system (CNS). They serve as important regulatory factors in signaling pathways and are essential for the formation of morphogen gradients. Furthermore, HSPGs are important molecules for the regulation of cell-cell interaction, cell motility and migration [2,3] and are therefore essential for normal brain development. Animal studies have shown that impairment of HS biosynthesis affects proliferation and differentiation of neural progenitor cells [4], axon guidance [5,6] and synapse formation and maintenance [7–9].

HS is one of the main accumulating glycosaminoglycans in mucopolysaccharidosis (MPS) types I (Hurler, Hurler/Scheie and Scheie syndromes), II, III (Sanfilippo syndrome) and VII [10]. In these disorders there is a deficiency of a lysosomal enzyme involved in the degradation of HS. This study will focus on MPS I and MPS III. In MPS I the absence of α-L-iduronidase (IDUA) leads to accumulation of both dermatan sulfate (DS) and HS. In all four MPS III subtypes only the lysosomal degradation of HS is impaired due to a deficiency of either heparan *N*-sulfatase (SGSH; MPS IIIA), *N*-acetyl-α-glucosaminidase (NAGLU; MPS IIIB), acetyl-CoA:a-glucosaminide *N*-acetyltransferase (HGSNAT; MPS IIIC) or *N*-acetylglucosamine 6-sulfatase (GNS; MPS IIID) [11].

There are significant differences in phenotype within and between MPS I and MPS III patients. Based on disease severity, MPS I used to be divided into three subtypes of which MPS I-Hurler (MPS I-H) represents the most severe, MPS I-Hurler/Scheie (MPS I-H/S) the intermediate and MPS I-Scheie (MPS I-S) the more attenuated phenotype. In contrast to the more attenuated MPS I phenotypes, patients with MPS I-H (and some MPS I-H/S patients) suffer from severe central nervous system (CNS) disease with progressive loss of cognitive functions [12,13]. Therefore, MPS I patients are nowadays preferably divided into groups with neuronopathic (NNP) versus non-neuronopathic disease (non-NNP). As in MPS I, a spectrum of disease severity can be distinguished in all four MPS III subtypes. Progressive loss of

neurocognitive function is, however, the predominant feature in all MPS III patients [14–16]. Based on the rate of disease progression two groups of patients can be distinguished: those with a severe and rapidly progressing phenotype (RP) and those with a more attenuated and slowly progressing (SP) phenotype.

Accumulation of HS and the subsequent cascade of pathophysiological events, including neuro-inflammation and apoptotic triggers, are held responsible for the CNS disease in both NNP MPS I and MPS III, since accumulation of only DS does not lead to CNS disease [17]. This is demonstrated by the fact that patients with MPS VI (Maroteaux-Lamy syndrome), in which only DS cannot be degraded, do not have CNS involvement. There are, however, several striking differences in CNS symptoms between NNP MPS I and MPS III. Whereas in MPS III patients neurocognitive decline is accompanied and even preceded by behavioral problems, hyperactivity, fearlessness and temper tantrums, these symptoms are absent in NNP MPS I patients [18,19]. The cause of these different CNS phenotypes is not clear but might be related to different chemical structures of the incompletely degraded HS molecules [20]. Differences in the sulfation pattern of HS derived disaccharides in brain tissue were indeed observed between MPS I and MPS III mice [21–24]. As modifications in the pattern of sulfation are important for the biological functions of HS, these differences may be related to the distinct nature of the CNS disease in MPS I and MPS III patients [2,3]. In order to further study the relation of total HS (tHS) levels and sulfation pattern with type and severity of CNS disease in these two disorders, we here analyze tHS concentrations and sulfation of HS derived disaccharides in plasma of patients with MPS I and MPS III.

MATERIALS AND METHODS

Patients

In this study 24 MPS I patients were included. Diagnosis was confirmed by enzymatic and genetic analyses in all patients. Based on predictive genotypes and/or clinical course of the disease, 12 patients were classified as NNP MPS I (or MPS I-H) and 12 as non-NNP MPS I (6 MPS I-H/S and 6 MPS I-S, respectively) by an experienced clinician (FAW)[25,26]. At time of plasma collection the median age of the NNP patients was 1.2 years (range 0.8 – 7.1 years) and of the non-NNP patients 10.6 years (range 1.0 – 35.3 years).

The cohort of MPS III patients consisted of 43 patients of which 23 had been diagnosed with MPS IIIA, 11 with MPS IIIB, and 9 with MPS IIIC. Diagnosis was based on enzymatic analysis in all patients and confirmed by mutation analysis in 42 patients. Based on the clinical course of the disease and genotype (if predictive), patients were categorized in two phenotypic subgroups by an experienced clinician (FAW) [14–16]. Thirteen patients were classified as having a severe, rapidly progressing (RP) phenotype of which 7 had MPS IIIA, 2 MPS IIIB and 4 MPS

IIIC. Median age at time of sample collection in the RP group was 11.7 years (range 3.4 – 17.6 years). The group with a more attenuated, slowly progressing (SP) phenotype consisted of 30 patients of which 16 had MPS IIIA, 9 MPS IIIB and 5 MPS IIIC patients. The median age at sample collection in this group was 21.2 years (range 4.4 – 66.9 years).
Blood samples of MPS I patients were collected for routine diagnostics before start of enzyme replacement therapy or hematopoietic stem cell transplantation. The MPS III patient plasma samples had been previously obtained for a study by de Ruijter *et al*. in 2013 and were re-analyzed [27]. All samples were collected after obtaining informed consent of parents and/or patients, and were encoded and stored in the biobank of the Academic Medical Center (AMC), Amsterdam, The Netherlands, according to the regulations of the Medical Ethics Review Committee of our center. These procedures followed the AMC Research Code on research using human materials.

Measurement of HS in plasma

Blood of patients was collected in EDTA tubes and centrifuged at 240 g for 10 minutes. Samples were stored at either -20°C or -80°C until further analysis. HS levels in patient plasma was determined using HPLC-MS/MS, as described previously [28].
tHS levels were determined as the sum of six HS derived disaccharides: D0A0, D0S0, D0A6&D2A0 and D0S6&D2S0 [29]. Because in controls only the D0A0 levels were above limit of quantification, D0A0 levels in patient plasma were corrected for age by subtracting the upper value of the 95% confidence interval for age of reference values. Age-corrected tHS levels were calculated using the ratio of age-corrected D0A0 over the absolute D0A0 concentration. This ratio was also used to correct the levels of the other HS disaccharides for age (HS disaccharide fractions were not dependent on the age of the patients (results not shown)). For compositional analysis, the fraction of each specific HS derived disaccharide was determined over the tHS level for each sample.

Data analysis

All data obtained in this study were analyzed using non-parametric Mann-Whitney U tests. A p-value of < 0.05 was considered statistically significant. All data analyses were performed using SPSS software for Windows (version 23.0, SPSS Inc., Chicago, IL).

RESULTS

Total HS levels and HS derived disaccharide fractions in plasma of MPS I patients with different disease severity

First, tHS levels and fractions of HS derived disaccharides were investigated separately for the two MPS subtypes. As described in previous studies, significantly higher tHS levels were found in NNP MPS I patients compared to non-NNP MPS I patients ($p < 0.01$]) (figure 1A) [30]. No significant differences in any of the fractions was seen between NNP and non-NNP MPS I patients (figure 1B).

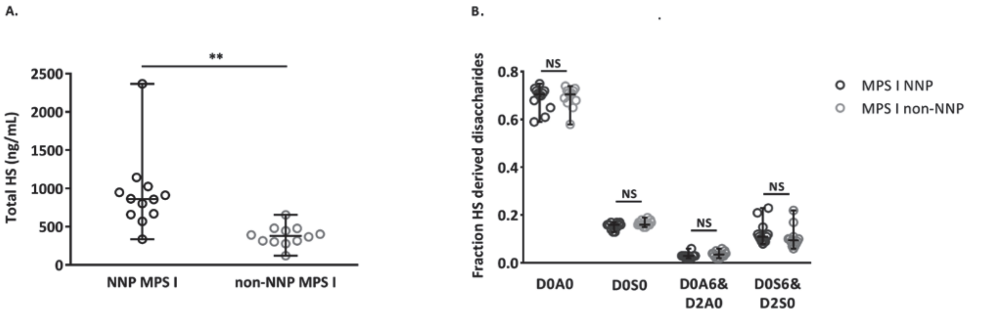


Figure 1. A. Levels of tHS concentrations in plasma corrected for age of NNP vs. non-NNP MPS I patients. B. Compositional analysis of HS derived disaccharides in plasma of NNP vs. non-NNP MPS I patients. Significance was determined using a Mann-Whitney U test. ** $p < 0.01$.

Total HS levels and HS derived disaccharide fractions in plasma of MPS III patients with different disease severity

As observed in NNP and non-NNP MPS I, and describer earlier by our group [27], significantly higher plasma tHS levels were found in the severely affected group of RP MPS III patients compared to the more attenuated group of SP patients (figure 2A). In contrast to MPS I, analyses of HS derived disaccharide fractions did show significant differences between patients with different disease severity in MPS III (figure 2B). D0A0 levels were significantly lower in RP MPS III patients plasma, whereas D0S0 and D0S6&D2S0 levels were significantly higher, suggesting that in RP MPS III patients fractions of sulfated disaccharides are overall more elevated than in SP patients.

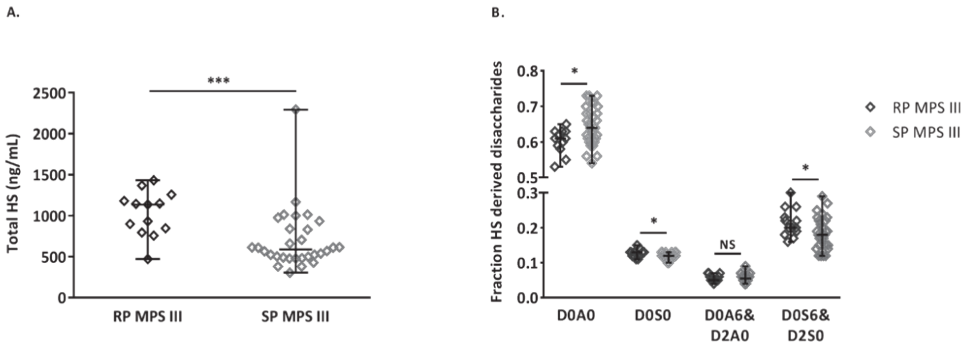


Figure 2. A. Levels of tHS concentrations in plasma corrected for age of RP vs. SP MPS III patients. B. Compositional analysis of HS derived disaccharides in plasma of RP vs. SP MPS III patients. In all cases the median and range are given. Significance was determined using a Mann-Whitney U test. * $p < 0.05$; *** $p < 0.001$.

Total HS levels and HS derived disaccharide fractions in plasma of MPS I versus MPS III patients

Then, tHS levels in plasma of MPS I (NNP MPS I and non-NNP MPS I) and MPS III patients (RP MPS III and SP MPS III) were analyzed (figure 3A). A significant difference was found between the two groups, with higher median tHS levels in the group of MPS III patients ($p < 0.05$). Nevertheless, a substantial overlap was seen between the two groups.

Further analyses of HS derived disaccharides were performed to assess whether disaccharide fractions differed between both patient groups (figure 3B). Despite considerable overlap in HS derived disaccharide fractions, significantly lower D0A0 and D0S0 fractions and higher D0A6&D2A0 and D0S6&D2S0 fractions were found in plasma of MPS III patients compared to MPS I patients ($p < 0.001$ for all HS derived disaccharide fractions). Overall, a trend towards higher levels of sulfated disaccharides was seen in patients with MPS III.

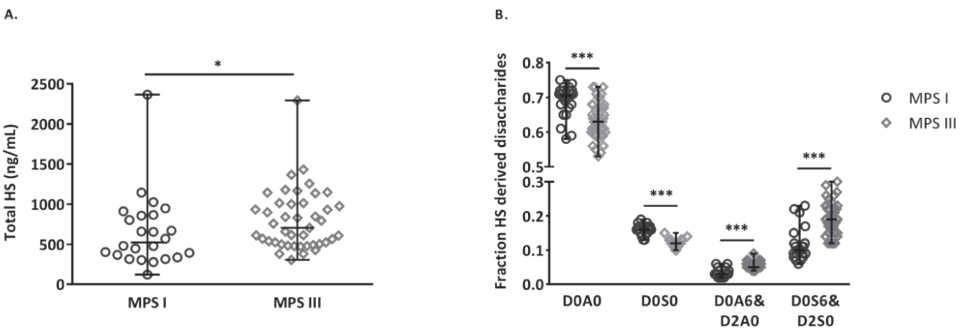


Figure 3. A. tHS levels in plasma of MPS I versus MPS III patients. B. Compositional analysis of HS derived disaccharides in plasma of MPS I and MPS III patients. Levels of HS derived disaccharides are depicted as fractions of tHS levels. In all cases the median and ranges are given. Significance was determined using a Mann-Whitney U test. * $p < 0.05$; *** $p < 0.001$.

HS derived disaccharide fractions in plasma of MPS I vs. MPS III patients with different disease severity

The nature and severity of CNS disease differs considerably between the subset of MPS I patients with a NNP phenotype, and RP and SP MPS III patients. Therefore, the level of sulfation of HS derived disaccharides in MPS I and MPS III patients with neuronopathic disease was compared. In addition to the differences between RP and SP MPS III patients shown in the previous figure, all HS derived disaccharide fractions in RP and SP MPS III patients differed significantly with those of NNP MPS I patients, with the most pronounced differences found in comparison to the RP MPS III group (figure 4). Despite considerable overlap, D0A0 levels were significantly lower in RP and SP MPS III patients compared to NNP MPS I patients ($p < 0.001$ and $p = 0.05$, respectively). In contrast, RP MPS III patients showed the highest D0S6&D2S0 fractions when compared to NNP MPS I patients ($p < 0.001$). For the D0S0 and D0A6&D2A0 fractions, small though significant differences were seen between all groups. It could well be that these differences in HS derived disaccharide fractions contribute to the differences in neuronopathic disease observed in these patients.

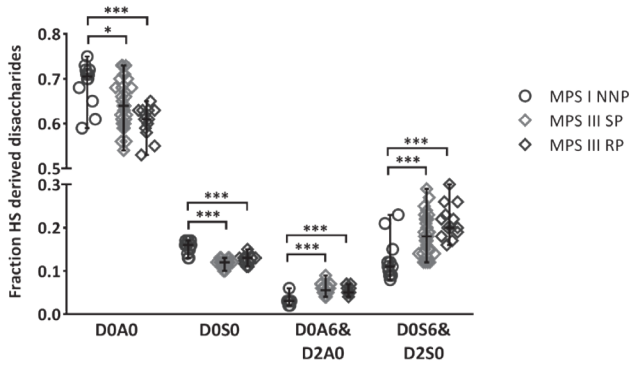


Figure 4. Compositional analysis of HS derived disaccharides in plasma of NNP MPS I, RP and SP MPS III patients. In all cases the median and range are given. Significance was determined using a Mann-Whitney U test. * $p < 0.05$; *** $p < 0.001$.

DISCUSSION

We show that fractions of specific HS derived disaccharides in plasma differ between MPS I and MPS III patients. This confirms previous studies in which differences in sulfation pattern of HS derived disaccharides were found in brain of MPS I and MPS III mice [22]. In addition, our study demonstrates that not only tHS levels [27], but also HS derived disaccharide fractions differ significantly between MPS III patients with different severity of disease. The same

correlation between tHS and disease severity was detected in MPS I. However, in MPS I no differences in sulfation pattern were detected between the different phenotypes.

The enzymatic degradation of HS is a well-ordered stepwise process that starts with cleavage of the large HS chains into smaller fragments by an endoglycosidase. The sequential action of exoglycosidases, sulfatases and an actyltransferase completes this process. In case of MPS I, the deficiency of IDUA results in impaired cleavage of an IdoA from the HS chain at the non-reducing end. The enzyme deficiencies in MPS III all lead to the impaired removal of a glucosamine [31]. As HS is a very heterogeneous molecule with different modifications along the chain, these differences in HS degradation could theoretically result in the accumulation of different storage products in MPS I and MPS III. Our data indeed show a different sulfation pattern of HS derived disaccharides in plasma of MPS I and MPS III patients and thereby provide evidence that the structure of stored HS differs between these diseases. These differences may be related to the remarkable differences in the type of CNS disease that are almost invariably observed between MPS I and MPS III patients.

Besides the findings of Wilkinson *et al*. in brain tissue of mice, there is no evidence yet that the differences observed in the sulfation of HS derived oligosaccharide fragments in human are indeed causing the observed differences in CNS disease between MPS I and MPS III patients [22]. However, it is known that the structure of HS, and especially the degree and pattern of HS modification and sulfation, determines the extensive and highly specific functions of HSPGs [2,3,32]. Since in the MPSs undegraded, accumulating HSPG fragments do not only remain intracellularly, but are also secreted to the cell's exterior this will influence the function of many HSPG-protein interactions [32]. The consequences of this intra- and extracellular HS storage have been best studied for MPS III. HS accumulation in MPS IIIB brain resulted in changes in expression of genes encoding for several fibroblast growth factors (FGFs) and their receptor (FGFR), that are responsible for overall neuroplasticity of the MPS IIIB mouse brain by affecting neuronal cell growth and function [33]. It has been shown that HS accumulation results in activation of integrin-based focal adhesions in astrocytes and neural stem cells in the ECM of MPS IIIB mice, leading to defective cell polarization and defects in oriented migration [34]. Recently published data further prove that abnormalities in HS content, including changes in HS derived oligosaccharides, and HS distribution, contribute to alterations in postsynaptic function in the developing somatosensory cortex of MPS IIIA mice [23]. Therefore, it is likely that structural changes in HS derived oligosaccharides determine the pattern of CNS disease in MPS patients.

The observation that the concentration of tHS in plasma correlates with disease severity in MPS I and MPS III was not surprising. As a result of the nature of the mutations in the relevant genes, differences in residual enzyme activity will lead to different tHS concentrations. For MPS I it was shown that different mutations and/or differences in residual activity of IDUA predict disease severity [25,35]. Correlations between genotype and disease severity could

also be established in MPS III [14–16,36]. These findings do indicate that both tHS and sulfation pattern of accumulating HS may play role in neuronopathic disease of these patients.

The observed difference in HS derived disaccharide fractions in plasma of patients with different phenotypic severity of MPS III is interesting. Differences in the underlying disease causing mutations might lead to small differences in the affinity of the enzyme for specific partially degraded HS molecules.

Our cohort mainly existed of MPS IIIA patients in whom the enzyme SGSH is deficient, which is responsible for the cleavage of sulfate groups attached to the nitrogen atom of glucosamine. It might be possible that a lower residual enzyme activity, that probably also determines the more severe phenotype, is responsible for the impaired cleavage of sulfate groups and thereby for the higher levels of sulfated disaccharides measured in patients with severe or attenuated MPS IIIA. The differences found between MPS III severity subgroups might only reflect differences between MPS IIIA patients. The groups of MPS IIIB and MPS IIIC patients in our cohort were too small to properly verify this. In MPS IIIB mice brain, however, higher overall levels of sulfated disaccharides are found compared to mice with MPS IIIA indicating that more studies into this subject are needed [22].

In conclusion, we show that tHS levels in plasma correlate with phenotypic severity in patients with MPS I and MPS III and that there are significant differences in HS derived disaccharide fractions in plasma between MPS I and MPS III patients. If indeed these differences found in plasma disaccharides also occur in the brain of patients, as was demonstrated for the brain of mice with MPS I and MPS III, this may well contribute to the differences in the CNS phenotypes of these two disorders. Further studies in mice models are needed to unravel the differences in pathophysiology of CNS disease between MPS I and MPS III.

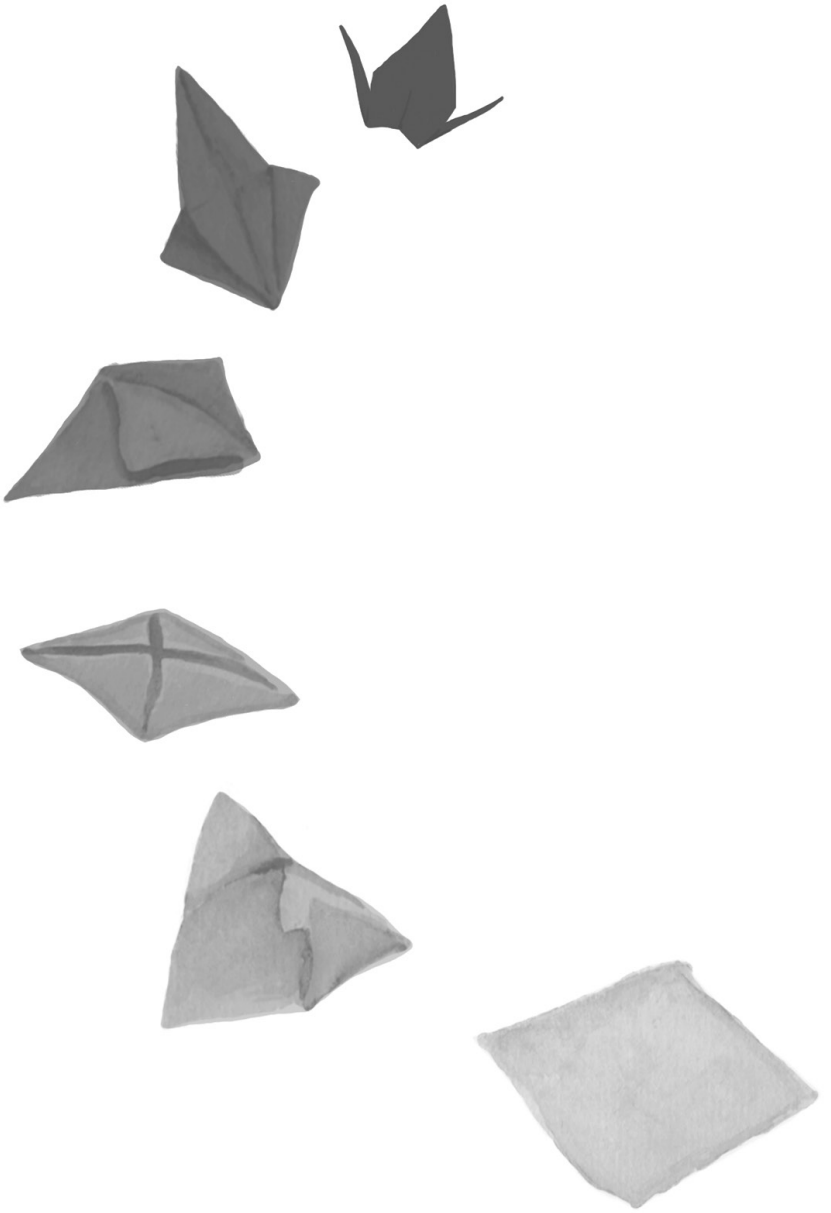
REFERENCES

1. Esko JD, Kimata K, Lindahl U. Chapter 16 Proteoglycans and Sulfated Glycosaminoglycans. In: Varki A, Cummings RD, Esko JD, eds. *Essentials of Glycobiology*. 2nd ed. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press; 2009.
2. Bishop JR, Schuksz M, Esko JD. Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature*. 2007;446(7139):1030-1037.
3. Sarrazin S, Lamanna WC, Esko JD. Heparan sulfate proteoglycans. *Cold Spring Harb Perspect Biol*. 2011;3(7):1-33.
4. McLaughlin D, Karlsson F, Tian N, et al. Specific modification of heparan sulphate is required for normal cerebral cortical development. *Mech Dev*. 2003;120(12):1481-1488.
5. Matsumoto Y, Irie F, Inatani M, Tessier-Lavigne M, Yamaguchi Y. Netrin-1/DCC signaling in commissural axon guidance requires cell-autonomous expression of heparan sulfate. *J Neurosci*. 2007;27(16):4342-4350.
6. Pratt T, Conway CD, Tian NMM-L, Price DJ, Mason JO. Heparan sulphation patterns generated by specific heparan sulfotransferase enzymes direct distinct aspects of retinal axon guidance at the optic chiasm. *J Neurosci*. 2006;26(26):6911-6923.
7. Allen NJ, Bennett ML, Foo LC, et al. Astrocyte glypicans 4 and 6 promote formation of excitatory synapses via GluA1 AMPA receptors. *Nature*. 2012;486(7403):410-4.
8. Ko JS, Pramanik G, Um JW, et al. PTPσ functions as a presynaptic receptor for the glypican-4/LRRTM4 complex and is essential for excitatory synaptic transmission. *Proc Natl Acad Sci USA*. 2015;112(6):1874-1879.
9. Irie F, Badie-Mahdavi H, Yamaguchi Y. Autism-like socio-communicative deficits and stereotypies in mice lacking heparan sulfate. *Proc Natl Acad Sci USA*. 2012;109(13):5052-5056.
10. Neufeld EF, Muenzer J. The Mucopolysaccharidoses. In: Valle D, Beaudet A, Vogelstein B, et al., eds. *The Metabolic and Molecular Basis of Inherited Disease*. 8th ed. New York, NY, USA: McGraw-Hill; 2001:3421-3452.
11. Muenzer J. Overview of the mucopolysaccharidoses. *Rheumatology*. 2011;50 Suppl 5:v4-12.
12. Pastores GM, Arn P, Beck M, et al. The MPS I registry: design, methodology, and early findings of a global disease registry for monitoring patients with Mucopolysaccharidosis Type I. *Mol Genet Metab*. 2007;91(1):37-47.
13. Beck M, Arn P, Giugliani R, et al. The natural history of MPS I: global perspectives from the MPS I Registry. *Genet Med*. 2014;16(10):759-765.
14. Valstar MJ, Neijs S, Bruggenwirth HT, et al. Mucopolysaccharidosis type IIIA: clinical spectrum and genotype-phenotype correlations. *Ann Neurol*. 2010;68(6):876-887.
15. Valstar MJ, Bruggenwirth HT, Olmer R, et al. Mucopolysaccharidosis type IIIB may predominantly present with an attenuated clinical phenotype. *J Inherit Metab Dis*. 2010;33(6):759-767.
16. Ruijter GJ, Valstar MJ, van de Kamp JM, et al. Clinical and genetic spectrum of Sanfilippo type C (MPS IIIC) disease in The Netherlands. *Mol Genet Metab*. 2008;93(2):104-111.
17. Archer LD, Langford-Smith KJ, Bigger BW, Fildes JE. Mucopolysaccharide diseases: a complex interplay between neuroinflammation, microglial activation and adaptive immunity. *J Inherit Metab Dis*. 2014;37(1):1-12.
18. Ahmed A, Whitley CB, Cooksley R, et al. Neurocognitive and neuropsychiatric phenotypes associated with the mutation L238Q of the α-L-iduronidase gene in Hurler-Scheie syndrome. *Mol Genet Metab*. 2014;111(2):123-127.
19. Rumsey RK, Rudser K, Delaney K, Potegal M, Whitley CB, Shapiro E. Acquired autistic behaviors in children with mucopolysaccharidosis type IIIA. *J Pediatr*. 2014;164(5):1147-1151.e1.
20. Węgrzyn G, Jakóbkiewicz-Banecka J, Narajczyk M, et al. Why are behaviors of children suffering from various neuronopathic types of mucopolysaccharidoses different? *Med hypotheses*. 2010;75(6):605-609.

21. Holley RJ, Deligny A, Wei W, et al. Mucopolysaccharidosis type I, unique structure of accumulated heparan sulfate and increased N-sulfotransferase activity in mice lacking α-l-iduronidase. *J Biol Chem*. 2011;286(43):37515-37524.
22. Wilkinson FL, Holley RJ, Langford-Smith KJ, et al. Neuropathology in mouse models of mucopolysaccharidosis type I, IIIA and IIIB. *PLoS One*. 2012;7(4):e35787.
23. Dwyer CA, Scudder SL, Lin Y, et al. Neurodevelopmental changes in excitatory synaptic structure and function in the cerebral cortex of Sanfilippo syndrome IIIA mice. *Sci Rep*. 2017;7:46576.
24. McCarty DM, Dirosario J, Gulaid K, et al. Differential distribution of heparan sulfate glycoforms and elevated expression of heparan sulfate biosynthetic enzyme genes in the brain of mucopolysaccharidosis IIIB mice. *Metab Brain Dis*. 2011;26(1):9-19.
25. Kingma SDK, Langereis EJ, de Klerk CM, et al. An algorithm to predict phenotypic severity in mucopolysaccharidosis type I in the first month of life. *Orphanet J Rare Dis*. 2013;8(1):99.
26. Bertola F, Filocamo M, Casati G, et al. IDUA mutational profiling of a cohort of 102 European patients with mucopolysaccharidosis type I: identification and characterization of 35 novel α-L-iduronidase (IDUA) alleles. *Hum Mutat*. 2011;32(6):E2189-210.
27. de Ruijter J, Ijlst L, Kulik W, et al. Heparan sulfate derived disaccharides in plasma and total urinary excretion of glycosaminoglycans correlate with disease severity in Sanfilippo disease. *J Inherit Metab Dis*. 2013;36(2):271-279.
28. de Ru MH, van der Tol L, van Vlies N, et al. Plasma and urinary levels of dermatan sulfate and heparan sulfate derived disaccharides after long-term enzyme replacement therapy (ERT) in MPS I: correlation with the timing of ERT and with total urinary excretion of glycosaminoglycans. *J Inherit Metab Dis*. 2013;36(2):247-255.
29. Lawrence R, Lu H, Rosenberg RD, Esko JD, Zhang L. Disaccharide structure code for the easy representation of constituent oligosaccharides from glycosaminoglycans. *Nat methods*. 2008;5(4):291-292.
30. Fuller M, Brooks D a, Evangelista M, Hein LK, Hopwood JJ, Meikle PJ. Prediction of neuropathology in mucopolysaccharidosis I patients. *Mol Genet Metab*. 2005;84(1):18-24.
31. Lawrence R, Brown JR, Al-Mafraji K, et al. Disease-specific non-reducing end carbohydrate biomarkers for mucopolysaccharidoses. *Nat Chem Biol*. 2012;8(2):197-204.
32. Xu D, Esko JD. Demystifying heparan sulfate-protein interactions. *Annu Rev Biochem*. 2014;83:129-157.
33. Li HH, Zhao H-Z, Neufeld EF, Cai Y, Gómez-Pinilla F. Attenuated plasticity in neurons and astrocytes in the mouse model of Sanfilippo syndrome type B. *J Neurosci Res*. 2002;69(1):30-38.
34. Bruyère J, Roy E, Ausseil J, et al. Heparan sulfate saccharides modify focal adhesions: Implication in mucopolysaccharidosis neuropathophysiology. *J Mol Biol*. 2015;427(4):775-791.
35. Terlato NJ, Cox GF. Can mucopolysaccharidosis type I disease severity be predicted based on a patient's genotype? A comprehensive review of the literature. *Genet Med*. 2003;5(4):286-294.
36. Meijer O, Welling L, Valstar M, et al. Residual N-acetyl-α-glucosaminidase activity in fibroblasts correlates with disease severity in patients with mucopolysaccharidosis type IIIB. *J Inherit Metab Dis*. 2016;39(3):437-445.

Part II

Treatment



Chapter 6

High-throughput screen fails to identify compounds that enhance residual enzyme activity of mutant *N*-acetyl-α-glucosaminidase in mucopolysaccharidosis type IIIB

O.L.M. Meijer, P. van den Biggelaar, R. Ofman,
F.A. Wijburg and N. van Vlies

JIMD Rep. 2017 [Epub ahead of print]

ABSTRACT

Background

In the severe neurodegenerative disorder mucopolysaccharidosis type IIIB (MPSIIIB or Sanfilippo disease type B), deficiency of the lysosomal enzyme *N*-acetyl-α-glucosaminidase (NAGLU) results in accumulation of heparan sulfate. Patients present with a severe, rapidly progressing phenotype (RP) or a more attenuated, slowly progressing phenotype (SP). In a previous study, residual NAGLU activity in fibroblasts of SP patients could be increased by culturing at 30°C, probably as a result of improved protein folding and lysosomal targeting under these conditions. Chaperones are molecules which influence protein folding and could therefore have therapeutic potential in SP MPSIIIB patients. Here we studied the effects of 1302 different compounds on residual NAGLU activity in SP MPSIIIB patient fibroblasts including 1280 approved compounds from the Prestwick Chemical Library.

Methods

Skin fibroblasts of healthy controls, an SP MPSIIIB patient (homozygous for the temperature sensitive mutation p.S612G) and an RP MPSIIIB patient (homozygous for the p.R297* mutation and non-temperature sensitive), were used. A high-throughput assay for measurement of NAGLU activity was developed and validated, after which 1302 different molecules were tested for their potential to increase NAGLU activity.

Results

None of the compounds tested were able to enhance NAGLU activity.

Conclusions

This high-throughput screen failed to identify compounds that could enhance residual activity of mutant NAGLU in fibroblasts of SP MPSIIIB patients with temperature sensitive mutations. To therapeutically simulate the positive effect of lower temperatures on residual NAGLU activity, first more insight is needed into the mechanisms underlying this temperature dependent increase.

INTRODUCTION

In mucopolysaccharidosis type IIIB (MPSIIIB or Sanfilippo disease type B; OMIM #252920), deficiency of the lysosomal enzyme *N*-acetyl-α-glucosaminidase (NAGLU; EC: 3.2.1.50) results in accumulation of the glycosaminoglycan (GAG) heparan sulfate [1]. Patients generally present between the age of 1 and 4 years with a delay in neurocognitive development, predominantly affecting speech and language skills, which is followed by a progressive neurocognitive decline accompanied by behavioral problems [2]. There is a wide spectrum of disease severity, ranging from a severe, rapidly progressing phenotype (RP) to a more attenuated, slowly progressing phenotype (SP). Whereas RP patients often die in their late teenage years or early adulthood, patients with an SP phenotype may show a stable developmental impairment for years [2,3]. No disease-modifying treatment is yet available.

Recently, we showed that culturing skin fibroblasts of MPSIIIB patients with an SP phenotype at 30°C significantly increased residual NAGLU activity, probably due to improved protein folding, decreased degradation and improved targeting to the lysosome [4]. Chaperones are molecules that could induce comparable effects and may be considered as potential therapeutic agents for SP MPSIIIB patients. Molecular chaperones, including the heat shock proteins, are endogenous chaperones that play an important role in protein stabilization and are key players in the intracellular protein quality control system [5]. Chemical chaperones on the other hand, are exogenous compounds that stimulate protein folding by nonspecific modes of action [6,7], whereas pharmacological chaperones stabilize proteins by more specific binding as they act as ligand to the enzyme or selectively bind a particular native conformation of the protein [8]. The use of pharmacological chaperones has been investigated for many diseases affecting protein folding, including LSDs, and several are now in clinical trials [9,10].

Suitable candidates for chaperone therapy in MPSIIIB are 2-acetamido-1,2-dideoxynojirimycin (2AcDNJ) and 6-acetamido-6-deoxycastanospermine, since they were found to be potent inhibitors of purified human NAGLU and its bacterial homolog [11,12]. Another compound of interest is glucosamine. Treatment of cultured fibroblasts from MPS IIIC patients (OMIM #252930) with glucosamine partially restored the activity of the deficient enzyme heparan acetyl-CoA:alpha-*glucosaminide N*-acetyltransferase (HGSNAT; EC:2.3.1.78). This could also be the case for MPSIIIB, as NAGLU binds glucosamine residues at the non-reducing end of the GAG chain [13,14].

Here we aimed to investigate the effects of known chemical and pharmacological chaperones on residual enzyme activity in an MPSIIIB fibroblast cell line in which residual enzyme activity can be increased by culturing at low temperature. Also, we investigated the effect of the 1280 approved compounds from the Prestwick Chemical Library, which have all proven their safety in humans.

MATERIAL AND METHODS

Cell culture

Cultured skin fibroblasts of healthy controls, an MPSIIIB patient with an SP phenotype, homozygous for the temperature sensitive missense mutation p.S612G, and of an MPSIIIB patient homozygous for the p.R297* mutation conveying an RP phenotype and previously demonstrated not to be temperature sensitive, were selected for validation of the assay and subsequent compound screen [4]. Fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 100 U/mL penicillin, 100 µg/mL streptomycin and 250 µg/mL amphotericin at 37°C (unless otherwise stated) in a humidified atmosphere containing 5% CO_2. Before adding FBS to the medium, bovine NAGLU was inactivated by incubation of FBS at 65°C for 35 minutes. All cell lines were found negative for mycoplasma contamination.

NAGLU activity assay

NAGLU activity in protein homogenates of skin fibroblasts was measured according to our previously described method [4]. Since this method is unsuitable for screening of a large number of compounds, an assay suitable for high-throughput screening was designed, based on the method described by Mauri *et al.* [15]. Fluorogenic 4-methylumbelliferyl-2-acetamido-2-deoxy-α-D-glucopyranoside (4MU-α-GlcNAc) (Moscerdam, Oegstgeest, The Netherlands) was used as substrate and dissolved to the required concentration in a 0.1 M citrate 0.2 M phosphate buffer pH 4.3. The assay was started by adding 50 µL reaction mixture (12.5 µL 4MU-α-GlcNAc, 37 µL 0.1 M citrate 0.2 M phosphate buffer pH 3.85 and 0.5 µL 10% Triton-X100) to each well which was incubated at 37°C for different time periods. The reaction was stopped by adding 150 µL 0.2 M sodium carbonate buffer pH 10.5. Fluorescence of released 4-methylumbelliferone was measured with a Fluostar Optima Microplate Reader (BMG Labtech, Ortenberg, Germany), using an excitation and emission wavelength of 360 nm and 450 nm, respectively. NAGLU activity was calculated using a calibration curve of 4-methylumbelliferone (Glycosynth Ltd., Warrington, Cheshire, UK).

Compound screen

Cells were harvested by trypsinization, counted using a Z™ series Coulter Counter (Beckman Coulter Inc., Brea, California, United States) and diluted in culture medium to the required concentrations. For each cell line, 100 µL cell suspension per well was plated in black, clear bottom 96-wells plates (Greiner Bio-One, Kremsmünster, Austria). Next day, culture medium was replaced with 200 µL culture medium containing one of the small compounds described below and incubated for 5 days following our standard protocol. After 5 days incubation, plates were washed 3 times with phosphate buffered saline (PBS) and NAGLU activity was measured as described above.

Chemicals

Taurine, D-arginine, L-homoarginine hydrochloride, saccharose, trimethylamine *N*-oxide (TMAO), dimethyl sulfoxide (DMSO ≥ 99.9%), ambroxol, D-glucosamine hydrochloride, *N*-acetylglucosamine, trichostatin A (TSA), bortezomib and ursodeoxycholic acid (UDCA) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). L-arginine monohydrochloride, trehalose and 4-phenylbutyrate (4-PBA) were from Merck (Darmstadt, Germany), β-alanine from BDH (Analytical Chemicals, VWR International, Radnor, PA, USA), glycerol and betaine were from Arcos Organics (Geel, Belgium) and glycine from Serva Electrophoresis GmbH (Heidelberg, Germany). Tauroursodeoxycholic acid (TUDCA) was from Calbiochem (Merck Millipore, Billerica, MA, USA), 2-acetamido-1,2-dideoxynojirimycin (2AcDNJ) from Bio-connect (Life Sciences, Huissen, The Netherlands) and suberanilohydroxamic acid (SAHA or Vorinostat) from Cayman Chemical Company (Ann Arbor, MI, USA).

The compounds used in our screen were first dissolved in milliQ (Synergy Water Purification System, Merck Millipore, Billerica, MA, USA), sterilized using a 0.45 µm syringe filter (Merck Millipore, Billerica, MA, USA) and diluted in culture medium to the required concentration. Except for UDCA, ambroxol, bortezomib, TSA and SAHA, for which stock solutions were prepared in DMSO and subsequently diluted in culture medium (final DMSO concentration 1.0%).

The Prestwick Chemical Library (Prestwick Chemical, Illkirch, France) consisted of 1280 compounds (2 mM stock solutions in DMSO), which were diluted in culture medium to a final concentration of 10 µM (final DMSO concentration 0.5%).

Western blot analysis

Cell pellets were dissolved in milliQ supplemented with cOmplete™ protease inhibitor cocktail (Roche, Mannheim, Germany) and disrupted by sonification using a Vibra Cell sonicator (Sonics & Materials Inc., Newtown, CT, USA). Protein concentration was measured in whole cell lysates as described by Lowry *et al.* [16]. For Western blot analysis of NAGLU, 50 µg of protein was loaded onto a NuPAGE Novex 4-12% Bis-Tris pre-cast polyacrylamide gel (Invitrogen, Carlsbad, CA, USA) that after electrophoresis, was transferred onto an Amersham Protran Nitrocellulose Blotting Membrane by semidry blotting (GE Healthcare Life Sciences, Little Chalfont, UK). Membranes were blocked in 30 g/L bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) in 0.1% Tween-20 in PBS (TPBS). Antibodies used were: rabbit anti-NAGLU antibody 1:800 (ab169874; Abcam, Cambridge, UK), mouse anti-β-actin antibody 1:10,000 (Sigma-Aldrich, St. Louis, MO, USA), goat anti-rabbit and donkey anti-mouse antibody 1:10,000 (IRDye 800CW and IRDye 680RD, respectively; LI-COR Biosciences, Lincoln, NE, USA). Primary antibodies were dissolved in TPBS and secondary antibodies in TPBS with Odyssey® blocking buffer and SDS 0.01%. Between antibody incubations membranes were washed 5 times with TPBS. Blots were analyzed using the Odyssey® CLx Infrared Imaging System (LI-COR Biosciences, NE, USA).

Statistical analysis

Data analyses were performed using SPSS software for Windows (version 23.0, SPSS Inc., Chicago, IL, USA). A p-value of < 0.05 was considered statistically significant.

RESULTS

Effects of culturing at 30°C on mutant NAGLU

Previously it has been shown that residual activity of NAGLU in fibroblasts of MPSIIIB patients with an SP phenotype can be increased by culturing at 30°C [4]. To further investigate the increase in activity of mutant NAGLU at low culture temperature, NAGLU protein and activity levels were determined in control and MPSIIIB fibroblast cell lines after culturing at 37°C and 30°C (figure 1A). Western blot analysis of fibroblasts from a healthy control cultured at 37°C showed that NAGLU consists of two forms: a precursor form with an apparent molecular weight of 85 kD and a mature form with an apparent molecular weight of 82 kD. In the SP p.S612G MPSIIIB cell line, only the precursor form was detected after culturing at 37°C, whereas after culturing at 30°C both NAGLU forms could be observed. This corresponded with an increase in NAGLU activity from 0.41 nmol.mg^{-1}.hr^{-1} after culturing at 37°C up to 4.06 nmol.mg^{-1}.hr^{-1} after culturing at 30°C (figure 1B; NAGLU activity in control fibroblasts cultured at 37°C: 19.71 nmol.mg^{-1}.hr^{-1}). In fibroblasts of the RP p.R297* MPSIIIB patient, no NAGLU protein was present under either of these conditions (measured NAGLU activities: 0.14 and 0.15 nmol.mg^{-1}.hr^{-1} after culturing at 37°C and 30°C, respectively).

Optimization and validation of the 96-wells NAGLU assay

Prior to the compound screen, a method suitable for high-throughput applications was developed based on the method described by Mauri *et al.* [15] and optimized for incubation time, 4MU-α-GlcNAc substrate concentration and cell density (supplementary figure 1). Based on these results we decided to use 10,000 cells/well and to measure NAGLU activity after 5 days of culturing using 1 mg/mL 4MU-α-GlcNAc substrate incubated at 37°C for 24 hours. Since at present no compound is known that can enhance residual NAGLU activity in MPSIIIB fibroblasts, fibroblasts of a healthy subject were used as a positive control and plated at a density of 2500 cells/well in each plate. As chaperones only act on missense variants, p.R297* MPS IIIB fibroblasts plated at a density of 10,000 cells/well were included as a negative control. Since this cell line contains a mutation resulting in a premature stop, no protein will be synthesized and no activity was expected to be measured.

Using these conditions, the Z-factor of the assay was determined, which is considered a reliable measure for evaluation and validation of high-throughput screens [17]. A calculated Z-factor of 0.69 classified this assay as “excellent”.

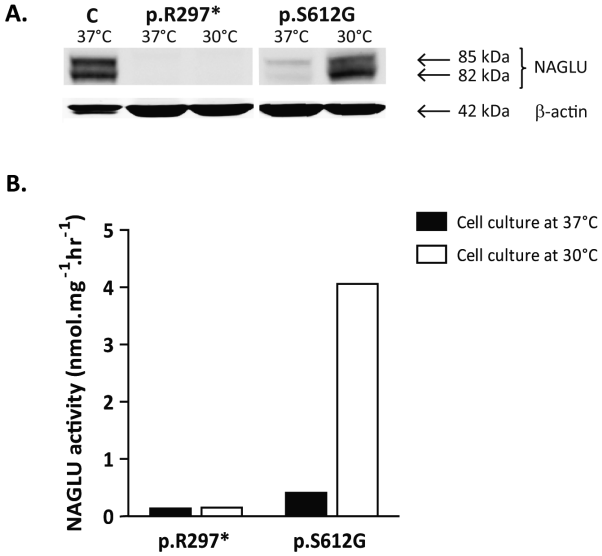


Figure 1. A. Western blot analysis of NAGLU protein levels and B. corresponding activity levels (nmol.mg^{-1}.hr^{-1}) after culturing MPSIIIB patient and control fibroblasts at 37°C and 30°C for 1 week. NAGLU activity was measured as was described previously. NAGLU activity in control fibroblasts cultured at 37°C was 19.71 nmol.mg^{-1}.hr^{-1}.

Effect of chemical chaperones

Chemical chaperones are generally divided into two subgroups: the osmolytes and hydrophobic compounds [7]. Several classes of osmolytes were studied: free amino acids and amino acid derivatives (β-alanine, glycine, taurine, D-arginine, L-homoarginine hydrochloride, L-arginine monohydrochloride), carbohydrates (trehalose and saccharose), polyols (glycerol), methylamines (betaine and TMAO) and organosulfur compounds (DMSO). In addition the effect of the hydrophobic chaperones 4-PBA and the bile acids UDCA and TUDCA was assessed.

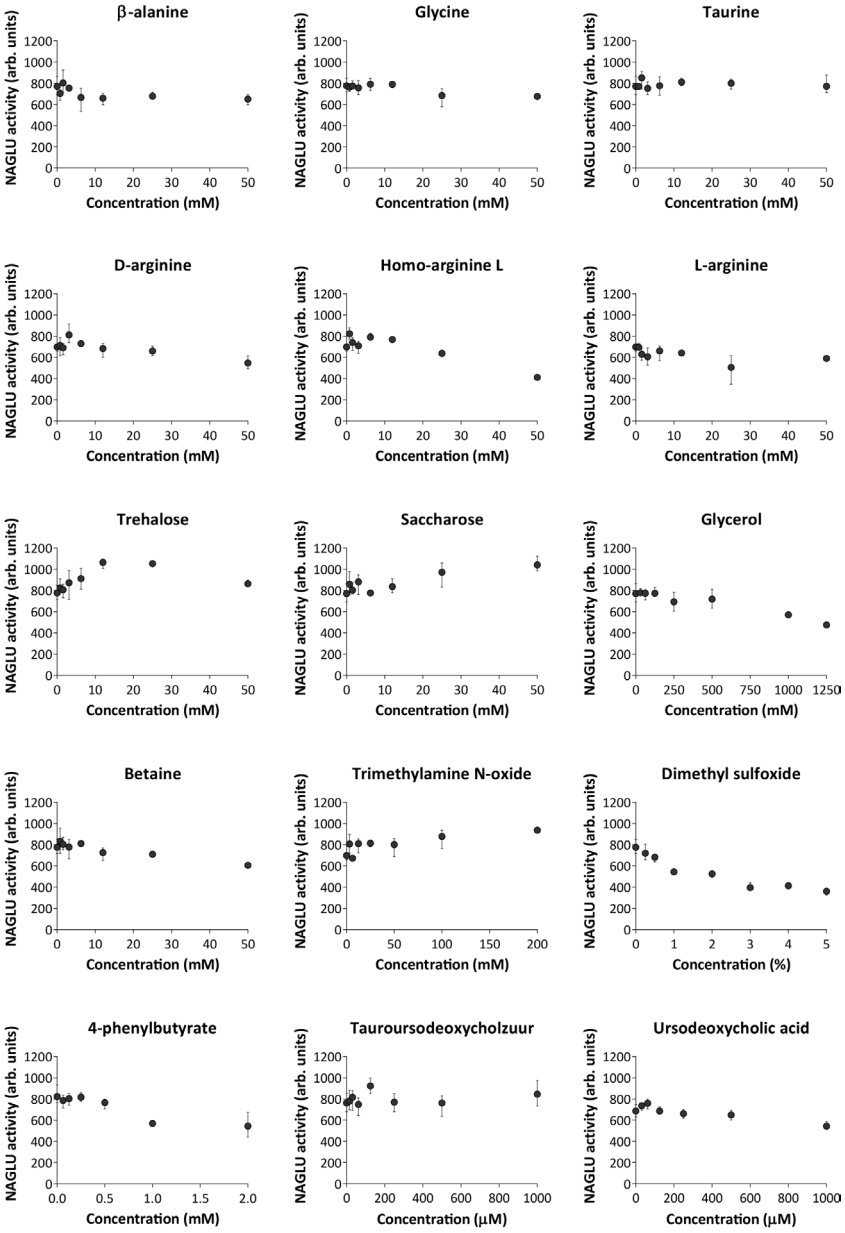
None of the 15 chemical chaperones tested, enhanced NAGLU activity in MPSIIIB fibroblasts after 5 days incubation at different concentrations (figure 2A).

Effect of pharmacological chaperones, previously investigated in LSDs

Pharmacological chaperones previously studied for potential effects in LDSs, were assessed and included ambroxol [18], the proteasome inhibitor bortezomib [19,20] and the HDAC inhibitors TSA and SAHA [21]. None of these compounds enhanced residual NAGLU activity in p.S612G MPSIIIB fibroblasts (figure 2B).

The (*N*-acetyl)glucosamine inhibitors D-glucosamine, *N*-acetylglucosamine and 2AcDNJ, which were previously shown to affect NAGLU and HGSNAT in MPSIIIB and MPS IIIC respectively, were also investigated [11–14]. As is shown in figure 2C, treatment with these

A.


β-alanine
Glycine
Taurine
D-arginine
Homo-arginine L
L-arginine
Trehalose
Saccharose
Glycerol
Betaine
Trimethylamine N-oxide
Dimethyl sulfoxide
4-phenylbutyrate
Tauroursodeoxycholzuur
Ursodeoxycholic acid
NAGLU activity (arb. units)
Concentration (mM)
Concentration (%)
Concentration (μM)

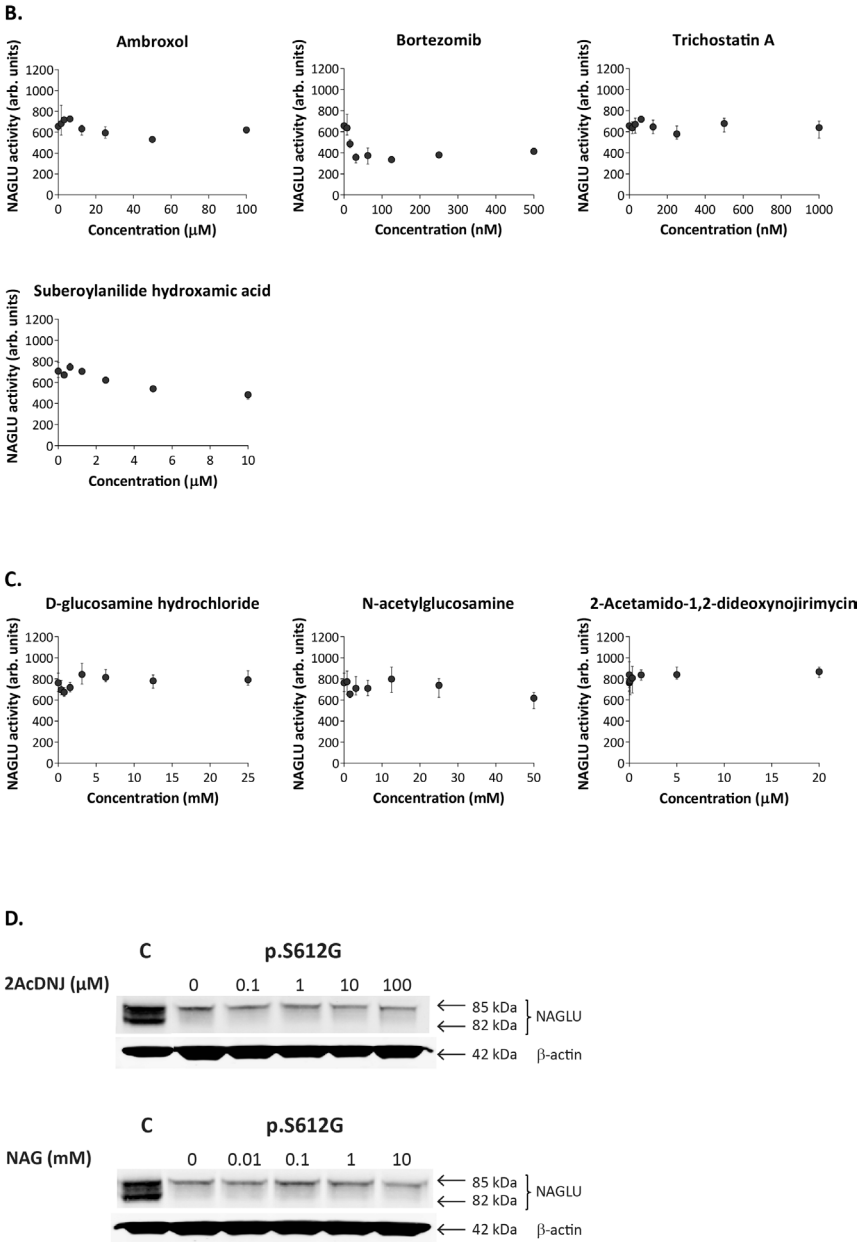


Figure 2. A. Effect of treatment with different classes of chemical chaperones on residual NAGLU activity in p.S612G MPSIIIB fibroblasts. B. Effect of treatment with pharmacological chaperones used in other protein folding diseases including LDSs, on residual NAGLU activity in p.S612G MPSIIIB fibroblasts. C. Effect of treatment with pharmacological chaperones used in MPS III on residual NAGLU activity in p.S612G MPSIIIB fibroblasts. NAGLU activity levels are shown in fluorescence (arb. units). All compound concentrations were tested in triplicate. Mean ± SD is given. D. Western blot analysis of the effect of treatment with *N*-acetylglucosamine (NAG) and 2AcDNJ for 5 days on NAGLU protein levels in p.S612G MPSIIIB fibroblasts.

compounds did not lead to significant enhancement of NAGLU activity levels.
To investigate whether any of these compounds did have an effect on protein levels, which, due to a too strong inhibitory effect, may not have resulted in detectable changes in enzyme activity, the effect of *N*-acetylglucosamine and 2AcDNJ on NAGLU protein was assessed on Western blot (figure 2D). Treatment with neither of these compounds led to higher expression of the precursor of NAGLU or the formation of the mature form of the enzyme.

Prestwick Chemical Library

So far none of the compounds tested showed any effect on residual activity of mutant NAGLU. Therefore the Prestwick Chemical Library was tested consisting of 1280 approved drugs (figure 3A). All compounds with a fluorescent signal of 500 arb. units or above the mean background were selected for further analyses. These included the antihypertensive drugs benzamil hydrochloride (PCL-657) and doxazosin mesylate (PCL-858), the anti-osteoporetic drug ibandronate sodium (PCL-1285) the xanthine oxidase inhibitor used for the treatment of gout, allopurinol (PCL-1213) and the antiseptic drug aminacrine (PCL-1717). To validate the results of the screen, MPSIIIB p.S612G fibroblasts were incubated for 5 days with the selected compounds at increasing concentrations. Both ibandronate sodium and allopurinol did not show any significant effect on NAGLU activity (figure 3B). For aminacrine, benzamil hydrochloride and doxasozin mesylate a dose dependent increase in fluorescent signal was observed. However, the same increase in fluorescence was obtained when the assay was repeated in the absence of the 4MU-α-GlcNAc substrate, indicating that there is no actual increase in residual NAGLU activity upon treatment with these compounds.

DISCUSSION

We assessed the effect of 1302 different molecules on residual enzyme activity in an MPSIIIB patient fibroblast cell line which responded with a significant increase in NAGLU activity when cultured at 30°C instead of 37°C [4]. As enzyme activity is related to the efficiency of protein folding, culturing at 30°C may improve folding of the mutant NAGLU enzyme [22,23]. Unfortunately, none of the molecules tested in our assay, including the 1280 compounds from the Prestwick Chemical Library, were effective.
The observed lack of effect may well be understood if protein misfolding is not, or only to a limited extend, involved in the SP MPSIIIB phenotype and if the observed increase in enzyme activity in fibroblasts cultured at 30°C is due to other mechanisms. Indeed, our observation on Western blot that at 37°C culture conditions only the 85 kDa precursor form of NAGLU is detected while at 30°C also the mature 82 kDa NAGLU protein is observed, rather suggests differences in protein synthesis and processing.

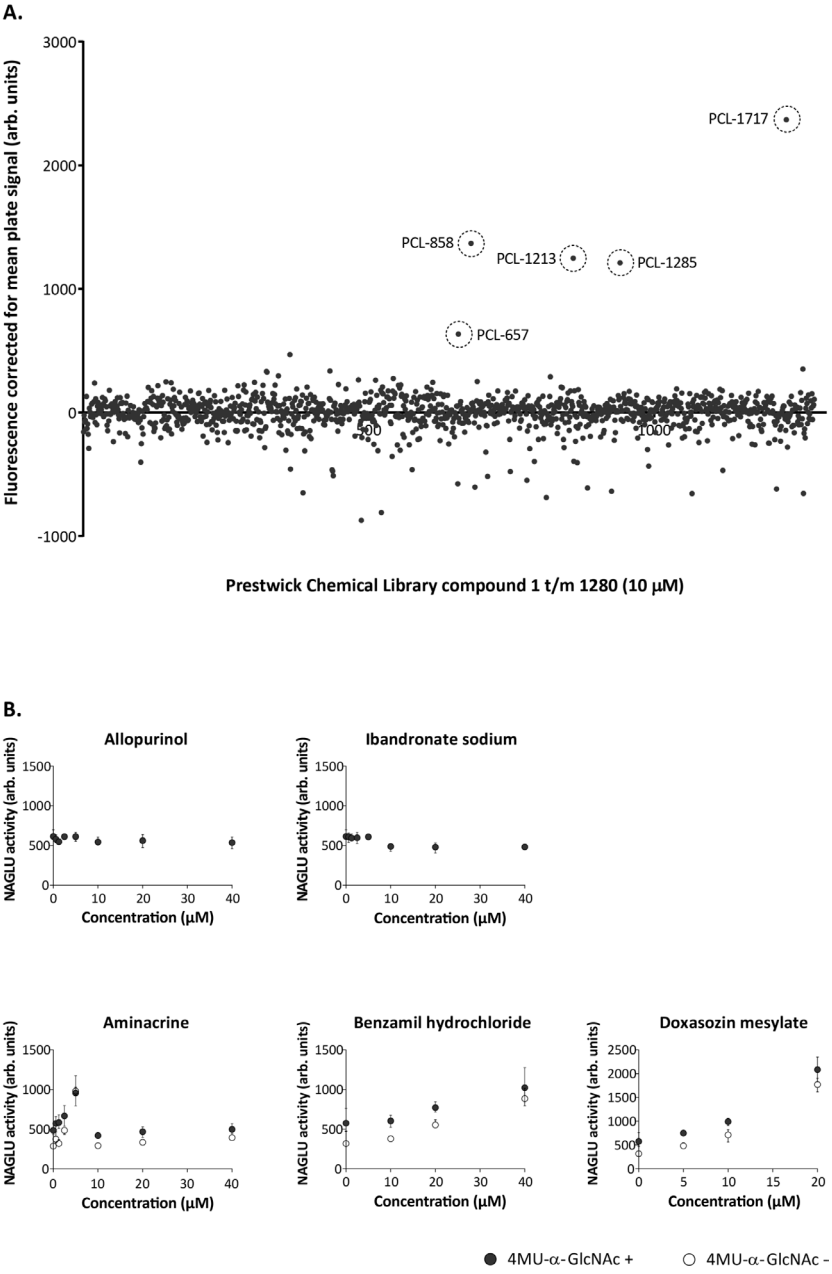


Figure 3. A. Effect of treatment with compounds from the Prestwick Chemical Library (10 µM) on NAGLU activity in p.S612G MPSIIIB fibroblasts. NAGLU activity levels are shown in fluorescence (arb. units) and were corrected for the mean plate signal. B. Validation of the compounds identified in the high-throughput screen of the Prestwick Chemical Library in p.S612G MPSIIIB fibroblasts. NAGLU activity levels are shown in fluorescence (arb. units) after incubation with or without 4MU-α-GlcNAc substrate. All compound concentrations were tested in triplicate. Mean ± SD is given.

The majority of chemical chaperones studied here, have remarkable general mechanisms of action and were shown to influence enzymatic activity in other protein folding diseases including LSDs [7,18–21]. We consider that, if protein misfolding is indeed involved in the MPSIIIB SP phenotype, some effect of these compounds would have been observed. Our observation that more NAGLU specific compounds such as the *N*-acetylglucosaminidase inhibitors *N*-acetylglucosamine and 2AcDNJ, also lacked effect on NAGLU protein and activity levels further supports the hypothesis that protein misfolding does not play a major role in MPSIIIB. In previous studies the binding capacity of 2AcDNJ has always been assessed using purified NAGLU [11,12]. As NAGLU is synthesized in the rough endoplasmic reticulum (ER), it is possible that these compounds do have the capacity to bind and stabilize mutant NAGLU, but cannot enter the ER in a cell culture model as used here. This may also have blocked potential effects of other compounds tested in this study, although it is unlikely that compounds which do not reach the target protein *in vitro,* would have therapeutic properties *in vivo*. Thus, despite the promising effects of chaperones in other LSDs such as Fabry disease, this approach may not serve all LSDs as was shown here for MPS IIIB [9,24,25].

A limitation of this study is that compounds were tested in one MPSIIIB cell line. Although this cell line was selected because its mutation conveys an SP phenotype and enzyme activity responded favorably to culturing at 30°C, we cannot exclude that this mutation is insensitive to the here tested compounds and that they might have had a positive effect on other mutations. However, allelic heterogeneity in MPSIIIB is large, and it would not be feasible to test all reported mutations [2]. A drawback of high-throughput screens in general is that compound libraries are often tested in a limited number of concentrations, so that an effect of any of the compounds at a different concentration cannot be ruled out.

Thus, despite a reliable and robust assay, this high-throughput screen failed to identify compounds that could enhance residual activity of mutant NAGLU in fibroblasts of an SP MPSIIIB patient homozygous for a temperature sensitive mutation. We conclude that to therapeutically simulate the positive effect of lower temperatures on residual NAGLU activity, first more insight is needed into the mechanisms underlying this temperature dependent increase in enzyme activity.

ACKNOWLEDGEMENTS

The authors would like to thank dr. S.F. van de Graaf of the Tytgat Institute for Liver and Intestinal Research / Department of Gastroenterology & Hepatology at the Academic Medical Center in Amsterdam, for being so kind to provide the Prestwick Chemical Library to us.

SUPPLEMENTARY DATA

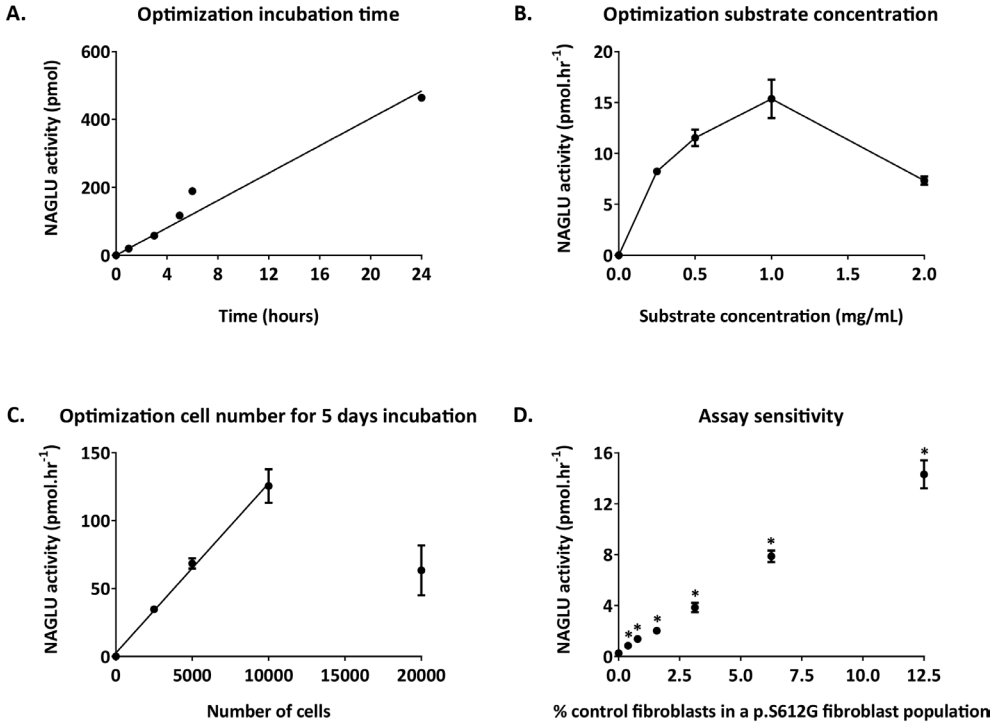


Figure S1. Optimization and validation of the 96-wells NAGLU high-throughput assay. A. Time dependency of NAGLU activity (pmol) after incubation with 1.5 mg/mL 4MU-α-GlcNAc substrate at 37°C for the indicated time points, in control fibroblasts plated at a cell density of 20,000 cells/well grown for 24 hours. NAGLU activity was linear up to an incubation time of 24 hours B. NAGLU activity (pmol.hr^{-1}) after incubation with various concentrations of 4MU-α-GlcNAc substrate at 37°C for 24 hours, in control fibroblasts plated at a cell density of 20,000 cells/well grown for 24 hours. Optimal enzyme activity was obtained at a substrate concentration of 1 mg/mL C. NAGLU activity (pmol. hr^{-1}) in control fibroblasts plated at different cell densities and cultured for 5 days. A 4MU-α-GlcNAc substrate concentration of 1 mg/mL was used and plates were incubated at 37°C for 24 hours. The increase in NAGLU activity was linear with cell density up to 10,000 cells/well. D. Determination of the sensitivity of the assay using cell populations which would show small incremental increases in NAGLU activity. NAGLU activity (pmol.hr^{-1}) is shown in populations of p.S612G MPSIIIB fibroblasts mixed with control fibroblasts in different ratios (total cell number 10,000 cells/well), after 5 days culturing. A 4MU-α-GlcNAc substrate concentration of 1 mg/mL was used and plates were incubated at 37°C for 24 hours. After 5 days culturing mean basal NAGLU activity in the population consisting of 100% p.S612G MPSIIIB fibroblasts was 0.26 pmol.hr^{-1} and in the population consisting of 100% control cells 85.05 pmol.hr^{-1}. In the wells containing only 0.391% control cells and 99.609% MPSIIIB cells, a significant increase in NAGLU activity could already be detected accurately (* $p < 0.001$). In all cases mean ± SD is given. If error bars would be shorter than the height of the symbol, no error bars were drawn.

Preliminary experiments showed that Triton X-100 at a final concentration of 0.1% had no adverse effect on NAGLU activity and could therefore be used for cell lysis (data not shown).

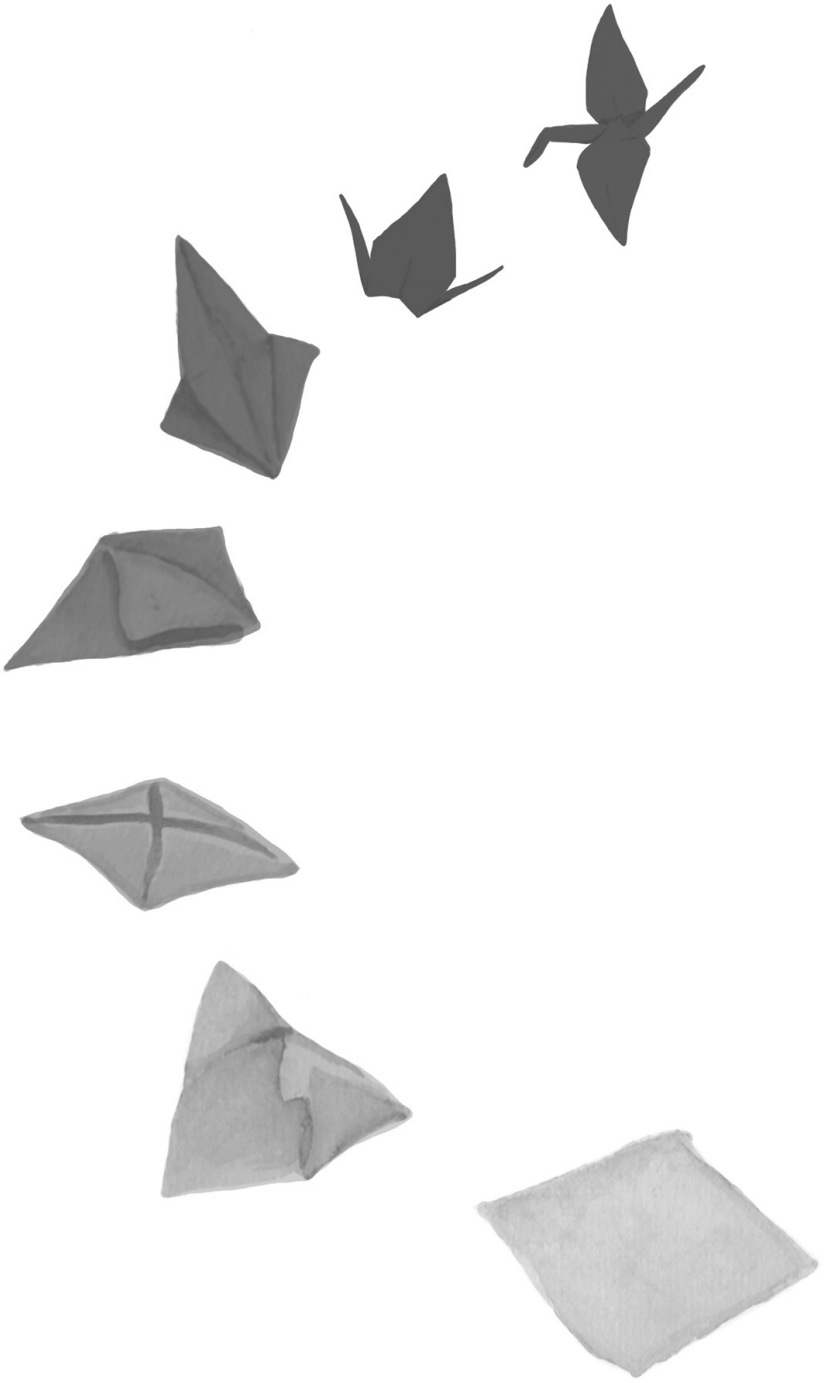
REFERENCES

1. Muenzer J. Overview of the mucopolysaccharidoses. *Rheumatology*. 2011;50 Suppl 5:v4-12.
2. Valstar MJ, Bruggenwirth HT, Olmer R, et al. Mucopolysaccharidosis type IIIB may predominantly present with an attenuated clinical phenotype. *J Inherit Metab Dis*. 2010;33(6):759-767.
3. Moog U, van Mierlo I, van Schrojenstein Lantman-de Valk HM, Spaapen L, Maaskant MA, Curfs LM. Is Sanfilippo type B in your mind when you see adults with mental retardation and behavioral problems? *Am J Med Genet C Semin Med Genet*. 2007;145C(3):293-301.
4. Meijer O, Welling L, Valstar M, et al. Residual N-acetyl-α-glucosaminidase activity in fibroblasts correlates with disease severity in patients with mucopolysaccharidosis type IIIB. *J Inherit Metab Dis*. 2016;39(3):437-445.
5. Hartl FU, Bracher A, Hayer-Hartl M. Molecular chaperones in protein folding and proteostasis. *Nature*. 2011;475(7356):324-332.
6. Engin F, Hotamisligil GS. Restoring endoplasmic reticulum function by chemical chaperones: An emerging therapeutic approach for metabolic diseases. *Diabetes Obes Metab*. 2010;12(suppl. 2):108-115.
7. Cortez L, Sim V. The therapeutic potential of chemical chaperones in protein folding diseases. *Prion*. 2014;8(2):197-202.
8. Parenti G. Treating lysosomal storage diseases with pharmacological chaperones: from concept to clinics. *EMBO Mol Med*. 2009;1(5):268-279.
9. Hollak C, Wijburg F. Treatment of lysosomal storage disorders: Successes and challenges. *J Inherit Metab Dis*. 2014;37(4):587-598.
10. Parenti G, Andria G, Valenzano K. Pharmacological chaperone therapy: Preclinical development, clinical translation, and prospects for the treatment of lysosomal storage disorders. *Mol Ther*. 2015;23(7):1138-1148.
11. Zhao KW, Neufeld EF. Purification and characterization of recombinant human α-N-acetylglucosaminidase secreted by Chinese hamster ovary cells. *Protein Expr Purif*. 2000;19(1):202-211.
12. Ficko-Blean E, Stubbs KA, Nemirovsky O, Vocadlo DJ, Boraston AB. Structural and mechanistic insight into the basis of mucopolysaccharidosis IIIB. *Proc Natl Acad Sci USA*. 2008;105(18):6560-6565.
13. Feldhammer M, Durand S, Pshezhetsky A V. Protein misfolding as an underlying molecular defect in mucopolysaccharidosis III type C. *PLoS One*. 2009;4(10):e7434.
14. Matos L, Canals I, Dridi L, et al. Therapeutic strategies based on modified U1 snRNAs and chaperones for Sanfilippo C splicing mutations. *Orphanet J Rare Dis*. 2014;9:180.
15. Mauri V, Lotfi P, Segatori L, Sardiello M. A rapid and rensitive method for measuring N-acetylglucosaminidase activity in cultured cells. *PLoS One*. 2013;8(6):1-9.
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193(1):265-275.
17. Zhang JH, Chung TD, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen*. 1999;4(2):67-73.
18. Maegawa GH, Tropak MB, Buttner JD, et al. Identification and characterization of ambroxol as an enzyme enhancement agent for Gaucher disease. *J Biol Chem*. 2009;284(35):23502-23516.
19. Shimada Y, Nishimura E, Hoshina H, et al. Proteasome inhibitor bortezomib enhances the activity of multiple mutant forms of lysosomal α-glucosidase in Pompe disease. *JIMD Rep*. 2015;18(1):33-39.
20. Macías-Vidal J, Girós M, Guerrero M, et al. The proteasome inhibitor bortezomib reduced cholesterol accumulation in fibroblasts from Niemann-Pick type C patients carrying missense mutations. *FEBS J*. 2014;281(19):4450-4466.
21. Pipalia NH, Cosner CC, Huang A, et al. Histone deacetylase inhibitor treatment dramatically reduces cholesterol accumulation in Niemann-Pick type C1 mutant human fibroblasts. *Proc Natl Acad Sci USA*. 2011;108(14):5620-5625.
22. Fan JQ. A contradictory treatment for lysosomal storage disorders: inhibitors enhance mutant enzyme activity. *Trends Pharmacol Sci*. 2003;24(7):355-360.

23. Gootjes J, Schmohl F, Mooijer PA, et al. Identification of the molecular defect in patients with peroxisomal mosaicism using a novel method involving culturing of cells at 40°C: Implications for other inborn errors of metabolism. *Hum Mutat*. 2004;24(2):130-139.
24. Germain DP, Hughes DA, Nicholls K, et al. Treatment of Fabry's disease with the pharmacologic chaperone Migalastat. *N Engl J Med*. 2016;375(6):545-555.
25. Hughes DA, Nicholls K, Shankar SP, et al. Oral pharmacological chaperone migalastat compared with enzyme replacement therapy in Fabry disease: 18-month results from the randomised phase III ATTRACT study. *J Med Genet*. 2017;54(4):288-296

Part III

Diagnosis



Chapter 7

Failure to shorten the diagnostic delay in two ultra-orphan diseases (mucopolysaccharidosis types I and III): potential causes and implications

G. Kuiper, O.L.M. Meijer, E.J. Langereis and F.A. Wijburg

These authors contributed equally

Orphanet J Rare Dis. 2018 [Accepted for publication]

ABSTRACT

Background

Rare diseases are often un- or misdiagnosed for extended periods, resulting in a long diagnostic delay that may significantly add to the burden of the disease. An early diagnosis is particularly essential if a disease-modifying treatment is available. The purpose of this study was to assess the extent of the diagnostic delay in the two ultra-rare diseases, i.e., mucopolysaccharidosis I (MPS I) and III (MPS III), both of which are lysosomal storage disorders with different phenotypic severities (MPS I is characterized by the severe Hurler and the more attenuated non-Hurler phenotypes, MPS III is characterized by the severe rapidly progressing (RP) phenotype and more attenuated slowly progressing (SP) phenotype). We investigated whether the diagnostic delay changed over the previous decades.

Results

The diagnostic delay, which is defined as the time between the first visit to a medical doctor for disease-related signs or symptom and the final diagnosis, was assessed using telephone interviews with patients diagnosed between 1988 and 2017 and/or their parents or legal guardian(s). In addition, the medical charts were reviewed. For MPS I (n = 29), the median diagnostic delay was 8 months (range 1 – 24 months) for Hurler patients and 28 months (range 2 – 147 months) for non-Hurler patients. For MPS III (n = 46), the median diagnostic delay was 33 months (range 1 – 365 months). No difference was observed between the RP and SP phenotypic groups. Comparing the diagnostic delay over time using 5-year time intervals, no reduction in the diagnostic delay was observed for MPS I or MPS III.

Conclusions

In the Netherlands, the time to diagnosis for patients with MPS I and MPS III has not changed between 1988 and 2017, and an extensive delay still exists between the first visit to a medical doctor for disease-related signs and symptoms and the final diagnosis. The numerous campaigns launched to increase awareness, leading to earlier diagnosis of these rare disorders, particularly of MPS I, have failed to achieve their goal. Robust selected screening protocols embedded in national guidelines and newborn screening for disorders that meet the criteria for population screening may be the only effective approaches for reducing the diagnostic delay.

BACKGROUND

Rare diseases with a prevalence of less than 1 in 2,000 citizens (as defined by the European Commission; EC) often carry a high physical and psychological burden and impact the quality of life of the patients, parents and caregivers. More than 6,000 rare diseases have been identified, and > 50% are present during childhood [1,2]. During previous decades, public and non-public organizations have launched numerous initiatives to increase the awareness of rare diseases, and in 1999, rare diseases first appeared on the agenda of the EC, resulting in a set of regulations and policies focusing on improving the recognition and visibility of rare diseases [1,3].

Due to their nature and the non-specific signs and symptoms at presentation and during the early phases of the disease, rare diseases are often un- or misdiagnosed for extended periods, leading to a long diagnostic delay [4–7]. Patients may visit many different healthcare professionals and undergo multiple unnecessary investigations before the correct diagnosis is finally achieved [4–7]. This diagnostic odyssey may significantly add to the burden of the disease [4,5,7]. An early diagnosis is particularly essential if a disease-modifying treatment is available because the patients' outcome often depends on the timely initiation of treatment [8–10]. Finally, because approximately 80% of rare diseases are inherited, an early diagnosis may allow genetic counseling and informed decision-making in family planning [2].

To prevent unnecessarily delayed diagnoses, numerous campaigns have been launched to increase awareness of rare diseases. Many campaigns, such as the 'rare diseases day' initiative, which has become a yearly event in many countries worldwide, are of a general nature, raising awareness of the existence of 'rare diseases'. Other initiatives focus on specific diseases and promoting an early diagnosis, thereby allowing the timely initiation of treatment [11–14]. These campaigns are organized by patient advocacy groups, health care providers and pharmaceutical companies.

However, to the best of our knowledge, no studies have specifically investigated whether these campaigns have reduced the diagnostic delay. We investigated the time to diagnosis of two very rare, invariable progressive and severe, inborn errors of metabolism: mucopolysaccharidosis type I (MPS I; estimated birth prevalence 1:100,000) for which treatment has been available for more than 15 years, and mucopolysaccharidosis type III (MPS III; estimated birth prevalence 1:60,000) for which treatment is under study. Both disorders belong to the group of lysosomal storage disorders. We assessed whether the diagnostic delay has decreased over recent decades.

Subtype	OMIM	Enzyme deficiency	Storage material	Main clinical features	Treatment	Birth prevalence
Mucopolysaccharidosis type I (MPS I)						
MPS I – Hurler (MPS I-H)	607014	α-L-iduronidase (IDUA)	Dermatan sulfate (DS) heparan sulfate (HS)	Progressive neurocognitive decline, hernias, facial dysmorphisms, corneal clouding, stiff joints, dysostosis multiplex, cardiac problems and hepatosplenomegaly. Death in childhood if untreated.	HSCT	1:100,000
MPS I – Hurler/Scheie (MPS I-H/S)	607015			Phenotype intermediate between MPS I-H and MPS I-S. Can present with or without neuronopathic disease.	HSCT or ERT	
MPS I – Scheie (MPS I-S)	607016			Corneal clouding, stiff joints, mild dysostosis multiplex. Normal intelligence en life expectancy.	ERT	
Mucopolysaccharidosis type III (MPS III)						
MPS IIIA	252900	Heparan N-sulfatase (SGSH)	Heparan sulfate (HS)	Progressive neurocognitive decline, behavioral problems, sleep disturbances, progressive loss of motor functions. Death in second or third decade of life. Broad spectrum of disease severity.	Not available	1:60,000
MPS IIIB	252920	*N*-acetyl-α-glucosaminidase (NAGLU)				
MPS IIIC	252930	Acetyl CoA:α-glucosaminide *N*-acetyltransferase (HGSNAT)				
MPS IIID	252940	*N*-acetylglucosamine 6-sulfatase (GNS)				

Table 1. Disease characteristics of mucopolysaccharidosis type I (MPS I) and type III (MPS III)

METHODS

Patients

This single center study was conducted at the Academic Medical Center (AMC) in Amsterdam and involved interviews with patients and/or parents or legal guardian(s) of patients with MPS I and MPS III with a confirmed diagnosis since 1988. Before 1988, reliable data were unavailable. The data were verified and/or supplemented with chart reviews or data inquiries from the general practitioner (GP) and the medical specialist(s) visited prior to diagnosis. Our center is a center of expertise for MPS I and MPS III in the Netherlands.

All MPS I and MPS III patients were included regardless of the phenotype. Table 1 presents the signs and symptoms frequently observed in MPS I and MPS III patients and information regarding the different phenotypes and enzymatic subtypes [15–19]. The phenotypes were assessed by an experienced clinician (FAW) based on the available clinical data. Only patients with a diagnosis confirmed by enzymatic testing and/or a mutation analysis were included. Patients were only included if the diagnostic studies leading to the final diagnosis were based on the clinical signs and symptoms. Patients who underwent diagnostic studies because of an affected family member were excluded. All patients and/or their parents or legal guardians provided informed consent for this study. The study proposal was reviewed by the Medical Ethics Committee of the AMC, who deemed that formal ethical approval was not necessary for this study.

Data collection

The data were collected using structured telephone interviews with patients and/or the patients' parents or legal guardian(s). The following variables were recorded:

- Year/month of first visit to the GP for a sign or symptom that was, in hindsight, likely related to MPS I/MPS III
- Year/month of first referral visit to a medical specialist for a sign or symptom that was, in hindsight, likely related to MPS I/MPS III
- Year/month of the confirmatory diagnosis, which was defined by the first demonstration of deficient enzyme activity or the presence of disease causing mutations

From each of these visits, the following data were recorded:

- MPS I/MPS III-related sign or symptom leading to the visit
- Other MPS I/MPS III-related symptoms present at that time point
- Type of medical specialist visited at first referral for a disease-related symptom
- Type of medical specialist who made the diagnosis

MPS I and MPS III disease-related signs and symptoms are presented in table 2.

Statistical analyses

The statistical analyses were performed using SPSS software for Windows (version 23.0, SPSS Inc., Chicago, Illinois, USA). Non-parametric Mann-Whitney U tests were performed to assess the significant differences in the time between the first visit to the GP and diagnosis and the time between the first visit to a medical specialist and the final diagnosis within the cohort of MPS I patients and between the Hurler and non-Hurler patients. The same analyses were performed for the RP and SP MPS III patients. To assess whether the diagnostic delay changed over time, the MPS I and MPS III patients were divided into different groups based on the year of diagnosis using a 5-year time interval. Non-parametric Kruskall-Wallis tests were performed to assess the significant differences among these groups.

Disease-related symptoms	
Mucopolysaccharidosis type I	**Mucopolysaccharidosis type III**
Hernias • Inguinal hernia • Umbilical hernia ***Ear, nose, throat problems*** • Frequent upper airway infections • Obstructive sleep apneas or excessive snoring during sleep • Tympanostomy tubes • Adenoidectomy • Tonsillectomy ***Gastro-intestinal problems*** • Hepatosplenomegaly ***Cardiac problems*** • Cardiomyopathy • Valvular dysfunction ***Skeletal and joint problems*** • Joint stiffness • Skeletal deformities • Kyphosis • Hip dysplasia • Bullet shaped metacarpals • Stunted growth of the long bones • Broad oar shaped ribs • Short stature • Carpal tunnel syndrome • Trigger fingers • Tendon shortening • Early arthrosis ***Hydrocephalus*** ***Corneal clouding*** ***Dysmorphic features*** • Frontal bossing • Depressed nasal bridge • Full lips and macroglossia ***Developmental delay***	***Developmental delay or decline*** • Neurocognitive functions • Motor functions ***Behavioral problems*** • Hyperactivity/restlessness • Aggression • Anxiety • Autistic behaviors • Other ***Dysmorphic features*** • Coarse facial features • Coarse hair • Hirsutism • Other ***Ear, nose, throat problems*** • Frequent upper airway infections • Frequent ear infections • Hearing problems • Tympanostomy tubes • Adenoidectomy • Tonsillectomy ***Gastro-intestinal problems*** • Frequent diarrhea • Hepatomegaly • Other ***Sleeping problems*** ***Seizures*** ***Hernias*** • Inguinal hernia • Umbilical hernia

Table 2. Disease-related signs and symptoms for MPS I and MPS III

RESULTS

MPS I and MPS III patient characteristics

Thirty-two MPS I patients met the inclusion criteria; of these patients, three were excluded (two patients did not consent, and one was lost to follow-up). From the group of MPS III patients, 53 patients met the inclusion criteria, and 7 of these patients were lost to follow-up. The characteristics of the patients included in the study are provided in table 3. At the time of this study, one male MPS IIIA patient (aged 4 years and 9 months) was considered too young to reliably predict the phenotypic severity.

Patient characteristics			
MPS I	**N**	**MPS III**	**N**
Total number of patients	29	**Total number of patients**	46
Male	15	*Male*	27
Female	14	*Female*	19
MPS I phenotype		**MPS III subtype**	
Hurler	20	*MPS IIIA*	28
Non-Hurler	9	*MPS IIIB*	9
		MPS IIIC	9
		MPS III phenotype	
		Rapidly progressing (RP) MPS III	16
		Slowly progressing (SP) MPS III	28
		Unknown	1

Table 3. Characteristics of the MPS I and MPS III patients. At the time of this study, one of the MPS III patients (aged 4 years and 9 months) was considered too young to determine the phenotypic severity.

MPS I: first visit to the GP for an MPS I-related symptom

Sixteen of the 29 MPS I patients first visited a GP for an MPS I-related symptom and were subsequently referred to a medical specialist. Eleven patients were directly seen by a medical specialist for MPS I-related symptoms without a prior visit to the GP, and this information was unclear for 2 patients. Due to the small group size, no further analyses of the first visit to the GP were performed.

MPS I: first visit to a medical specialist for an MPS I-related symptom

The MPS I patients first visited a medical specialist for an MPS I-related symptom at a median age of 4 months (range 0 – 54 months; median age: MPS I Hurler patients 3 months (range 0 – 20 months) and MPS I non-Hurler patients 12 months (range 0 – 54 months) (table 4). Both the Hurler and non-Hurler patients were first seen by a general pediatrician (69%), and

recurrent airway infections were the most common reason for these visits. Additional MPS I-related signs and symptoms that were present at the time of the first visit to a medical specialist are presented in table 4.

	First visit to a medical specialist					
	All MPS I		**Hurler**		**Non-Hurler**	
Number of patients	29		20		9	
Age at first visit (months)						
Median	4		3		12	
Range	0 – 54		0 – 20		0 – 54	
Specialism of 1st referral	**Nr.**	**%**	**Nr.**	**%**	**Nr.**	**%**
Ear, nose, and throat specialist	3	10%	2	10%	1	11%
General pediatrician	20	69%	14	70%	6	67%
Orthopedic surgeon	2	7%	1	5%	1	11%
Pediatric surgeon	3	10%	2	10%	1	11%
Pediatric cardiologist	1	3%	1	5%	0	0%
Sign or symptom leading to 1st referral	**Nr.**	**%**	**Nr.**	**%**	**Nr.**	**%**
Recurrent airway infections	7	24%	5	25%	2	22%
Upper airway obstruction	3	10%	2	10%	1	11%
Inguinal/umbilical hernia	4	14%	3	15%	1	11%
Hydrocephalus	2	7%	1	5%	1	11%
Hepatosplenomegaly	1	3%	1	5%	0	0%
Kyphosis/hip dysplasia	3	10%	2	10%	1	11%
Joint stiffness	1	3%	0	0%	1	11%
Facial features	4	14%	4	20%	0	0%
Hearing problems	1	3%	1	5%	0	0%
Growth delay	2	7%	1	5%	1	11%
Developmental delay	1	3%	0	0%	1	11%
Other MPS I-related signs & symptoms at 1st referral	**Nr.**	**%**	**Nr.**	**%**	**Nr.**	**%**
Recurrent airway infections	7	24%	4	20%	3	33%
Upper airway obstruction	10	34%	9	45%	1	5%
Inguinal/umbilical hernia	8	28%	6	30%	2	10%
Hydrocephalus	2	7%	2	10%	0	0%
Hepatosplenomegaly	2	7%	1	5%	1	5%
Joint stiffness	3	10%	2	10%	1	11%
Facial features	4	14%	2	10%	2	10%
Hearing problems	6	21%	4	20%	2	10%
Vision problems	1	3%	0	0%	1	5%
Developmental delay	5	5%	2	10%	3	15%
Growth delay	1	3%	0	0%	1	5%

Table 4. Characteristics of the entire group of MPS I patients at the first visit to a medical specialist for an MPS I-related symptom as specified for the MPS I Hurler and non-Hurler patients. The sums of the percentages of each item may not equal 100% because the percentages represent rounded values.

MPS I: time to diagnosis

The median age at diagnosis of all MPS I patients was 12 months (range 5 – 151 months) (table 5). The Hurler patients were diagnosed at a significantly younger age (11 months (range 5 – 31 months) than the non-Hurler patients (57 months (range 5 – 151 months) (p = 0.005) (figure 1A). The diagnosis of MPS I was most often made by a general pediatrician (45%), followed by a pediatrician specialized in inborn errors of metabolism (IEM) (31%).
The median delay between the first visit to a medical specialist and the final diagnosis for the entire group was 9 months (range 1 – 147; median delay: Hurler patients 8 months (range 1 – 24 months) and non-Hurler patients 28 months (range 2 – 147 months; the difference between the Hurler and non-Hurler patients was not significant) (figure 1B).

Final diagnosis of MPS I						
	All MPS I		**Hurler**		**Non-Hurler**	
Number of patients	29		20		9	
Age at diagnosis (months)						
Median	12		11		57	
Range	5 – 151		5 – 31		5 – 151	
Delay medical specialist - diagnosis (months)						
Median	9		8		28	
Range	1 – 147		1 – 24		2 – 147	
Diagnosing specialist	**Nr.**	**%**	**Nr.**	**%**	**Nr.**	**%**
General pediatrician	13	45%	10	50%	3	33%
Clinical geneticist	4	14%	3	15%	1	11%
Pediatrician specialized in IEM	9	31%	5	25%	4	44%
Ophthalmologist	2	7%	2	10%	0	0%
Rheumatologist	1	3%	0	0%	1	11%

Table 5. Characteristics of the entire group of MPS I patients, MPS I Hurler patients and non-Hurler patients at the time of diagnosis. The sums of the percentages of each item may not equal 100% because the percentages represent rounded values. IEM: inborn errors of metabolism

To assess whether the diagnostic delay changed over time, the patients were divided into different subgroups based on the year of the diagnosis using a 5-year time interval. Over the study period from 1988 to 2017, no significant reduction in the diagnostic delay was observed (figure 2A). In addition, no significant differences were observed in the time between the first visit to the medical specialist and the final diagnosis (figure 2B). When performing the same analyses only for the group of Hurler patients, no differences were observed in the median age at diagnosis and the median time between the first visit to the medical specialist and diagnosis over time (figure 2B, 2D).

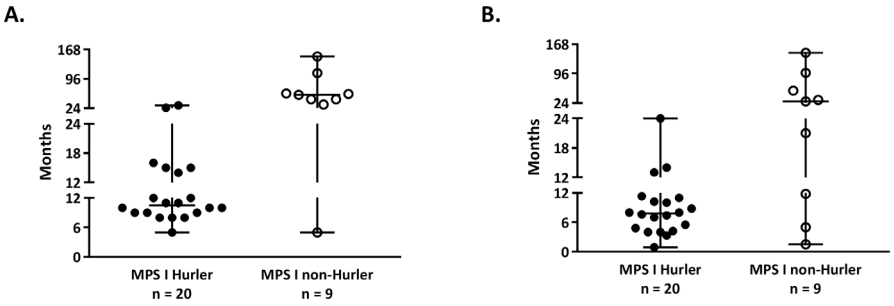


Figure 1. A. Age at diagnosis of the MPS I Hurler and non-Hurler patients. B. Time between the first visit to a medical specialist for an MPS I-related symptom and final diagnosis in MPS I Hurler and non-Hurler patients. In all figures, time is presented in months.

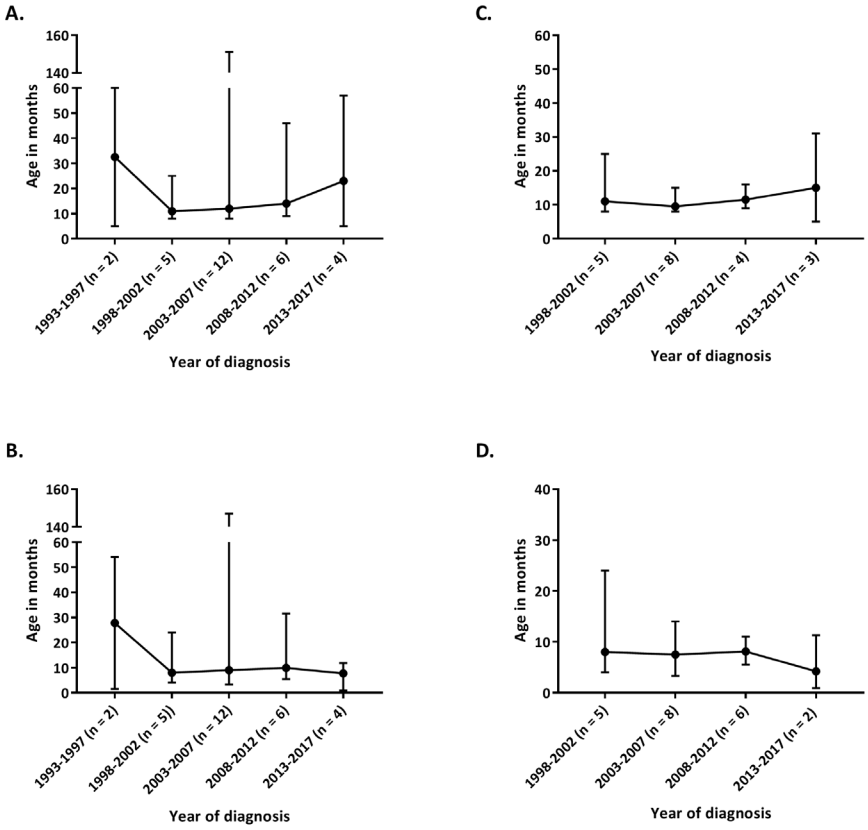


Figure 2. A. Age at diagnosis of the entire group of MPS I patients. B. Time between the first visit to a medical specialist for an MPS I-related symptom and final diagnosis in the entire group of MPS I patients. C. Age at diagnosis of the group of MPS I Hurler patients. D. Time between the first visit to a medical specialist for an MPS I-related symptom and final diagnosis in the group of MPS I Hurler patients. In all figures, time is presented in months. Both MPS I and MPS I Hurler patients were divided into groups based on the year of diagnosis.

MPS III: first visit to the GP for an MPS III-related symptom

Almost all MPS III patients (45 of the 46) first visited a GP for an MPS III-related symptom (table 6). The median age at the first visit for the entire group was 22 months (range 1 – 84 months): 16 months for the RP patients (range 1 – 33 months) and 24 months for the SP patients (range 1 – 84 months). Upper airway infections and middle ear problems were the most frequent symptoms leading to the visit to the GP. Other signs and symptoms leading to the visit to the GP and additional MPS III-related signs and symptoms present at that time are presented in table 6.

	First visit to a general practitioner					
	All MPS III		**RP MPS III**		**SP MPS III**	
Number of patients	45		16		28	
Age at 1st visit (months)						
Median	22		16		24	
Range	1 – 84		1 – 33		1 – 84	
Sign or symptom leading to 1st visit	**Nr.**	**%**	**Nr.**	**%**	**Nr.**	**%**
Developmental delay	9	20%	2	13%	6	21%
Upper airway problems	30	67%	12	75%	18	64%
Diarrhea	1	2%	0	0%	1	4%
Liver problems	1	2%	0	0%	1	4%
Seizures	1	2%	0	0%	1	4%
Inguinal/umbilical hernia	3	7%	2	13%	1	4%
Other MPS III-related signs & symptoms at 1st presentation	**Nr.**	**%**	**Nr.**	**%**	**Nr.**	**%**
Developmental delay	15	33%	3	19%	12	43%
Behavioral problems	29	64%	10	63%	18	64%
Dysmorphic features	27	60%	11	69%	15	54%
Upper airway problems	10	22%	2	13%	7	25%
Diarrhea	23	51%	11	69%	11	39%
Sleeping disturbances	21	47%	9	56%	11	39%
Inguinal/umbilical hernia	11	24%	8	50%	3	11%

Table 6. Characteristics of the entire group of MPS III patients, RP MPS III patients and SP MPS III patients at the first visit to the GP for an MPS III-related symptom. One of the SP MPS III patients did not visit the GP before receiving a referral to a medical specialist. One of the patients was considered too young to determine the phenotypic severity at the time of this study. The sums of the percentages of each item may not equal 100% because the percentages represent rounded values.

MPS III: first visit to a medical specialist for an MPS III-related symptom

The median age at the first visit to a medical specialist for an MPS III-related symptom was 28 months in the MPS III patients (range 2 – 171 months; median age: 19 months in RP patients (range 6 – 39 months) and 30 months in SP patients (range 2 – 171 months)) (table 7).

First visit to a medical specialist						
	All MPS III		**RP MPS III**		**SP MPS III**	
Number of patients	46		16		29	
Age at 1st visit (months)						
Median	28		19		30	
Range	2 – 171		6 – 39		2 – 171	
Specialism of 1st referral	**Nr.**	**%**	**Nr.**	**%**	**Nr.**	**%**
Ear, nose, and throat specialist	30	65%	13	81%	16	55%
General pediatrician	7	15%	1	6%	6	21%
Pediatric neurologist	2	4%	0	0%	2	7%
Pediatric surgeon	4	9%	2	13%	2	7%
Pediatric cardiologist	1	2%	0	0%	1	3%
Pediatric psychiatrist	2	4%	0	0%	2	7%
Sign or symptom leading to 1st referral	**Nr.**	**%**	**Nr.**	**%**	**Nr.**	**%**
Developmental delay	7	15%	0	0%	7	24%
Upper airway problems	2	4%	1	6%	1	3%
Adenotonsillectomy/tympanostomy tubes	29	63%	13	81%	15	52%
Diarrhea	1	2%	0	0%	1	3%
Liver problems	1	2%	0	0%	1	3%
Seizures	1	2%	0	0%	1	3%
Correction Inguinal/umbilical hernia	4	9%	2	13%	2	7%
Cardiac murmur	1	2%	0	0%	1	3%
Other MPS III-related signs & symptoms at 1st referral	**Nr.**	**%**	**Nr.**	**%**	**Nr.**	**%**
Developmental delay	21	46%	10	63%	10	34%
Behavioral problems	35	76%	14	88%	20	69%
Dysmorphic features	29	63%	11	69%	17	59%
Upper airway problems	10	22%	1	6%	9	31%
Diarrhea	23	50%	11	69%	11	38%
Sleeping disturbances	21	46%	9	56%	12	41%
Inguinal/umbilical hernia	11	24%	9	56%	2	7%

Table 7. Characteristics of the entire group of MPS III patients, RP MPS III patients and SP MPS III patients at the first visit to a medical specialist for an MPS III-related symptom. The sums of the percentages of each item may not equal 100% because the percentages represent rounded values.

The patients were most often referred to an ear, nose, and throat (ENT) specialist (65%), and 61% of the cases subsequently underwent an adenotonsillectomy or placement of tympanostomy tubes (81% of the RP patients and 52% of SP patients). In most patients, other MPS III-related symptoms were already present at the time of the first visit to the medical specialist, including developmental delay, behavioral and sleeping problems, dysmorphic features, hernias and recurrent episodes of unexplained diarrhea.

MPS III: time to diagnosis

The final diagnosis was established at a median age of 62 months, with a range of 20 to 522 months (table 8). As shown in figure 3A, the RP patients were significantly younger at the time of diagnosis (54 months, range 34 – 79 months) than the SP patients (71 months, range 20 – 522) ($p < 0.05$). The patients were most often diagnosed by a clinical geneticist, followed by a general pediatrician or a pediatrician specializing in IEM.

	Final diagnosis of MPS III					
	All MPS III		**RP MPS III**		**SP MPS III**	
Number of patients	46		16		29	
Age at diagnosis (months)						
Median	62		54		71	
Range	20 – 522		34 – 79		20 – 522	
Delay general practitioner – diagnosis (months)*						
Median	39		39		42	
Range	2 – 438		6 – 76		3 – 438	
Delay medical specialist – diagnosis (months)						
Median	33		33		41	
Range	1 – 365		2 – 66		5 – 365	
Diagnosing specialist	**Nr.**	**%**	**Nr.**	**%**	**Nr.**	**%**
Clinical geneticist	16	35%	5	31%	11	38%
General pediatrician	13	28%	5	31%	8	28%
Pediatrician specialized in IEM	12	26%	5	31%	6	21%
Pediatric neurologist	4	9%	1	6%	3	10%
Specialist for the mentally disabled	1	2%	0	0%	1	3%

Table 8. Characteristics of the entire group of MPS III patients, RP MPS III patients and SP MPS III patients at the time of diagnosis. The sums of the percentages of each item may not equal 100% because the percentages represent rounded values. IEM: inborn errors of metabolism.

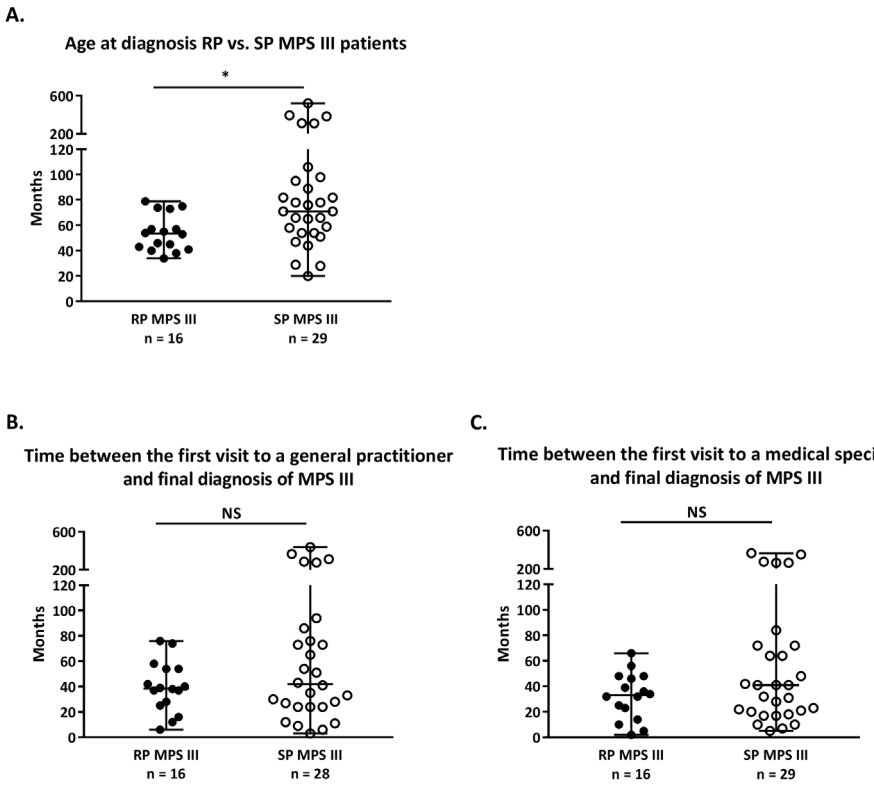


Figure 3. A. Age at diagnosis in the RP and SP MPS III patients. B. Time between the first visit to the GP for an MPS III-related symptom and the final diagnosis (in months) in the RP and SP MPS III patients. C. Time between the first visit to a medical specialist for an MPS III-related symptom and the final diagnosis (in months) in the RP and SP MPS III patients. * $p < 0.05$; NS = non-significant.

The median delay between the first visit to the GP for an MPS III-related symptom and the final diagnosis in the entire group of MPS III patients was 39 months (range 2 – 438 months), and no difference was observed among patient groups with varying disease severities (figure 3B). The median time between the first visit to a medical specialist for an MPS III-related symptom and the final diagnosis was 33 months (range 1 – 365 months). Similarly, no difference in delay was observed between the two phenotypic groups (figure 3C).

To assess whether the diagnostic delay changed over time, the MPS III patients were divided into different groups based on the year of the diagnosis using a 5-year time interval. Although a trend of diagnosing at a younger age was observed over time (figure 4A), no significant differences were observed between the cohorts of patients diagnosed in different time intervals. Similarly, the time between the first visit to the GP for an MPS III-related symptom and the time of the final diagnosis (figure 4B) and the time between the first visit

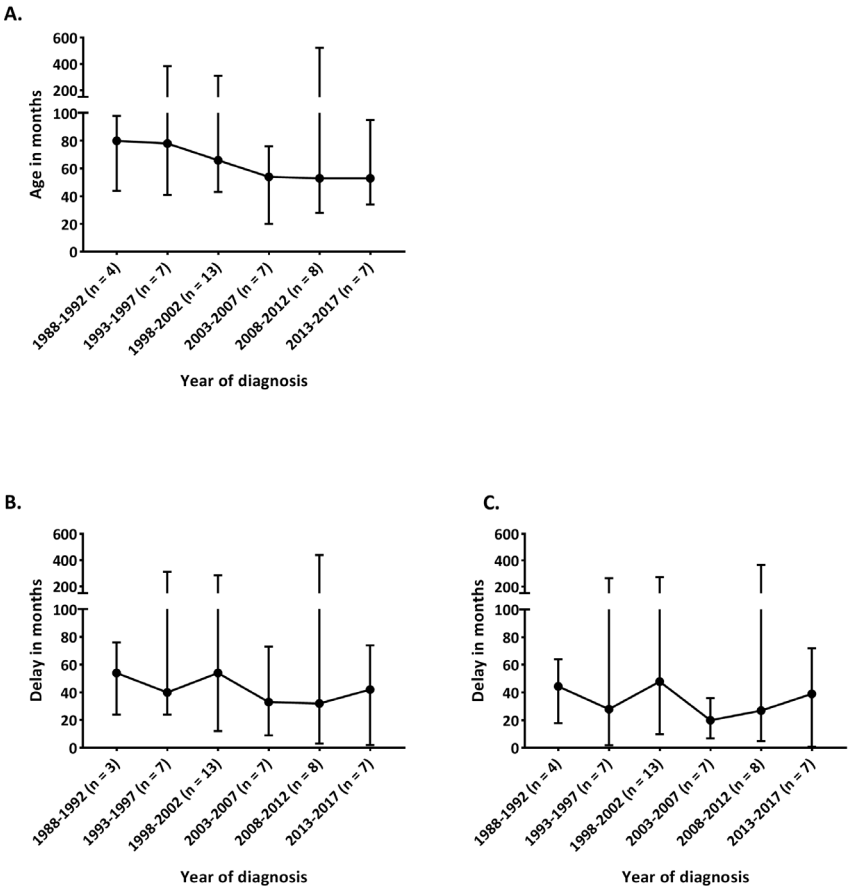


Figure 4. A. Age at diagnosis in the MPS III patients. B. Time between the first visit to a GP for an MPS III-related symptom and the final diagnosis. C. Time between the first visit to a medical specialist for an MPS III-related symptom and the final diagnosis. In all figures, time is presented in months. The MPS III patients were divided into groups based on the year of diagnosis. One patient never visited the GP for an MPS III-related symptom.

to a medical specialist and the time of the final diagnosis (figure 4C) were not significantly reduced during the study period from 1988 to 2017. Further analyses of the RP and SP patients did not reveal any differences over time (data not shown).

DISCUSSION

This study is the first to report the diagnostic odyssey in MPS I and MPS III patients in the Netherlands. We demonstrate the presence of a substantial diagnostic delay in both MPS

I and MPS III patients without a reduction in the time between the first consultation with a medical doctor (GP or medical specialist) for disease-related signs or symptoms and the time of the final diagnosis over a 20-year period.

In the Dutch healthcare system, patients, including children, are typically first seen by a GP, who may refer the patient to a medical specialist. Thus, the time to diagnosis after the visit to the GP was longer than the time between the visit to a medical specialist and the diagnosis. Remarkably, the longest diagnostic delay was observed after the first visit to a medical specialist, particularly in the MPS III patients.

The MPS I patients were diagnosed at a significantly younger age than the MPS III patients, which is most likely due to the early manifestation of the somatic symptoms [15,20], leading to earlier medical attention and referral. In MPS I, patients with the severe Hurler phenotype were diagnosed at a significantly younger age than the non-Hurler patients. The median age at diagnosis in the Hurler patients was comparable to that reported in previous studies [5,21–23]. However, the more attenuated non-Hurler patients in our cohort were diagnosed at an earlier age than that reported in other studies [18,21–24]. This finding may be due to the relatively small sample size of non-Hurler patients in our cohort. The lack of a decrease in the time to diagnosis over the previous two decades is disappointing and worrisome for two reasons. First, an early diagnosis allows for the early initiation of treatment and better disease outcomes. Treatment with hematopoietic stem cell transplantation (HSCT) for MPS I Hurler was first shown to be effective in halting or preventing the cognitive decline in the early 1980s and is currently the treatment of choice for this group of patients. Earlier HSCT leads to better outcomes [25–27]. In addition, intravenous enzyme replacement therapy (ERT) is the treatment of choice for MPS I patients with a non-Hurler phenotype, and studies have demonstrated that an early start of treatment is beneficial [9,10,28,29]. Second, to reduce the diagnostic delay and promote early diagnosis, numerous MPS I awareness campaigns have been launched, particularly after the introduction of ERT for the treatment of the somatic symptoms in 2003. These campaigns included direct mailings to health care professionals in the Netherlands presenting the typical features of MPS I patients, expert lectures on early signs and symptoms of MPS I at scientific meetings of relevant medical specialists (including pediatricians, ENT specialists, pediatric rheumatologists and pediatric neurologists) and exhibit booths of a pharmaceutical company commercially marketing ERT for MPS I (Genzyme Sanofi) providing educational material on lysosomal storage disorders, including MPS I, at major relevant medical conferences in the Netherlands. Our data indicate that these efforts have not led to a significant reduction in the time to an MPS I diagnosis.

In our cohort of MPS III patients, the diagnosis was established at a significantly younger age in the severe RP patients (age 54 months; 4 years and 6 months) than in the SP patients (age 71 months; 5 years and 11 months). However, the diagnostic process preceding the diagnosis did not differ between the two groups, and the age at final diagnosis is comparable

to observations reported in other studies [30–32]. Although no disease-modifying treatment is currently available, several clinical trials, including intrathecal ERT and gene therapy, have recently been initiated for MPS III types A and B [33,34]. An early diagnosis and early start of treatment before the onset of progressive cognitive deterioration are considered essential. Given that patients with the RP phenotype plateau in development by 30 months and exhibit rapid cognitive decline at 40 – 50 months, a diagnosis should be made before the age of 3 years to allow the initiation of therapy at the optimal timing [17]. This goal, however, was only achieved in 9% of the patients in this study, and no decrease in age at diagnosis was observed over the previous 20 years.

Our study has some limitations. First, we defined diagnostic delay as the time between the first visit to a GP or medical specialist for a potential disease-related symptom and the final diagnosis, whereas diagnostic delay generally refers to the time between the onset of symptoms and diagnosis in other studies[18,35,36]. However, we consider the use of the time of symptom onset susceptible to a significant recall bias, whereas the time of the first visit to a medical doctor can be verified, thereby providing more reliable data. Second, our study has a retrospective design. Nevertheless, the amount of missing data was small, and the data could be verified in the medical records. In addition, due to the rarity of both disorders, a prospective design is not feasible. Third, the number of patients included in our study was small. Given that we were able to recruit almost all patients from the Netherlands diagnosed with MPS I and MPS III between 1988 and 2017, we assume that our data reliably represent the situation in our country. Finally, MPS I and MPS III are ultra-rare (ultra-orphan) diseases because they affect less than one person per 50,000 people [37]. The results of our study might not be applicable to relatively more common rare diseases affecting one person per 2,000 – 50,000 people.

The lack of a reduction in the diagnostic delay over time was previously reported for MPS I by d'Aco *et al.* based on data from an observational international MPS I registry [38]. In addition, a study investigating the time of diagnosis in Pompe disease, which is a lysosomal storage disease in which the timing of the start of therapy (ERT) is essential, to the surprise of the authors, also failed to demonstrate a reduction in the diagnostic delay despite improved diagnostic laboratory techniques allowing for a rapid diagnosis [39]. Multiple efforts to increase awareness of Pompe disease and expedite its diagnosis have been exerted globally over recent decades.

Determining why awareness campaigns for rare diseases fail to reduce the diagnostic delay in MPS I and III and Pompe disease is challenging. Due to the very low birth prevalence of these disorders, many specialists, including GPs, general pediatricians, orthopedic surgeons and ENT specialists, may visit with no or only one undiagnosed patient during their entire career. Awareness of specific (combinations of) signs and symptoms of a (ultra) rare disease may be lacking when confronted with a patient (many) years after exposure to an awareness

campaign. Long-lasting knowledge regarding the symptoms of (ultra) rare diseases can likely only be achieved by intensive repetitive learning, which is not a feasible option for all medical specialists. Furthermore, because most signs and symptoms at presentation are not specific, considerable time is generally spent excluding more common disorders.

Several alternative strategies are possible. One strategy involves the selective screening of groups of patients with certain signs and symptoms but without a diagnosis of the rare disease of interest. Such studies have been performed for MPS I and included studies investigating MPS screening in patients with previous surgical repair or the presence of inguinal and/or umbilical hernia in combination with pediatric ENT surgery and children visiting rheumatology, hand or skeletal dysplasia clinics (clinicaltrials.gov identifiers: NCT02095015, NCT01675674). Both trials have been terminated. To the best of our knowledge, these results have not been published, suggesting a failure to identify significant numbers of otherwise unrecognized patients. A study investigating screening patients under the age of 18 years with carpal tunnel syndrome for MPS also failed to detect patients with MPS [40]. The extremely low yield of screening certain groups of patients for an ultra-rare disorder likely discourages participation, leading to the discontinuation of these programs. The yields of selective screening may improve when groups of patients are screened for a multitude of disorders, thus obviating the need of knowledge regarding specific rare disorders. Because the diagnostic approach in children with impaired cognitive development may significantly differ among health care systems in different regions of the world and obtaining an early diagnosis in patients with MPS III is very difficult, screening of children with an intellectual developmental disorder for several rare diseases may significantly reduce the diagnostic delay. A diagnostic algorithm for the identification of treatable causes of cognitive impairment has been proposed [41], and several publications have demonstrated the importance of an early metabolic screening in all patients with unexplained developmental delay [42,43]. In addition, a review by Cleary and Green [44] provided a guideline for the metabolic screening of patients with a developmental delay. The authors emphasize that IEMs can present with isolated developmental delay and that any regression of skills is suggestive of an IEM and warrants an intensive metabolic investigation. The slowing of cognitive development with a speech delay is one of the first symptoms of MPS III and often occurs before the age of 2.5 years; these symptoms could lead to an early diagnosis if these guidelines are followed. However, as the median age at diagnosis of patients with the most common RP phenotype is 54 months (range 34 – 79 months) in our study, it is clear that these guidelines are not used in the Netherlands. Indeed, the current guideline by the Dutch Society for Pediatrics (NvK, 2005) recommends screening for IEMs only if additional signs and symptoms are present and not in in the presence of isolated cognitive delay [45]. Fortunately, a new guideline is currently under development.

An interesting option for the (near) future is computer-assisted diagnosis, which can expedite the diagnosis of rare diseases. Artificial intelligence, deep learning and even a 3D facial analysis may assist clinicians during the diagnostic process, suggesting both diagnoses and appropriate investigations based on information in the electronic patient records [46–48]. Finally, newborn population screening (NBS) may ensure very early diagnosis in patients with rare diseases and should be considered if a disease meets at least the following criteria (first proposed by Wilson and Jungner in 1968) [49]: (a) the condition is an important health problem; (b) a suitable test for diagnosis is available; (c) a latent or early symptomatic state is recognizable; (d) the understanding of the condition's natural history is adequate; and (e) an acceptable treatment for patients with a recognized disease is available. Because MPS I is considered to meet these criteria, this disorder has been introduced in NBS programs in the USA and Taiwan [50] and will be introduced in the NBS panel in the Netherlands [51]. MPS III is currently not considered eligible for NBS because no disease-modifying therapy is yet available.

CONCLUSIONS

In conclusion, we demonstrate that the time to diagnosis in patients with MPS I and MPS III has not changed between 1988 and 2017 in the Netherlands and a long delay between the first visit to a medical doctor for signs and symptoms related to the disease and the final diagnosis is common. Therefore, campaigns to increase the awareness of rare diseases in general, and of MPS I specifically, failed to achieve this goal. This finding is likely due to the non-specific initial signs and symptoms and the ultra-rare nature of both disorders. Because most medical doctors will probably visit with patients with these disorders never or only once during their entire career, it is questionable whether education of combinations of signs and symptoms of specific (ultra) rare diseases will ever be effective. Robust selected screening protocols embedded in national guidelines may be the best alternative. Such guidelines may include urinary screening for GAGs in all children with kyphosis and extensive screening for IEMs in all children with developmental delay, thus obviating the need for detailed knowledge regarding specific (ultra) rare diseases. Finally, NBS should be considered for those disorders that meet the criteria for population screening because this may be the only approach to guarantee a timely initiation of therapy in all patients with specific rare diseases.

ACKNOWLEDGMENTS

We thank all families for their willingness to contribute to this study and participate in our telephone survey.

FUNDING

This study was partially funded by a grant from Sanofi Genzyme. The funder had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

REFERENCES

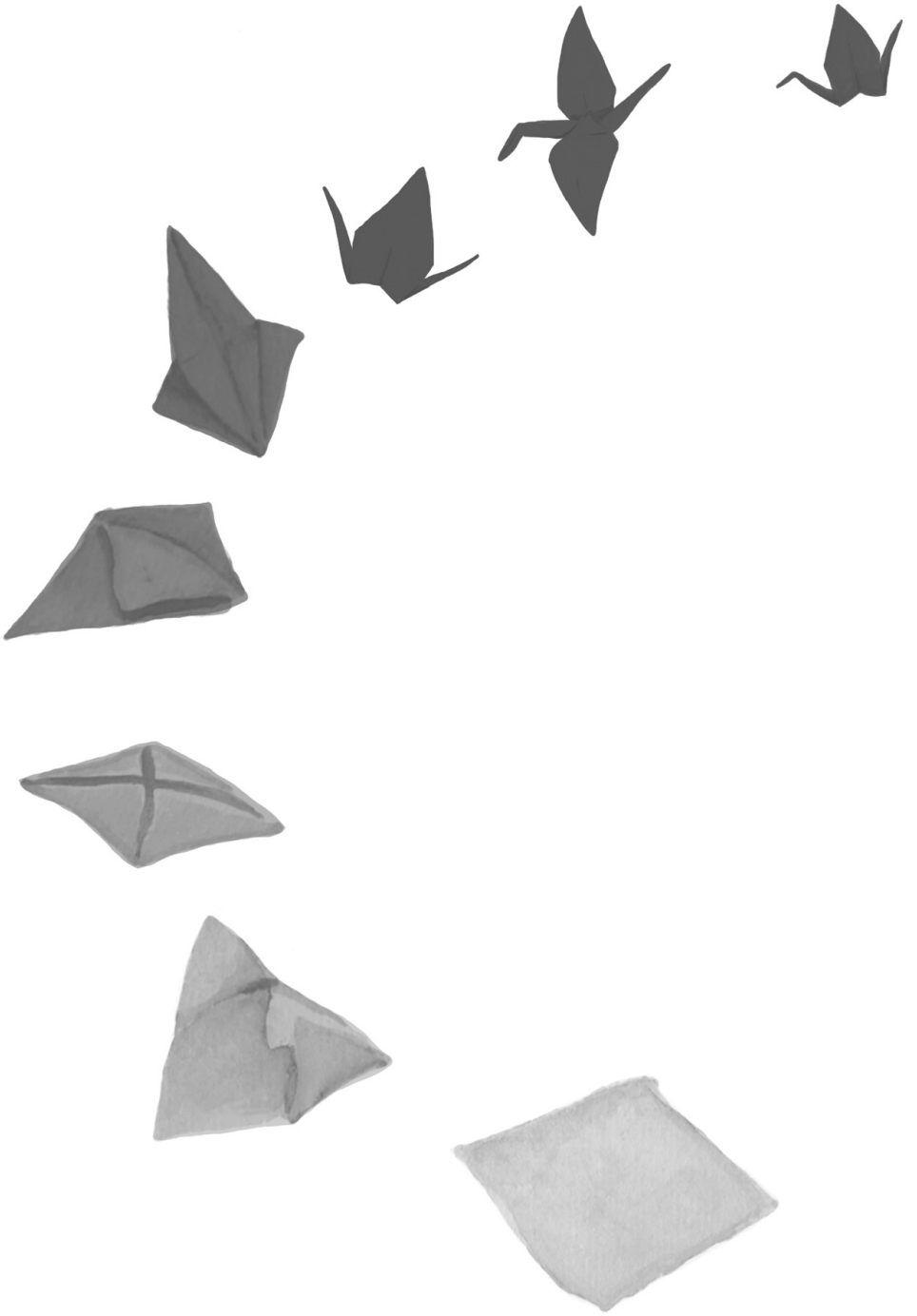
1. https://ec.europa.eu/health/rare_diseases/policy.
2. http://www.eurordis.org/sites/default/files/publications/Fact_Sheet_RD.pdf.
3. Regulation (EC) No 1295/1999 of the European Parliament and of the Council of 29 April 1999 adopting a programme of Community action on rare diseases within the framework for action in the field of public health (1999-2003) Official Journal of the Europea.
4. Lisi EC, Gillespie S, Laney D, Ali N. Patients' perspectives on newborn screening for later-onset lysosomal storage diseases. *Mol Genet Metab*. 2016;119(1):109-114.
5. de Ru MH, Bouwman MG, Wijburg FA, van Zwieten MCB. Experiences of parents and patients with the timing of Mucopolysaccharidosis type I (MPS I) diagnoses and its relevance to the ethical debate on newborn screening. *Mol Genet Metab*. 2012;107(3):501-507.
6. Bruni S, Lavery C, Broomfield A. The diagnostic journey of patients with mucopolysaccharidosis I: A real-world survey of patient and physician experiences. *Mol Genet Metab Reports*. 2016;8:67-73.
7. Zurynski Y, Deverell M, Dalkeith T, et al. Australian children living with rare diseases: experiences of diagnosis and perceived consequences of diagnostic delays. *Orphanet J Rare Dis*. 2017;12(1):68.
8. Aldenhoven M, Wynn RF, Orchard PJ, et al. Long-term outcome of Hurler syndrome patients after hematopoietic cell transplantation: an international multicenter study. *Blood*. 2015;125(13):2164-2172.
9. Gabrielli O, Clarke LA, Bruni S, Coppa G V. Enzyme-replacement therapy in a 5-month-old boy with attenuated presymptomatic MPS I: 5-year follow-up. *Pediatrics*. 2010;125(1):e183-7.
10. Laraway S, Breen C, Mercer J, Jones S, Wraith JE. Does early use of enzyme replacement therapy alter the natural history of mucopolysaccharidosis I? Experience in three siblings. *Mol Genet Metab*. 2013;109(3):315-316.
11. Zurynski Y, Frith K, Leonard H, Elliott E. Rare childhood diseases: how should we respond? *Arch Dis Child*. 2008;93(12):1071-1074.
12. Hampton T. Rare disease research gets boost. *JAMA*. 2006;295(24):2836-2838.
13. Schieppati A, Henter JI, Daina E, Aperia A. Why rare diseases are an important medical and social issue. *Lancet*. 2008;371(9629):2039-2041.
14. http://www.rarediseaseday.org/events/world.
15. Neufeld EF, Muenzer J. The Mucopolysaccharidoses. In: Valle D, Beaudet A, Vogelstein B, et al., eds. *The Metabolic and Molecular Basis of Inherited Disease*. 8th ed. New York, NY, USA: McGraw-Hill; 2001:3421-3452.
16. Valstar MJ, Ruijter GJG, van Diggelen OP, Poorthuis BJ, Wijburg FA. Sanfilippo syndrome: a mini-review. *J Inherit Metab Dis*. 2008;31(2):240-252.
17. Shapiro EG, Nestrasil I, Delaney KA, et al. A Prospective Natural History Study of Mucopolysaccharidosis Type IIIA. *J Pediatr*. 2016;170:278-287e4.
18. Vijay S, Wraith JE. Clinical presentation and follow-up of patients with the attenuated phenotype of mucopolysaccharidosis type I. *Acta Paediatr*. 2005;94(7):872-877.
19. Cleary M, Wraith JE. The presenting features of mucopolysaccharidosis type IH (Hurler syndrome). *Acta Paediatr*. 1995;84(3):337-339.
20. Muenzer J. Overview of the mucopolysaccharidoses. *Rheumatology*. 2011;50 Suppl 5:v4-12.
21. Pastores GM, Arn P, Beck M, et al. The MPS I registry: Design, methodology, and early findings of a global disease registry for monitoring patients with Mucopolysaccharidosis Type I. *Mol Genet Metab*. 2007;91(1):37-47.
22. D'Aco K, Underhill L, Rangachari L, et al. Diagnosis and treatment trends in mucopolysaccharidosis I: findings from the MPS I Registry. *Eur J Pediatr*. 2012;171(6):911-919.
23. Beck M, Arn P, Giugliani R, et al. The natural history of MPS I: global perspectives from the MPS I Registry. *Genet Med*. 2014;16(10):759-765.

24. Thomas JA, Beck M, Clarke JTR, Cox GF. Childhood onset of Scheie syndrome, the attenuated form of mucopolysaccharidosis i. *J Inherit Metab Dis*. 2010;33(4):421-427.
25. Muenzer J, Wraith JE, Clarke LA. Mucopolysaccharidosis I: management and treatment guidelines. *Pediatrics*. 2009;123(1):19-29.
26. de Ru MH, Boelens JJ, Das AM, et al. Enzyme replacement therapy and/or hematopoietic stem cell transplantation at diagnosis in patients with mucopolysaccharidosis type I: results of a European consensus procedure. *Orphanet J Rare Dis*. 2011;6:55.
27. Aldenhoven M, Wynn RF, Orchard PJ, et al. Long-term outcome of Hurler syndrome patients after hematopoietic cell transplantation: an international multi-center study. *Blood*. 2015;125(13):2164-2172.
28. Al-Sannaa NA, Bay L, Barbouth DS, et al. Early treatment with laronidase improves clinical outcomes in patients with attenuated MPS I: a retrospective case series analysis of nine sibships. *Orphanet J Rare Dis*. 2015;10(1):131.
29. Gabrielli O, Clarke LA, Ficcadenti A, et al. 12 year follow up of enzyme-replacement therapy in two siblings with attenuated mucopolysaccharidosis I: the important role of early treatment. *BMC Med Genet*. 2016;17(1):19.
30. Meyer A, Kossow K, Gal A, et al. Scoring evaluation of the natural course of mucopolysaccharidosis type IIIA (Sanfilippo syndrome type A). *Pediatrics*. 2007;120(5):e1255-61.
31. Héron B, Mikaeloff Y, Froissart R, et al. Incidence and natural history of mucopolysaccharidosis type III in France and comparison with United Kingdom and Greece. *Am J Med Genet A*. 2011;155(1):58-68.
32. Delgadillo V, O'Callaghan M del M, Gort L, Coll MJ, Pineda M. Natural history of Sanfilippo syndrome in Spain. *Orphanet J Rare Dis*. 2013;8:189.
33. Hollak CEM, Wijburg FA. Treatment of lysosomal storage disorders: Successes and challenges. *J Inherit Metab Dis*. 2014;37(4):587-598.
34. Parenti G, Andria G, Ballabio A. Lysosomal Storage Diseases: From Pathophysiology to Therapy. *Annu Rev Med*. 2015;66(1):471-486.
35. Valstar MJ, Bruggenwirth HT, Olmer R, et al. Mucopolysaccharidosis type IIIB may predominantly present with an attenuated clinical phenotype. *J Inherit Metab Dis*. 2010;33(6):759-767.
36. Valstar MJ, Neijs S, Bruggenwirth HT, et al. Mucopolysaccharidosis type IIIA: clinical spectrum and genotype-phenotype correlations. *Ann Neurol*. 2010;68(6):876-887.
37. REGULATION (EU) No 536/2014 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 April 2014 on clinical trials on medicinal products for human use, and repealing Directive 2001/20/EC).
38. D'Aco K, Underhill L, Rangachari L, et al. Diagnosis and treatment trends in mucopolysaccharidosis I: findings from the MPS I Registry. *Eur J Pediatr*. 2012;171(6):911-919.
39. Kishnani PS, Amartino HM, Lindberg C, Miller TM, Wilson A, Keutzer J. Timing of diagnosis of patients with pompe disease: Data from the pompe registry. *Am J Med Genet A*. 2013;161(10):2431-2443.
40. Nørmark MB, Kjaer N LA. Prevalence of Mucopolysaccharidosis Types I, II, and VI in the Pediatric and Adult Population with Carpal Tunnel Syndrome (CTS). Retrospective and Prospective Analysis of Patients Treated for CTS. *JIMD Rep*. 2017 [Epub ahead of print]
41. Van Karnebeek CDM, Shevell M, Zschocke J, Moeschler JB, Stockler S. The metabolic evaluation of the child with an intellectual developmental disorder: Diagnostic algorithm for identification of treatable causes and new digital resource. *Mol Genet Metab*. 2014;111(4):428-438.
42. Engbers HM, Berger R, Van Hasselt P, et al. Yield of additional metabolic studies in neurodevelopmental disorders. *Ann Neurol*. 2008;64(2):212-217.
43. Sayson B, Popurs MAM, Lafek M, Berkow R, Stockler-Ipsiroglu S, van Karnebeek CDM. Retrospective analysis supports algorithm as efficient diagnostic approach to treatable intellectual developmental disabilities. *Mol Genet Metab*. 2015;115(1):1-9.
44. Cleary MA, Green A. Developmental delay: when to suspect and how to investigate for an inborn error of metabolism. *Arch Dis Child*. 2005;90(11):1128-1132.

45. https://www.nvk.nl/Portals/0/richtlijnen/mentale%20retardatie/mentaleretardatie.pdf.
46. Cahan A, Cimino JJ. A learning health care system using computer-aided diagnosis. *J Med Internet Res*. 2017;19(3):1-12.
47. Chen Y, Argentinis E, Weber G. IBM Watson: How Cognitive Computing Can Be Applied to Big Data Challenges in Life Sciences Research. *Clin Ther*. 2016;38(4):688-701.
48. Gripp KW, Baker L, Telegrafi A, Monaghan KG. The role of objective facial analysis using FDNA in making diagnoses following whole exome analysis. Report of two patients with mutations in the BAF complex genes. *Am J Med Genet A*. 2016;170(7):1754-1762.
49. Wilson JM, Jungner G. Principles and practice of screening for disease. WHO, Geneva; 1968. Available from: http://whqlibdoc.who.int/php/WHO_PHP_34.pdf
50. Paciotti S, Persichetti E, Pagliardini S, et al. First pilot newborn screening for four lysosomal storage diseases in an Italian region: Identification and analysis of a putative causative mutation in the GBA gene. *Clin Chim Acta*. 2012;413(23):1827-1831.
51. https://zoek.officielebekendmakingen.nl/blg-775624.pdf.

Discussion & Future perspectives

Summaries



Chapter 8

Discussion and future perspectives

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Mucopolysaccharidosis type III (MPS III or Sanfilippo disease) is a multisystem disease that most severely affects the central nervous system (CNS). Its pathophysiology is complex and several disease causing factors play a role: the primary enzyme deficiency, impairment of breakdown of the glycosaminoglycan (GAG) heparan sulfate (HS) and subsequent storage; the secondary effects of HS accumulation; changes in HS structure and function; and overall lysosomal dysfunction resulting from HS storage. More knowledge on each aspect is needed to fully understand the pathophysiology of the disease. Along with better understanding, disease-modifying treatment options for MPS III need to be developed.
This thesis unfolds new mechanisms underlying the pathophysiology of MPS III, its diagnosis, including the preceding diagnostic Odyssey and phenotype prediction, and investigates potential treatment options for MPS III.

UNFOLDING THE PATHOPHYSIOLOGY OF MPS III: WHAT IS NEW?

Mild cold stress has a beneficial effect on enzyme activity in MPS III patients' fibroblasts

Under normal assay conditions enzyme activity in MPS IIIB patients is very low when compared to activity levels in healthy controls. Despite a small but significant difference in activity levels of *N*-acetyl-α-glucosaminidase (NAGLU) between patients with a rapidly progressing (RP) and slowly progressing (SP) phenotype, there is still a considerable overlap between the two patient groups, as was shown in **chapter 3**. We demonstrate that culturing fibroblasts of patients with SP MPS IIIB at 30°C can restore residual enzyme activity to 10% of control activity, which is associated with an attenuated course of the disease or even absence of symptoms [1,2], in contrast to RP MPS IIIB fibroblasts in which enzyme activity remained very low, at the level of background activity, under all conditions. Interestingly, the same trend was recently observed in MPS IIIA fibroblasts by Knottnerus *et al.* [3]. In both studies higher residual enzyme activities in fibroblasts resulted in lower levels of HS storage, indicating that the enzyme formed is biochemically active. Besides serving as an important tool for phenotype prediction (which will be discussed later), these data suggest that the capacity of a mutant enzyme to be processed into an enzymatically active form, is a major determinant for the clinical course of the disease. Moreover, as was shown in **chapter 4**, it provides evidence that the MPS III cell contains its own internal mechanisms to increase residual enzyme activity which can, *in vitro*, easily be 'switched on' by cold stress resulting in higher mRNA levels, increased levels of precursor and mature NAGLU protein and better processing of the precursor protein into the mature form. The underlying mechanisms responsible for these changes, however, remain unknown.

Culturing cells at lower temperatures is a well-established method used to interfere with protein stability and activity [4–6]. It is generally considered that mild cold stress (27-32°C) improves protein folding, although the exact underlying mechanism(s) have not yet been unraveled. In several mammalian cell systems it has been shown that mild cold stress influences the expression of a wide variety of genes leading to the induction of cold-shock proteins, including cold-inducible RNA binding protein and RNA binding motif protein 3 [7,8]. These proteins seem to modulate transcription and translation, and to function as RNA chaperones [9,10] which can explain the higher NAGLU mRNA levels found in SP patients' fibroblasts. Because these seem quite general responses, it is striking that the positive effect of cold shock on mRNA levels was mainly observed in SP MPS IIIB cell lines and was less pronounced in control fibroblast cell lines. Moreover, the higher levels of functional NAGLU protein found in these patients can only be partially explained by higher mRNA levels and, so far, do not provide an explanation for the changes in protein processing found in the here tested MPS IIIB cell lines.

Future studies should focus on the effect of low culturing temperatures on gene-expression in MPS III cell lines, addressing those genes involved in mRNA transcription and translation as well as protein processing. From this perspective, transcription factor EB (TFEB) is a target of great interest. This transcriptional factor has shown to be a key regulator of lysosomal biogenesis and function [11]. Overexpression of TFEB in HeLa cells resulted in higher (activity) levels of lysosomal enzymes, including NAGLU [12]. TFEB is activated by stress and reacts to external signals such as starvation [13]. It might well be that cold stress positively affects TFEB expression. As lysosomal accumulation of HS derived oligosaccharides is a stressor in itself, this might, directly or indirectly, influence TFEB expression even more. This could also explain the differences in mRNA expression and NAGLU protein levels found between SP MPS IIIB patients and healthy controls. Better understanding of these processes will potentially facilitate the development of new therapeutic approaches which, in theory, may benefit more protein folding and / or processing diseases.

The positive effects of low culture temperature were limited to SP MPS IIIB fibroblasts carrying missense mutations. Future studies should therefore also include MPS III fibroblast cell lines that contain missense mutations that comprise an RP phenotype. These were not present in our cohort of MPS IIIB patients´ fibroblast cell lines, but have been described by others for MPS IIIB [14] as well as for MPS IIIA [3,15]. The latter study showed that the beneficial effect of cold stress on enzyme activity was not found in MPS IIIA fibroblasts carrying missense mutations associated with an RP phenotype. We hypothesize that the same is true for MPS IIIB. If indeed so, culturing at 30°C would provide a good and rather simple tool for determining the phenotypic consequences of new missense mutations of unknown severity.

Changes in HS structure and function may play a role in neuronopathic disease

HS is a molecule that is a widely distributed throughout the body and has a wide variety of biological functions. It is the only primary accumulating substrate in MPS III, but together with DS it also accumulates in MPS I, MPS II and MPS VII, in the latter accompanied by KS accumulation. Of these MPS subtypes, MPS III features the most pronounced neuronopathic phenotype of which the nature considerably differs from the other MPS subtypes. These differences might be related to differences in accumulating HS derived oligosaccharides.

The structure of HS is heterogeneous and contains multiple modifications. These modifications, including the pattern of HS sulfation, determine the broad range of functions of HS containing proteoglycans [16–18]. As was described in detail in the introduction of this thesis, HS breakdown consists of a multi-step process. Therefore differences in the underlying MPS causing enzyme deficiencies, will result in different accumulating HS derived oligosaccharides, both intra- and extracellularly which will influence interactions between HS containing proteoglycans and other proteins and thereby influence overall cell function [18].We therefore hypothesized that the type and sulfation pattern of the accumulating HS derived oligosaccharides determines the differences in neuronopathic disease.

In line with the findings of Wilkinson *et al.*, who found differences in sulfation pattern of HS derived disaccharides between MPS I and MPS III mouse brains [19], differences in the fractions of specific HS derived disaccharides in plasma were found between MPS I and MPS III patients, as was shown in **chapter 5**. Moreover, HS derived disaccharide fractions differed significantly between MPS III patients with different severity of disease. Nevertheless, differences found in our cohort are small.

Although it is unclear to which extent the differences in HS sulfation account for differences in CNS disease between patients with different MPS subtypes and patients with different disease severity, there is evidence that changes in HS sulfation do play a role in the overall pathophysiology of the disease. Accumulation of different HS derived oligosaccharides was shown to result in defective cell polarization and migration in the ECM [20] and to alter postsynaptic function in the developing somatosensory cortex [21]. To be able to further support the hypothesis that structural changes in HS derived oligosaccharides also contribute to differences in CNS disease, more studies are needed in patients and other MPS III disease models with different severity of disease.

DIAGNOSIS OF MPS III AND PHENOTYPE PREDICTION

Mucopolysaccharidosis: a diagnostic Odyssey

MPS III belongs to the large group of more than 6000 rare disease that have been identified so far [22,23]. Due to the rather non-specific signs and symptoms in the early phase of the

disease in combination with its low prevalence resulting in a lack of familiarity among medical doctors, MPS III often remains un- or misdiagnosed for a long period of time, despite the fact that with the current knowledge and recent developments in laboratory techniques, the diagnosis can be established with high specificity and sensitivity [24,25]. For several MPSs it has been shown that this diagnostic odyssey is a considerable burden for patients, their parents and caregivers [26–29].

In **chapter 7** the time to diagnosis was investigated for the two (ultra-)rare diseases MPS I and MPS III, and it was assessed whether the diagnostic delay had changed over time. Remarkably, no change in time between the onset of disease related symptoms and final diagnosis was found for MPS I and MPS III patients diagnosed in the Netherlands between 1988 and 2017. Interestingly, the most pronounced delay was found for the time between the first visit to a medical specialist and final diagnosis. These findings suggest that rare disease awareness campaigns initiated by different stakeholders including patient advocacy groups and pharmaceutical companies and the availability of therapy (as is the case for MPS I), have not resulted in earlier diagnosis.

Nonetheless, early diagnosis is essential for timely initiation of therapy and, as shown for MPS I, will likely allow for better treatment outcome [30–32]. Although there is no disease-modifying treatment available yet, it seems realistic, in view of all current efforts, that an effective treatment will be developed for MPS III in the near future. Patients with an RP phenotype reach a plateau in their development by 30 months of age after which a rapid decline sets in from the age of 40-50 months [33]. Diagnosis should thus best be made before the age of 3 years which in our cohort was the case in only a minority of patients (9%).

Early diagnosis will thus optimize timing of treatment initiation of disease-modifying therapies and is likely to improve treatment outcome. As long as such therapies are not yet available, early diagnosis allows for the early start of supportive therapies, and the accurate monitoring of disease related complications. It will also enable the inclusion in clinical trials, which especially applies to MPS III patients, for whom therapies are still only available in trial setting and often only include children diagnosed at young age. Furthermore, and extremely important, shortening of the diagnostic delay and prevention of the long odyssey by which the diagnosis is often preceded, will hopefully diminish the great emotional and psychosocial impact of this process on patients, their parents and caregivers, and will allow for genetic counseling of parents on family planning [27,34].

Since most medical doctors will most probably only encounter one (or even none) of these patients during their entire career, focusing on educational programs for clinicians on rare diseases including inborn errors of metabolism such as MPS I and MPS III, will probably harbor little effect. To improve the time to diagnosis and shorten the diagnostic odyssey of patients with MPS III, the most feasible strategy will be to ensure that targeted screening for inborn errors of metabolism is embedded in national protocols for children presenting

with a developmental delay. Eventually, newborn screening (NBS) will be the only method to ensure timely diagnosis. However, as long as there is no disease-modifying treatment available, MPS III does not meet the criteria for population screening which hampers the introduction in the most, and certainly the Dutch, NBS programs [35].

Mild cold stress as a tool for phenotype prediction in MPS III

Until quite recently, parents of children newly diagnosed with MPS III, received the prognosis that due to the rapidly progressive neurodegenerative nature of the disease, their child would lose most cognitive functions before the age of 10 years and pass away in the second or third decade of life. However, natural history studies in all MPS III subtypes have taught us that the disease comprises a spectrum, ranging from a severe and rapidly progressing (RP) phenotype, to a more attenuated, slowly progressing (SP) phenotype, for which survival into the seventh decade has been reported [15,33,36–38]. For MPS IIIA and MPS IIIB genotype-phenotype correlations have been established [14,15,38–41]. Nevertheless, new mutations of which the phenotypic implications are unknown, are found on a frequent basis. Especially in MPS IIIB allelic heterogeneity is high. For both MPS IIIA [3] and MPS IIIB **(chapter 3)** it has been shown that culturing patient fibroblasts at 30°C is a reliable and robust tool for phenotype prediction and that residual enzyme activity after culturing at 30°C can reliably distinguish RP from SP MPS III patients.

Prediction of disease phenotype of individual patients will become essential in the near future for several reasons. First, it will allow for proper design of clinical trials for new disease-modifying therapies and to determine outcome measures that will help to reliably assess the efficacy of these therapies. Second, for possible future inclusion of MPS III in NBS programs, phenotype prediction will be essential, especially to help decision making on timing of treatment initiation and the type of treatment. Third, in the absence of a disease-modifying therapy, reliable phenotype prediction will allow for better counseling of parents and caregivers and will help them in decisions regarding supportive therapies and interventions, together with life and family planning.

WHY THERE IS STILL NO TREATMENT FOR MPS III AND WHY THERE IS REASON FOR HOPE

´There is yet no disease-modifying treatment for MPS III´, were the key words of the research proposal resulting in this thesis. After more than four years this conclusion has not significantly changed.

The development of therapies for rare diseases including MPS III, is challenging. First of all, drug development is costly and financial resources are limited especially because the

number of patients that will eventually benefit from these therapies is small. The small patient numbers often require international, multi-center studies with study designs that suit relative small patient groups and make use of representative (and often strict) inclusion criteria as well as reliable and clinically relevant outcome measures [42]. Therefore, a good insight in the natural history and the phenotypic spectrum of the disease is needed. Finally, in MPS III the CNS is the most severely affected organ which requires for drug delivery strategies that circumvent or allow for direct transport through the blood-brain barrier (BBB). This makes the translation of potential new therapies from an *in vitro* setting to animal models and eventually patients, even more challenging [43].

Nevertheless, new developments hold many promises, particularly in those cases where drug development is patient driven and patient advocacy groups, academic research centers and the pharmaceutical industry join forces, as is for instance the case in the development of gene therapy for MPS IIIA by the pharmaceutical company Lysogene (www.lysogene.com). Despite the fact that several challenges need to be overcome before a well-established therapy enters the clinic, which is disappointing for those children and (young) adults already diagnosed with MPS III, the current developments in this field are promising and yield more potential than five years ago.

Upcoming treatment approaches

As was delineated in the introduction of this thesis, potential therapies for MPS III can interfere on different levels of the pathophysiological process: the level of the genetic defect by gene therapy; the level of the deficient enzyme, by either replacing the enzyme via ERT or HSCT, or by enhancing the residual activity of the mutant enzyme via chaperone therapy; by reduction of the synthesis of the accumulating substrate (SRT) and by optimizing overall lysosomal function and cellular clearance via TFEB activation.

Gene therapy

The most intensively studied approach for the treatment of MPS III at this moment is probably gene therapy which now has been developed for both MPS IIIA and MPS IIIB. Intracerebral gene therapy for MPS IIIA patients has shown to be safe and well tolerated (clinicaltrials.gov identifier: NCT01474343; clinicaltrials.gov identifier: NCT02053064) and a phase II/III study is planned to start soon [44]. Also for MPS IIIB, intracerebral gene therapy has shown to be safe and well-tolerated, and cognitive benefits were found during follow-up, especially in children treated at young age (ISRCTN19853672)[45]. Intravenous gene therapy has been developed for MPS IIIA and MPS IIIB, and phase I/II studies have recently been started (clinicaltrials.gov Identifier: NCT02716246; http://abeonatherapeutics.com/research-and-development/).

Enzyme replacement therapy

Several clinical studies have assessed or are currently assessing the use of enzyme replacement therapy (ERT) for the different MPS III subtypes, either by an intravenous, intrathecal or intracerebroventricular approach. To circumvent the BBB, ERT has been developed that could be administered directly to the CNS via an intrathecal drug delivery device. Over the past year this approach has been studied for MPS IIIA and expectations were high. Disappointingly, recent outcomes of the phase II study showed insufficient effect on neurocognitive decline (clinicaltrials.gov identifier: NCT02060526). Although, clinical trials on intravenous ERT for MPS IIIB are currently ongoing (clinicaltrials.gov identifier: NCT02324049; clinicaltrials.gov identifier: NCT02618512), the further development of this program was recently stopped by the involved pharmaceutical company. Unfortunately, no reasons were provided. Another trial studies ERT for MPS IIIB dosed via an intracerebroventricular device (clinicaltrials.gov identifier: NCT02754076). No ERT has yet been developed for the other MPS III subtypes. Since the underlying defect in MPS IIIC comprises a membrane bound lysosomal enzyme and ERT is most suitable for replacing soluble enzymes, MPS IIIC is not an optimal candidate for ERT.

Hematopoietic stem cell transplantation

The rationale behind the use of hematopoietic stem cell transplantation (HSCT) for the treatment of the MPSs is based on the principle of cross-correction: donor derived HSCs with normal enzyme activity can cross the BBB, where they transform into microglia cells and secrete lysosomal enzymes to the cells in their surroundings, where it can be taken up via receptor mediated uptake into the deficient cells [46]. In the severe phenotype of MPS I (MPS I-Hurler) HSCT is the treatment of choice [47–52]. So far, HSCT has shown no substantial effect in MPS III patients [53–56]. This is probably due to the fact that in case of MPS III insufficient enzyme is produced by donor derived microglia in the brain [57]. Therefore, new forms of HSCT have been developed for MPS III that make use of lentiviral gene therapy resulting in higher enzyme expression (*ex-vivo* autologous gene therapy) [58]. For MPS IIIA a clinical trial is on its way (www.orchard-tx.com/programmes/#mps-iiia).

Small molecules and chaperone therapy

Due to their low-molecular weight and, at least theoretical, potential to cross the BBB, small molecules functioning as chemical or pharmacological chaperones are of interest as potential treatment option for MPS III patients, especially for those with an SP phenotype. Chaperones are compounds that bind and stabilize mutant proteins and thereby increase residual enzyme activity. This approach has shown promising results in MPS IIIC cell models [59] and chaperone therapy is currently investigated in clinical trials for several other LSDs [60–62]. Recently, the pharmacological chaperone Migalastat was granted marketing approval in the US and Europe for treatment of Fabry disease [63,64].

Based on our findings that residual enzyme activity in fibroblasts of SP MPS IIIB patients could be significantly increased by culturing at 30°C, we hypothesized that residual enzyme activity could be enhanced via chaperone treatment. Therefore we assessed the effect of 1302 different molecules, including the 1280 molecules of the Prestwick Chemical Library, on residual enzyme activity in an MPS IIIB fibroblast cell line in which enzyme activity increased significantly upon culturing at 30°C (**chapter 6**). None of the compounds showed to have any beneficial effect on NAGLU activity. This might be due to fact the beneficial effect of mild cold shock on residual NAGLU activity is a multifactorial process that is much more complex than was generally assumed and involves protein processing, folding and trafficking (**chapter 4**).

More studies on the genetic basis of these processes are needed, as well as on the confirmation and stability of the enzymes involved in MPS III and the effect that different mutations have on these processes [65]. Many missense mutations have been reported for the different MPS III subtype, but it remains uncertain to which extend these result in alterations in protein confirmation, although studies on a bacterial homologue of NAGLU [66] and the results described in **chapter 4** point in that direction. Computational analyses could help to unravel the effect of these mutations on protein confirmation and folding, which could also allow for the discovery of new treatment approaches. Until then chaperone therapy appears to be a suboptimal approach for the treatment of MPS III(B) and other treatment modalities should be considered.

Transcription factor EB as therapeutic target

Finally, an interesting approach that is currently studied in *in vitro* models for several LSDs, is the induction of transcription factor EB (TFEB) [65]. As was previously mentioned, TFEB regulates the expression of genes involved in lysosomal biogenesis and function as well as genes responsible for autophagy induced cellular clearance. TFEB overexpression in glia differentiated neuronal stem cells derived from MPS IIIA mice resulted in higher clearance of accumulating GAGs and the restoration of cell morphology [67]. In fibroblasts of Gaucher disease patients carrying missense mutations, sucrose (saccharose) mediated TFEB induction resulted in higher expression of the gene encoding for glucocerebrosidase and those involved in protein folding and lysosomal trafficking, resulting in increased levels of mutant enzyme, improved processing and overall higher activity levels [68]. Sucrose was included in the compound screen described in **chapter 6**, but did not have any effect on NAGLU activity. However, other compounds such as hydroxypropyl-β- cyclodextrin have shown to interfere with TFEB and to be able to reduce lysosomal storage [69]. This compound has already been studied in a clinical trial for the LSD Niemann Pick C [70]. Therefore, genetic and chemical stimulation of TFEB as a therapeutic target for MPS III should definitely be further explored.

Substrate reduction therapy

Substrate reduction therapy (SRT) aims to reduce HS accumulation by reducing GAG synthesis. A compound extensively studied for this purpose is the isoflavone genistein [71]. So far, findings of these studies are contradictory. Whereas *in vitro* studies in MPS IIIA and MPS IIIB fibroblasts were promising and animal studies found lower GAG levels and normalization of behavior in MPS IIIB mice treated with high dose genistein (160 mg/kg/day)[72], a double blind placebo controlled cross-over trial did not detect any clinical effects in MPS III patients treated with genistein at 10 mg/kg/day [73–75]. However, oral bioavailability in humans is low, which makes it considerably more difficult to dose genistein within a therapeutic range in patients[76]. Therefore the final results of a phase III study on the efficacy of high dose oral genistein in patients with different MPS subtypes that has recently been completed, are expected with much interest (EudraCT number: 2013-001479-18).

FUTURE PERSPECTIVES

Unfolding the pathophysiology of MPS III, including its diagnosis and potential treatment options, reveals, once more, how tremendously complex the underlying disease mechanisms are. It is by studying inborn errors of metabolism such as MPS III that we actually understand how ingenious the cellular metabolic machinery in the human body works. From a researcher's perspective this is fascinating. From the perspective of a patient and his or her family it may be experienced as a verdict, implying a life determined by, in case of MPS III, an invariably progressive and devastating disease. Bringing those two perspectives together and ensuring that research serves the patient as well as the community, remains one of the biggest challenges in de field of rare diseases and requires the collaboration of patient advocacy groups, medical doctors, researchers and funders.

The focus of future research in MPS III should be in line with the subjects addressed in this thesis: pathophysiology, diagnosis and treatment. On top of the agenda will remain the validation and implementation of a treatment for MPS III that can successfully treat the CNS. Based on recent advances in this field, gene therapy, potentially in a combined strategy with autologous HSCT, may well have the greatest chance of succeeding, especially when taking into account the disappointing outcomes of the phase II/III study on intrathecal ERT for MPS IIIA (clinicaltrials.gov identifier: NCT02060526). Although there are considerable risks and long-term side effects are still unknown, one of the great advantages of gene therapy is that, if successful, only one single treatment is needed. However, since MPS III is a progressive multisystem disease, long term follow-up is needed to determine whether brain directed gene therapy sufficiently treats other organs than the CNS. This is also an issue in

MPS I-Hurler patients for whom HSCT has demonstrated to successfully treat neurological disease by halting disease progression, but remains unable to sufficiently ameliorate bone disease, which still has a great impact on the quality of life of these patients [30,77]. For MPS III the success of gene therapy will probably depend on the route of administration and will not only require a intracerebral or intrathecal, but also a systemic approach. Moreover, a multimodal treatment approach will most likely be needed in which ERT, TFEB inducers, small molecules and SRT may all play a role for enhancing residual enzyme activity and reducing substrate accumulation.

To shorten the diagnostic odyssey and, in the future, allow for the best treatment outcome, early diagnosis is essential. As long as there is no treatment, MPS III is unsuitable for implementation in the (Dutch) NBS program. Therefore, better and early targeted screening for all children presenting with a developmental delay is needed. Only by this means, it can be achieved that children newly diagnosed with MPS III receive optimal supportive care and therapies. Moreover, it will give them the opportunity to be included in clinical trials that often handle strict age-related inclusion criteria. Early access to an investigational drug may benefit the patient and being able to include patients at a young age before neurological decline has set in, will highly strengthen the quality of these trials.
In addition to early targeted screening and future newborn population screening, preconception screening offers a different approach that aims for assessing carrier status of future parents and determining the risk of conceiving a child with an inheritable, autosomal recessive disease or X-linked disease. Preconception screening is already offered in high risks populations, but is slowly emerging to be implemented on a community-based scale [78]. Considering its severe, neurodegenerative course and the fact that there is still no treatment, MPS III would be a suitable candidate to be included in preconception screening programs. Naturally, these kind of interventions are subject to various ethical considerations. Therefore, the experiences of patients and their parents and caregivers are of high importance. Eventually, they are the experts on what it means to raise and take of care a child with a devastating and invariably deathly disease and how it affects the quality of life of their child and family. A community based study on preconception screening including MPS III is currently performed under the unaffected population and parents and healthy siblings of MPS III patients. These results will further fuel the debate on this topic.

For overall disease understanding and counseling of parents, as well as the assessment of the effect of experimental therapies for MPS III in clinical trials and the discussion on screening programs (by whatever means), knowledge on the natural history of MPS III and its clinical spectrum is of high importance. Although the natural course MPS III has been described extensively in a more narrative manner, prospective longitudinal data are relatively scarce

and often restricted to a limited period of time [33]. Therefore, international and independent, long-term longitudinal disease registries for MPS III are desirable.
A profound insight in the natural history of MPS III, will also allow for a consensus on the relevant cognitive measures as endpoints for clinical trials. For a neurodegenerative disease such as MPS III it is extremely difficult to determine when a therapy is considered to be effective. In case of slowing of disease progression, stabilization, or only in case of reversal of decline[79] ? Recently, the first consensus recommendations on this matter were published as well as on the preferred measuring instruments [79,80]. To move clinical research forward and closer towards a therapy for MPS III an open and systematic collaboration is needed by all stakeholders including patient groups, researchers, medical doctors and the industry, which should also take into account the considerations of those parties that will eventually decide on marketing approval and reimbursement of these new therapies such as the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) as well as health insurers. For other orphan diseases it has happened that, despite marketing approval granted by the regulating authorities, the 'payers' (e.g. health insurance companies or national regulating committees) subsequently declined to reimburse the newly approved drug due to a lack of what they considered to be clinically relevant outcome measures in combination with the often (extremely) high costs of these therapies reaching up to €200.000-500.000, or even in excess of € 1 million, per patient per year.

In fundamental or translational research, research findings often "happen while being busy making other plans". It was by that means that during the process of this thesis the search for small molecules that could improve protein folding of NAGLU, eventually resulted in a robust method for phenotype prediction in MPS IIIB and later also in MPS IIIA. Although the initial outcomes of this high-throughput screen seemed disappointing, they led to new hypotheses essential for unravelling the pathophysiology of MPS III as well as for the development of new therapies and the determination of treatment effects. Therefore, fundamental research in MPS III remains essential and should focus on further unravelling the functions of the lysosome, the synthesis and processing of lysosomal enzymes affected in MPS III and the (genetic) factors by which these processes are influenced. Recent discoveries in the field of genome editing including CRISPR-cas9 may dramatically change the field of fundamental and translational research in the near future [81].

In conclusion, the questions unanswered remain numerous and the work to be done extensive, and that for a disease that is called rare or even ultra-rare. For some this may lead to raising the question: why put in so much effort?
All patients deserve our greatest efforts and disease prevalence should never determine for which diseases a treatment should be developed. MPS III is a devastating neurodegenerative

disorder that severely affects the quality of life of patients and their families. Altogether, all those rare disease are actually not that rare and have a great impact on our society. Eventually, we can only unravel the miraculous and fascinating mechanisms that are captured in the human body by studying this kind of rare conditions and finding a disease-modifying treatment for one these disorders may open new windows for many others.

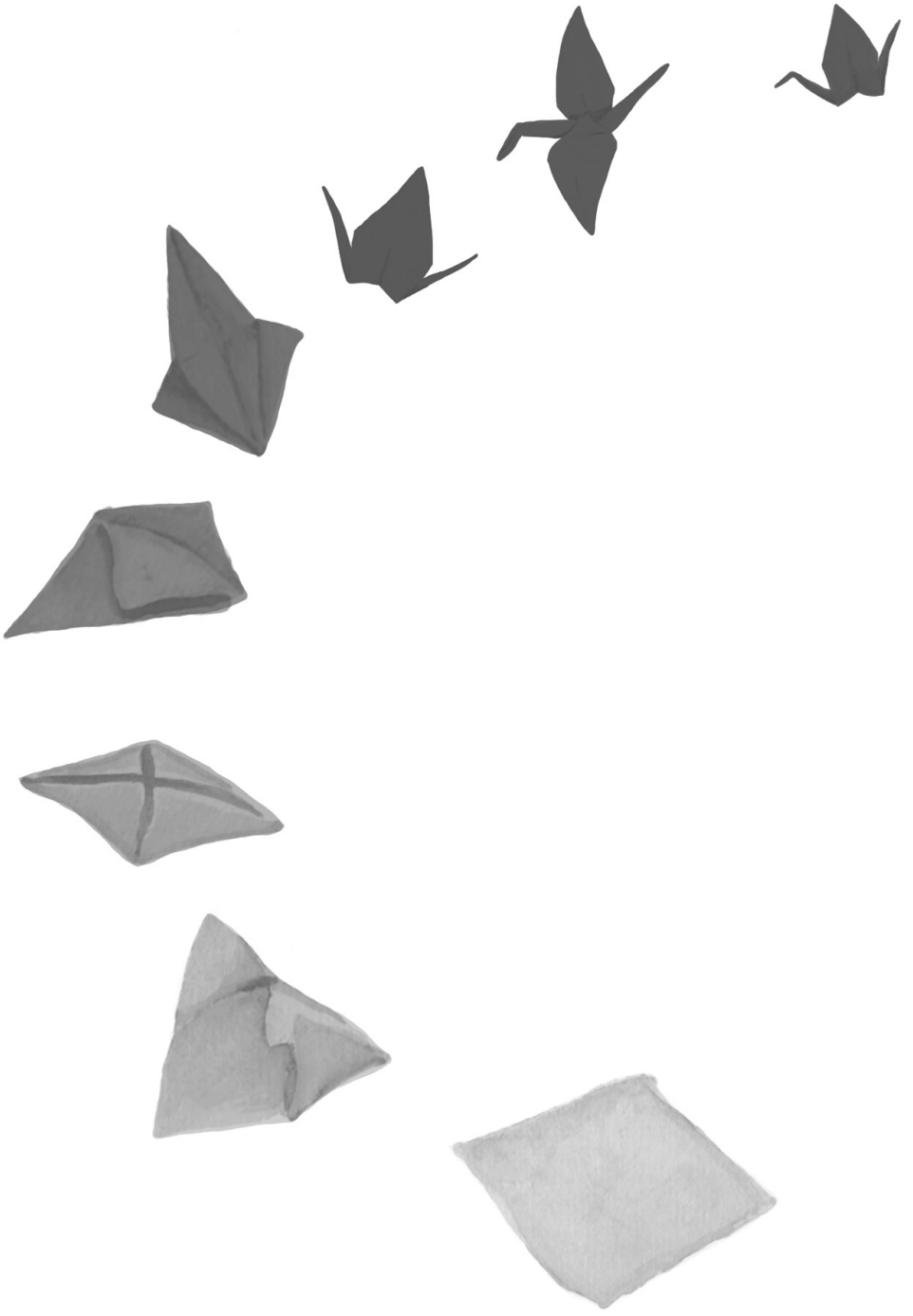
REFERENCES

1. Leinekugel P, Michel S, Conzelmann E, Sandhoff K. Quantitative correlation between the residual activity of β-hexosaminidase A and arylsulfatase A and the severity of the resulting lysosomal storage disease. *Hum Genet*. 1992;88(5):513-523.
2. Schueler UH, Kolter T, Kaneski CR, Zirzow GC, Sandhoff K, Brady RO. Correlation between enzyme activity and substrate storage in a cell culture model system for Gaucher disease. *J Inherit Metab Dis*. 2004;27(5):649-658.
3. Knottnerus SJG, Nijmeijer SCM, IJlst L, Te Brinke H, van Vlies N, Wijburg FA. Prediction of phenotypic severity in mucopolysaccharidosis type IIIA. *Ann Neurol*. 2017 [Epub ahead of print]
4. Gootjes J, Schmohl F, Mooijer PA, et al. Identification of the molecular defect in patients with peroxisomal mosaicism using a novel method involving culturing of cells at 40°C: Implications for other inborn errors of metabolism. *Hum Mutat*. 2004;24(2):130-139.
5. Diekman EF, Ferdinandusse S, van der Pol L, et al. Fatty acid oxidation flux predicts the clinical severity of VLCAD deficiency. *Genet Med*. 2015;17(12):989-994.
6. Houten SM, Frenkel J, Rijkers GT, Wanders RJ a, Kuis W, Waterham HR. Temperature dependence of mutant mevalonate kinase activity as a pathogenic factor in hyper-IgD and periodic fever syndrome. *Hum Mol Genet*. 2002;11(25):3115-3124.
7. Al-Fageeh MB, Smales CM. Control and regulation of the cellular responses to cold shock: the responses in yeast and mammalian systems. *Biochem J*. 2006;397(2):247-259.
8. Roobol A, Carden MJ, Newsam RJ, Smales CM. Biochemical insights into the mechanisms central to the response of mammalian cells to cold stress and subsequent rewarming. *FEBS J*. 2009;276(1):286-302.
9. Fujita J. Cold shock response in mammalian cells. *J Mol Microbiol Biotechnol*. 1999;1(2):243-255.
10. Dresios J, Aschrafi A, Owens GC, Vanderklish PW, Edelman GM, Mauro VP. Cold stress-induced protein Rbm3 binds 60S ribosomal subunits, alters microRNA levels, and enhances global protein synthesis. *Proc Natl Acad Sci USA*. 2005;102(6):1865-1870.
11. Sardiello M, Palmieri M, di Ronza A, et al. A gene network regulating lysosomal biogenesis and function. *Science*. 2009;11(11):4607-4611.
12. Mauri V, Lotfi P, Segatori L, Sardiello M. A rapid and sensitive method for measuring N-acetylglucosaminidase activity in cultured cells. *PLoS One*. 2013;8(6):1-9.
13. Settembre C, Fraldi A, Jahreiss L, et al. A block of autophagy in lysosomal storage disorders. *Hum Mol Genet*. 2008;17(1):119-129.
14. Yogalingam G, Hopwood JJ. Molecular genetics of mucopolysaccharidosis type IIIA and IIIB: Diagnostic, clinical, and biological implications. *Hum Mutat*. 2001;18(4):264-281.
15. Valstar MJ, Neijs S, Bruggenwirth HT, et al. Mucopolysaccharidosis type IIIA: clinical spectrum and genotype-phenotype correlations. *Ann Neurol*. 2010;68(6):876-887.
16. Bishop JR, Schuksz M, Esko JD. Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature*. 2007;446(7139):1030-1037.
17. Sarrazin S, Lamanna WC, Esko JD. Heparan sulfate proteoglycans. *Cold Spring Harb Perspect Biol*. 2011;3(7):1-33.
18. Xu D, Esko JD. Demystifying heparan sulfate-protein interactions. *Annu Rev Biochem*. 2014;83:129-157.
19. Wilkinson FL, Holley RJ, Langford-Smith KJ, et al. Neuropathology in mouse models of mucopolysaccharidosis type I, IIIA and IIIB. *PLoS One*. 2012;7(4):e35787.
20. Bruyère J, Roy E, Ausseil J, et al. Heparan sulfate saccharides modify focal adhesions: Implication in mucopolysaccharidosis neuropathophysiology. *J Mol Biol*. 2015;427(4):775-791.
21. Dwyer CA, Scudder SL, Lin Y, et al. Neurodevelopmental changes in excitatory synaptic structure and function in the cerebral cortex of Sanfilippo syndrome IIIA mice. *Sci Rep*. 2017;7:46576.
22. https://ec.europa.eu/health/rare_diseases/policy.

23. http://www.eurordis.org/sites/default/files/publications/Fact_Sheet_RD.pdf.
24. Langereis EJ, Wagemans T, Kulik W, et al. A multiplex assay for the diagnosis of mucopolysaccharidoses and mucolipidoses. *PLoS One*. 2015;10(9):1-13.
25. de Ruijter J, de Ru MH, Wagemans T, et al. Heparan sulfate and dermatan sulfate derived disaccharides are sensitive markers for newborn screening for mucopolysaccharidoses types I, II and III. *Mol Genet Metab*. 2012; 107(4):705-710.
26. Lisi EC, Gillespie S, Laney D, Ali N. Patients' perspectives on newborn screening for later-onset lysosomal storage diseases. *Mol Genet Metab*. 2016;119(1):109-114.
27. de Ru MH, Bouwman MG, Wijburg FA, van Zwieten MCB. Experiences of parents and patients with the timing of Mucopolysaccharidosis type I (MPS I) diagnoses and its relevance to the ethical debate on newborn screening. *Mol Genet Metab*. 2012;107(3):501-507.
28. Bruni S, Lavery C, Broomfield A. The diagnostic journey of patients with mucopolysaccharidosis I: A real-world survey of patient and physician experiences. *Mol Genet Metab Reports*. 2016;8:67-73.
29. Zurynski Y, Deverell M, Dalkeith T, et al. Australian children living with rare diseases: experiences of diagnosis and perceived consequences of diagnostic delays. *Orphanet J Rare Dis*. 2017;12(1):68.
30. Aldenhoven M, Wynn RF, Orchard PJ, et al. Long-term outcome of Hurler syndrome patients after hematopoietic cell transplantation: an international multi-center study. *Blood*. 2015;125(13):2164-2172.
31. Gabrielli O, Clarke LA, Bruni S, Coppa G V. Enzyme-replacement therapy in a 5-month-old boy with attenuated presymptomatic MPS I: 5-year follow-up. *Pediatrics*. 2010;125(1):e183-7.
32. Laraway S, Breen C, Mercer J, Jones S, Wraith JE. Does early use of enzyme replacement therapy alter the natural history of mucopolysaccharidosis I? Experience in three siblings. *Mol Genet Metab*. 2013;109(3):315-316.
33. Shapiro EG, Nestrasil I, Delaney KA, et al. A Prospective Natural History Study of Mucopolysaccharidosis Type IIIA. *J Pediatr*. 2016;170:278-287e4.
34. Bouwman MG, de Ru MH, Linthorst GE, Hollak CEM, Wijburg FA, van Zwieten MCB. Fabry patients' experiences with the timing of diagnosis relevant for the discussion on newborn screening. *Mol Genet Metab*. 2013;109(2):201-207.
35. Wilson J, Jungner G. Principles and practice of screening for disease. *Public Health Pap*. 1968;(34). http://whqlibdoc.who.int/php/WHO_PHP_34.pdf.
36. Ruijter GJ, Valstar MJ, van de Kamp JM, et al. Clinical and genetic spectrum of Sanfilippo type C (MPS IIIC) disease in The Netherlands. *Mol Genet Metab*. 2008;93(2):104-111.
37. Moog U, van Mierlo I, van Schrojenstein Lantman-de Valk HM, Spaapen L, Maaskant MA, Curfs LM. Is Sanfilippo type B in your mind when you see adults with mental retardation and behavioral problems? *Am J Med Genet C Semin Med Genet*. 2007;145C(3):293-301.
38. Valstar MJ, Bruggenwirth HT, Olmer R, et al. Mucopolysaccharidosis type IIIB may predominantly present with an attenuated clinical phenotype. *J Inherit Metab Dis*. 2010;33(6):759-767.
39. Di Natale P, Balzano N, Esposito S, Villani GRD. Identification of molecular defects in Italian Sanfilippo a patients including 13 novel mutations. *Hum Mutat*. 1998;11(4):313-320.
40. Muschol N, Storch S, Ballhausen D, et al. Transport, enzymatic activity, and stability of mutant sulfamidase (SGSH) identified in patients with mucopolysaccharidosis type III A. *Hum Mutat*. 2004;23(6):559-566.
41. Muschol N, Pohl S, Meyer A, Gal A, Ullrich K, Braulke T. Residual activity and proteasomal degradation of p.Ser298Pro sulfamidase identified in patients with a mild clinical phenotype of Sanfilippo A syndrome. *Am J Med Genet A*. 2011;155A(7):1634-1639.
42. Griggs RC, Batshaw M, Dunkle M, et al. Clinical research for rare disease: Opportunities, challenges, and solutions. *Mol Genet Metab*. 2009;96(1):20-26.

43. van Gelder CM, Vollebregt AAM, Plug I, van der Ploeg AT, Reuser AJJ. Treatment options for lysosomal storage disorders: developing insights. *Expert Opin Pharmacother*. 2012;13(16):2281-2299.
44. Tardieu M, Zérah M, Husson B, et al. Intracerebral Administration of Adeno-Associated Viral Vector Serotype rh.10 Carrying Human SGSH and SUMF1 cDNAs in Children with Mucopolysaccharidosis Type IIIA Disease: Results of a Phase I/II Trial. *Hum Gen Ther*. 2014;25(6):506-516.
45. Tardieu M, Zérah M, Gougeon M-L, et al. Intracerebral gene therapy in children with mucopolysaccharidosis type IIIB syndrome: an uncontrolled phase 1/2 clinical trial. *Lancet Neurol*. 2017;4422(17):1-9.
46. Krivit W, Sung JH, Shapiro EG, Lockman LA. Microglia: the effector cell for reconstitution of the central nervous system following bone marrow transplantation for lysosomal and peroxisomal storage diseases. *Cell Transpl*. 1995;4(4):385-392.
47. Hobbs JR, Hugh-Jones K, Barrett AJ, et al. Reversal of clinical features of Hurler's disease and biochemical improvement after treatment by bone-marrow transplantation. *Lancet*. 1981;2(8249):709-712.
48. Peters C, Shapiro EG, Anderson J, et al. Hurler syndrome: II. Outcome of HLA-genotypically identical sibling and HLA-haploidentical related donor bone marrow transplantation in fifty-four children. The Storage Disease Collaborative Study Group. *Blood*. 1998;91(7):2601-2608.
49. Souillet G, Guffon N, Maire I, et al. Outcome of 27 patients with Hurler's syndrome transplanted from either related or unrelated haematopoietic stem cell sources. *Bone Marrow Transpl*. 2003;31(12):1105-1117.
50. Guffon N, Souillet G, Maire I, Straczek J, Guibaud P. Follow-up of nine patients with Hurler syndrome after bone marrow transplantation. *J Pediatr*. 1998;133(1):119-125.
51. Malm G, Gustafsson B, Berglund G, et al. Outcome in six children with mucopolysaccharidosis type IH, Hurler syndrome, after haematopoietic stem cell transplantation (HSCT). *Acta Paediatr*. 2008;97(8):1108-1112.
52. Boelens JJ, Aldenhoven M, Purtill D, et al. Outcomes of transplantation using various hematopoietic cell sources in children with Hurler syndrome after myeloablative conditioning. *Blood*. 2013;121(19):3981-3987.
53. Vellodi A, Young E, New M, Pot-Mees C, Hugh-Jones K. Bone marrow transplantation for Sanfilippo disease type B. *J Inherit Metab Dis*. 1992;15(6):911-918.
54. Güngör N, Tunçbilek E. Sanfilippo disease type B. A case report and review of the literature on recent advances in bone marrow transplantation. *Turk J Pediatr*. 1995;37(2):157-163.
55. Shapiro EG, Lockman LA, Balthazor M, Krivit W. Neuropsychological outcomes of several storage diseases with and without bone marrow transplantation. *J Inherit Metab Dis*. 1995;18(4):413-429.
56. Sivakumur P, Wraith JE. Bone marrow transplantation in mucopolysaccharidosis type IIIA: a comparison of an early treated patient with his untreated sibling. *J Inherit Metab Dis*. 1999;22(7):849-850.
57. Langford-Smith A, Wilkinson FL, Langford-Smith KJ, et al. Hematopoietic stem cell and gene therapy corrects primary neuropathology and behavior in mucopolysaccharidosis IIIA mice. *Mol Ther*. 2012;20(8):1610-1621.
58. Sergijenko A, Langford-Smith A, Liao AY, et al. Myeloid/Microglial driven autologous hematopoietic stem cell gene therapy corrects a neuronopathic lysosomal disease. *Mol Ther*. 2013;21(10):1938-1949.
59. Feldhammer M, Durand S, Pshezhetsky A V. Protein misfolding as an underlying molecular defect in mucopolysaccharidosis III type C. *PLoS One*. 2009;4(10):e7434.
60. Bernier V, Lagacé M, Bichet DG, Bouvier M. Pharmacological chaperones: potential treatment for conformational diseases. *Trends Endocrinol Metab*. 2004;15(5):222-228.
61. Parenti G. Treating lysosomal storage diseases with pharmacological chaperones: from concept to clinics. *EMBO Mol Med*. 2009;1(5):268-279.
62. Hollak C, Wijburg F. Treatment of lysosomal storage disorders: Successes and challenges. *J Inherit Metab Dis*. 2014;37(4):587-598.

63. Germain DP, Hughes DA, Nicholls K, et al. Treatment of Fabry's disease with the pharmacologic chaperone Migalastat. *N Engl J Med*. 2016;375(6):545-555.
64. Hughes DA, Nicholls K, Shankar SP, et al. Oral pharmacological chaperone migalastat compared with enzyme replacement therapy in Fabry disease: 18-month results from the randomised phase III ATTRACT study. *J Med Genet*. 2017;54(4):288-296.
65. Matalonga L, Gort L, Ribes A. Small molecules as therapeutic agents for inborn errors of metabolism. *J Inherit Metab Dis*. 2017;40(2):177-193.
66. Ficko-Blean E, Stubbs KA, Nemirovsky O, Vocadlo DJ, Boraston AB. Structural and mechanistic insight into the basis of mucopolysaccharidosis IIIB. *Proc Natl Acad Sci USA*. 2008;105(18):6560-6565.
67. Medina DL, Fraldi A, Bouche V, et al. Transcriptional activation of lysosomal exocytosis promotes cellular clearance. *Dev Cell*. 2011;21(3):421-430.
68. Song W, Wang F, Savini M, et al. TFEB regulates lysosomal proteostasis. *Hum Mol Genet*. 2013;22(10):1994-2009.
69. Song W, Wang F, Lotfi P, Sardiello M, Segatori L. 2-Hydroxypropyl-β-cyclodextrin promotes transcription factor EB-mediated activation of autophagy: implications for therapy. *J Biol Chem*. 2014;289(14):10211-10222.
70. Ory DS, Ottinger EA, Farhat NY, et al. Intrathecal 2-hydroxypropyl-β-cyclodextrin decreases neurological disease progression in Niemann-Pick disease, type C1: a non-randomised, open-label, phase 1-2 trial. *Lancet*. 2017 [Epub ahead of print]
71. Wegrzyn G, Jakóbkiewicz-Banecka J, Gabig-Cimińska M, et al. Genistein: a natural isoflavone with a potential for treatment of genetic diseases. *Biochem Soc Trans*. 2010;38(2):695-701.
72. Malinowska M, Wilkinson FL, Langford-Smith KJ, et al. Genistein improves neuropathology and corrects behaviour in a mouse model of neurodegenerative metabolic disease. *PLoS One*. 2010;5(12):e14192.
73. Piotrowska E, Jakóbkiewicz-Banecka J, Tylki-Szymanska A, et al. Genistin-rich soy isoflavone extract in substrate reduction therapy for Sanfilippo syndrome: an open-label, pilot study in 10 pediatric patients. *Curr Ther Res Clin Exp*. 2008;69(2):166-179.
74. Piotrowska E, Jakobkiewicz-Banecka J, Maryniak A, et al. Two-year follow-up of Sanfilippo Disease patients treated with a genistein-rich isoflavone extract: assessment of effects on cognitive functions and general status of patients. *Med Sci Monit*. 2011;17(4):CR196-202.
75. de Ruijter J, Valstar MJ, Narajczyk M, et al. Genistein in Sanfilippo disease: a randomized controlled crossover trial. *Ann Neurol*. 2012;71(1):110-120.
76. Yang Z, Kulkarni K, Zhu W, Hu M. Bioavailability and pharmacokinetics of genistein: mechanistic studies on its ADME. *Anticancer Agents Med Chem*. 2012;12(10):1264-1280.
77. Langereis EJ, Os MM Den, Breen C, et al. Progression of hip dysplasia in mucopolysaccharidosis type i hurler after successful hematopoietic stem cell transplantation. *J Bone Joint Surg Am*. 2016;98(5):386-95.
78. Henneman L, Borry P, Chokoshvili D, et al. Responsible implementation of expanded carrier screening. *Eur J Hum Genet*. 2016;24(6):e1-e12.
79. Ghosh A, Shapiro E, Rust S, et al. Recommendations on clinical trial design for treatment of Mucopolysaccharidosis Type III. *Orphanet J Rare Dis*. 2017;12(1):117.
80. van der Lee JH, Morton J, Adams HR, et al. Cognitive endpoints for therapy development for neuronopathic mucopolysaccharidoses: Results of a consensus procedure. *Mol Genet Metab*. 2017;121(2):70-79.
81. Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. *Science*. 2014;346(6213):1258096-1258096.



Chapter 9

(Dutch) summary

SUMMMARY

Mucopolysaccharidosis type III (MPS III), also known as Sanfilippo disease, is a rare autosomal recessive lysosomal storage disease (LSD) and is caused by the deficiency of one of the lysosomal enzymes responsible for the degradation of the glycosaminoglycan heparan sulfate (HS). Based on the enzyme involved, four subtypes are recognized that are clinically indistinguishable: MPS IIIA (heparan *N*-sulfatase (SGSH)), MPS IIIB (*N*-acetyl-α-glucosaminidase (NAGLU)), MPS IIIC (acetyl CoA:α-glucosaminide *N*-acetyltransferase (HGSNAT)) and MPS IIID (*N*-acetylglucosamine 6-sulfatase (GNS)). MPS III belongs to the group of seven mucopolysaccharidoses in which the deficiency of different lysosomal enzymes all result in impaired breakdown of particular glycosaminoglycans.
In case of MPS III, the impaired degradation and subsequent storage of HS, triggers a complex pathophysiological cascade that most severely affects the central nervous system (CNS). Consequently, patients generally present between the age of 1 and 4 years with a developmental delay that initially primarily affects speech and language development. After reaching a maximum level of development a gradual neurocognitive decline sets in that is often accompanied by severe behavioral problems, seizures and sleep disturbances. The disease spectrum comprises a continuum, but in general two groups can be distinguished: patients with a rapidly progressing (RP) phenotype and those with a slowly progressing (SP) phenotype. RP patients suffer from a rapid neurocognitive decline and often decease in their (late) teenage years or early twenties. In contrary, patients with an SP phenotype may show a stable neurocognitive impairment for years and in exceptional cases survival into the seventh decade has been reported.

There is yet no disease-modifying therapy available for MPS III. As part of the introduction of this thesis, **chapter 2** presents more background information on the pathophysiological processes involved in MPS III and on potential therapies that may modulate the course of disease by interfering on different levels of the pathophysiological cascade. First of all on the level of the genetic defect by gene therapy, potentially in a combined strategy with hematopoietic stem cell transplantation. Replacing the missing or defective enzyme by enzyme replacement therapy, provides another and logical strategy that warrants further investigation, especially since it has proven to be successful for the treatment of other LSDs. Another and still more experimental strategy, that is also further discussed in **chapter 6,** could be the use of chaperone therapy that aims for the enhancement of residual enzyme activity of the mutant enzyme. Since the CNS is predominantly affected in MPS III, overcoming the blood brain barrier provides the biggest challenge for a treatment to be successful. Nevertheless, the recent advances in this field are promising, especially those involving gene therapy.

To come closer towards a successful treatment for MPS III, a better understanding of the underlying disease mechanisms is needed. The first part of this thesis therefore focusses on the pathophysiology of MPS III. As was described above, a spectrum of disease severity is recognized in MPS III that comprises patients with an RP phenotype and patients with an SP phenotype. For MPS IIIA, there are rather good genotype-phenotype correlations. Due to a large allelic heterogeneity and the frequent occurrence of new mutations, establishing genotype-phenotype correlations for MPS IIIB is difficult. However, for the assessment of the efficacy of potential new therapies, a method able to reliably predict the natural course of the disease is necessary. In **chapter 3** it is shown that enzyme activity after culturing patient fibroblasts at 30°C can reliably discriminate RP from SP MPS IIIB patients. Enzyme activity in RP MPS IIIB patients remains undetectably low under all culturing conditions. In SP patients, culturing at 30°C results in a pronounced increase in NAGLU activity levels, up to 10% of controls which is generally assumed to be sufficient for symptom alleviation. In addition, lower HS levels are found indicating that the higher NAGLU levels measured *in vitro*, comprise a form of the enzyme that is also intracellularly active. Moreover, there seems a correlation between the capacity of patients' fibroblasts to increase NAGLU activity at 30°C and the time that patients lose specific functions such as the ability to speak and to walk. Thus, culturing fibroblasts at low culture temperatures provides a robust and reliable method to discriminate between RP and SP MPS IIIB patients and can be used for the assessment of treatment effects as well as for counseling of parents of newly diagnosed MPS IIIB patients. Moreover, mimicking the effect of mild cold shock could be an interesting new target for the treatment of SP MPS IIIB patients.
Therefore, the mechanisms underlying this phenomenon are further studied in **chapter 4.** Fibroblast studies show that in healthy controls, NAGLU is present as a precursor and a mature protein. In fibroblasts of RP patients no NAGLU protein is detected under either 37°C or 30°C culture conditions. In SP patients' fibroblasts cultured at 37°C, only the precursor of NAGLU is present. Culturing at lower temperatures results in higher NAGLU mRNA levels, increased levels of the precursor and mature form of NAGLU and improved protein processing. The formation of mature NAGLU corresponds with higher NAGLU activity levels. Increasing the levels of NAGLU precursor in SP MPS IIIB fibroblasts cultured at 37°C via proteasome inhibition, does not result in better processing or higher levels of the mature and enzymatically active form of NAGLU. This indicates that the beneficial effects of culturing at low temperatures result from a multifactorial process.

Heparan sulfate is the main accumulating glycosaminoglycan in MPS III, but its degradation is also impaired in several other MPSs, including MPS I. As in MPS III, MPS I is characterized by a spectrum of disease severity ranging from a severe phenotype (MPS I-Hurler) to a more attenuated phenotype (MPS I-Scheie), and also comprises an intermediate phenotype

(MPS I-Hurler/Scheie). In both MPS I and MPS III the CNS is involved, although remarkable differences in the type of symptoms are seen between the two MPSs and between MPS I and MPS III patients with different severity of disease. In **chapter 5** it is assessed whether total HS levels and sulfation pattern of the accumulating HS derived disaccharides in plasma correlate with differences in disease severity observed between the MPS subtypes and between MPS I and MPS III patients with different disease severity. As is shown in previous studies, total HS levels are higher in plasma of RP MPS III patients compared to SP patients. In addition, a higher degree of sulfation of the HS derived disaccharide fractions is found in RP MPS III patients. A correlation between total HS levels and disease severity is also seen in plasma of MPS I patients. Moreover, fractions of HS derived disaccharides in plasma differ between MPS I and MPS III. As sulfation pattern to a great extend determines HS function, differences in incompletely degraded HS molecules may relate to differences in CNS phenotype observed between the two MPSs.

The second part of this thesis concentrates on the treatment of MPS III. Based on the findings in chapter 3 and chapter 4, **chapter 6** studies the effect of a large amount of compounds for their ability to enhance residual enzyme activity in an SP MPSIIIB patient fibroblast cell line that responds favorably to culturing at low temperature. The compounds tested include several types of chaperones that influence protein folding and stability and have shown to have beneficial effects on other protein folding diseases including LSDs. In addition, the 1280 approved compounds from the Prestwick Chemical Library are studied. To study the selected molecules for their potential to increase NAGLU activity, first a robust high-throughput assay for the measurement of NAGLU activity is developed and validated. Nonetheless, none of the 1302 tested compounds is able to therapeutically simulate the positive effects of lower culture temperatures and to increase NAGLU activity.

The third part of this thesis addresses the diagnosis of MPS III. Rare diseases such as MPS III often remain undiagnosed, or even misdiagnosed, for a long period of time. This delay and the diagnostic odyssey by which the diagnosis is often preceded, may further increase the disease burden experienced by patients and their parents or caregivers. An early diagnosis is particularly important if a disease-modifying treatment is available, and, as long that is not the case, to ensure the initiation of supportive therapies, to allow for the inclusion in clinical trials and for genetic counseling of parents. Therefore, over the past years multiple initiatives have been launched to increase awareness on rare diseases. In **chapter 7** the diagnostic delay, defined as the time between the first visit to a medical doctor for an MPS-related sign or symptom and the final diagnosis, is studied in Dutch patients diagnosed with MPS I and MPS III between 1988 and 2017, and is assessed if the time to diagnosis has changed over time.

The findings of this study show that children with MPS I are generally diagnosed at an earlier age than patients with MPS III, which is not very surprising, since MPS III patients present with rather aspecific symptoms that are often first attributed to more common childhood conditions. Nevertheless, for both MPS subtypes, there is no substantial decrease observed over the past three decades in the age at which the diagnosis is established, nor in the time to diagnosis. RP MPS III patients are diagnosed at an earlier age (4 years and 6 months) than SP patients (5 years and 11 months). Although there is no treatment for MPS III yet, the best outcome of a future therapy will most likely be achieved if initiated before irreversible neurocognitive decline sets in, approximately around the age of 3 years. In our cohort this is the case in only 9% of patients. For reducing the diagnostic delay in both patients groups and to ensure that the diagnosis is established at an age that allows for the best treatment outcome, better selected screening protocols included in national guidelines are needed as well as the implementation of newborn screening for those disorders that meet the criteria for population screening.

Finally, in **chapter 8** the findings that are described in this thesis are further discussed. Promising new therapies are explained in more detail and considerations are given regarding further studies on the pathophysiology, diagnosis and treatment of MPS III. Much effort will be needed, but recent developments in all fields are promising. It will require the collaboration of patient advocacy groups, medical doctors, researchers, funders, health insurers and governments, to develop a therapy for MPS III that will benefit the patients and at the same time will not put an excessive financial burden on the community. Consensus should be reached on when a treatment for MPS III is considered effective. Nonetheless, to ensure the best treatment outcome, diagnosis of MPS III should be established at an earlier stage.
Critics may raise the question why so much research is directed towards a disease that is so extremely rare. First of all, MPS III is a devastating neurodegenerative disorder that severely limits the quality of life of children suffering from this disease and thereby also affects the lives of their parents, brothers and sisters. Moreover, when taking together all those orphan diseases they are actually not that rare and altogether have a major impact on our society. Ultimately, we can only unravel the fascinating and miraculous mechanisms that are captured in the human body by studying this kind of rare conditions, and being able to treat one, will also allow for better treatments for other rare diseases.

SAMENVATTING

Mucopolysacharidose type III (MPS III), ook de ziekte van Sanfilippo genoemd, is een zeldzame maar ernstige, erfelijke stofwisselingsziekte. MPS III behoort tot de mucopolysacharidosen, een groep van zeven aandoeningen die op hun beurt weer deel uitmaken van de grotere groep van meer dan 60 verschillende lysosomale stapelingsziekten. Het lysosoom is een organel, een afgebakend onderdeel dat in elke cel van het menselijk lichaam, behalve de rode bloedcel, in veelvoud voorkomt en waarin een groot aantal verschillende soorten lichaamseigen moleculen wordt afgebroken, waarbij nieuwe grondstoffen vrijkomen. Voor dat afbraakproces gebruikt het lysosoom verschillende enzymen (eiwitten). In het geval van een lysosomale stapelingsziekte is er sprake van een mutatie, een fout in het DNA, het erfelijk materiaal waarin het bouwplan voor ons lichaam ligt opgeslagen, die ertoe leidt dat één (of meerdere) van de enzymen van het lysosoom onvoldoende of niet in de goede vorm wordt aangemaakt. Als gevolg hiervan kunnen bepaalde stoffen niet goed worden afgebroken en stapelen ze zich op in het lysosoom. Cellen raken overladen met deze onverwerkte stoffen waardoor ze niet meer goed kunnen functioneren. Dit leidt tot het in gang zetten van een cascade van pathologische processen met progressieve schade en dus een progressieve ziekte, tot gevolg.

In het geval van MPS III kan het molecuul heparansulfaat niet worden afgebroken doordat één van de vier enzymen die gezamenlijk verantwoordelijk zijn voor dat afbraaakproces, ontbreekt. Afhankelijk van het ontbrekende enzym worden er vier subtypen onderscheiden: MPS IIIA (heparan *N*-sulfatase (SGSH)), MPS IIIB (*N*-acetyl-α-glucosaminidase (NAGLU)), MPS IIIC (acetyl CoA:α-glucosaminide *N*-acetyltransferase (HGSNAT)) en MPS IIID (*N*-acetylglucosamine 6-sulfatase (GNS)).

Heparansulfaat is een polysacharide, een groot suikermolecuul dat ook wel een glycosaminoglycaan wordt genoemd. Heparansulfaat is als actief biologisch molecuul gebonden aan eiwitten, als glycoproteïne (proteïne = eiwit). Heparansulfaat bevindt zich aan de buitenzijde van de cel waar het een belangrijke functie heeft in de signaaloverdracht tussen cellen. Stapeling van heparansulfaat leidt tot progressieve schade aan cellen en ondanks het feit dat heparansulfaat in vrijwel alle weefsels voorkomt, is in het geval van MPS III het centraal zenuwstel het meest ernstig aangedaan.

Na een initieel normale ontwikkeling, presenteren kinderen met MPS III zich meestal tussen de leeftijd van 1 en 4 jaar met een ontwikkelingsachterstand, waarbij vooral de taal- en spraakontwikkeling is aangedaan. Rond 4-jarige leeftijd bereiken ze een maximaal ontwikkelingsniveau, waarna de cognitieve ontwikkeling stopt en geleidelijk achteruitgaat. De fase van achteruitgang gaat vaak gepaard met ernstige gedragsstoornissen, slaapproblemen en epilepsie. Uiteindelijk zijn kinderen in zowel hun cognitieve als motorisch functies en vaardigheden volledig beperkt en geheel zorgafhankelijk.

Er is een groot verschil in ziekte-ernst, met aan één zijde van het spectrum kinderen met een snel progressieve vorm van de ziekte ("rapidly progressing (RP) phenotype") en aan de andere kant kinderen met een langzaam progressieve vorm ("slowly progressing (SP) phenotype"). Kinderen met een snel progressieve vorm gaan snel achteruit en overlijden gedurende hun tienerjaren of op jongvolwassenleeftijd, terwijl kinderen met een langzaam progressieve vorm gedurende een lange tijd een stabiel ontwikkelingsniveau behouden. In deze groep is overleving tot op 50-60 jarige leeftijd beschreven.
Naast de neurologische symptomen, hebben kinderen met MPS III vrijwel altijd opvallende, grove gelaatstrekken en is er daarnaast vaak sprake van lies- of navelbreuken, een vergrote lever, een wisselend ontlastingspatroon en maken kinderen met MPS III in hun eerste levensjaren vaak opvallend veel bovenste luchtweginfecties door.

Er werd en wordt veel onderzoek gedaan naar een mogelijke behandeling voor MPS III. Desondanks is de ziekte nog altijd onbehandelbaar. In **hoofdstuk 2**, als onderdeel van de introductie van dit proefschrift, wordt een overzicht gegeven van de mogelijke bandelingen voor MPS III en in welke fase van onderzoek die zich bevinden. De grootste uitdaging bij de ontwikkeling van een therapie voor MPS III is dat het medicijn in de juiste hoeveelheid op de goede plaats in het lichaam terecht moet komen: de hersenen. Dit wordt bemoeilijkt door de bloed-hersen barrière (BHB), een beschermende laag tussen de bloedvaten in de hersenen en de hersenen zelf, die voorkomt dat niet zomaar alle grote moleculen, waaronder giftige stoffen, maar ook medicijnen, de hersenen binnen kunnen komen. Een potentiële behandeling voor MPS III kan in theorie op verschillende punten van het onderliggende ziekteproces aangrijpen, mits de BHB kan worden omzeild of stoffen zo worden ontwikkeld dat ze over de BHB heen kunnen worden getransporteerd.
Een eerste aangrijpingspunt is op genetisch niveau door middel van gentherapie, waarbij een goede kopie van het gen dat codeert voor het enzym dat de patiënt zelf niet goed kan maken, via de bloedbaan of direct in de hersenen wordt toegediend. Door het stukje DNA te verpakken in een onschadelijk gemaakt viruspartikel, kan het door de cel worden opgenomen en worden afgelezen waarbij een normaalwerkend enzym wordt gevormd. In patiënten met MPS IIIA en MPS IIIB is aangetoond dat deze methode relatief veilig kan worden uitgevoerd en studies naar effectiviteit zullen op korte termijn van start gaan.
Gentherapie kan ook worden toegepast in combinatie met stamceltransplantatie. Bij een gewone stamceltransplantatie worden de voorlopers van de eigen bloedcellen (die het enzym niet goed kunnen maken) vervangen door gezonde donorcellen. Na de transplantatie produceren deze donorcellen het missende enzym, ook voor omliggende cellen. De getransplanteerde cellen kunnen zich over de BHB naar de hersenen verplaatsen om daar het enzym te maken en uit te scheiden naar de omliggende hersencellen. Deze therapie heeft de voorkeur in de behandeling van patiënten met de ernstige vorm van MPS

I (MPS I-Hurler), maar is niet effectief gebleken voor de behandeling van MPS III. Dat komt waarschijnlijk doordat in gezonde stamcellen de uitscheiding van de enzymen betrokken bij MPS III veel lager is dan die van het enzym betrokken bij MPS I. Door stamceltransplantatie te combineren met gentherapie kan in de stamcellen een speciale kopie van het gen worden ingebouwd dat het enzym extra hoog tot expressie brengt. Het voordeel van deze methode is dat hiervoor de eigen stamcellen van de patiënt gebruikt kunnen worden wat de risico's van de behandeling verlaagt.
Een andere methode voor de behandeling van MPS III, is het direct toedienen van het ontbrekende enzym, zogenaamde enzym vervangende therapie. Voor verschillende lysosomale stapelingsziekten is deze benadering effectief gebleken, maar wordt in het geval van MPS III bemoeilijkt door het feit dat enzymen te groot zijn voor passage over de BHB. Een recente studie, waarbij het ontbrekende enzym intrathecaal (direct in het hersenvocht) werd toegediend, is niet effectief gebleken om cognitieve achteruitgang bij patiënten met MPS IIIA te voorkomen. Een studie naar enzymtherapie voor MPS IIIB is op dit moment nog gaande.
Het reduceren van de aanmaak en daarmee de stapeling van heparansulfaat, is een andere benadering voor de behandeling van MPS III en wordt ook substraatreductietherapie genoemd. Het uit soja-afkomstige genisteïne is in dit kader een veel onderzochte stof voor MPS III. Vooralsnog zijn de uitkomsten tegenstrijdig. Er wordt dan ook uitgekeken naar de resultaten van een studie die het effect van hoge dosis genisteïne bij een relatief groot aantal MPS patiënten onderzoekt.
Een behandeling die ook in dit proefschrift verder wordt onderzocht, omvat het gebruik van stoffen die chaperonnes worden genoemd (**hoofdstuk 6**). Nadat DNA in de celkern is afgelezen wordt in het endoplasmatisch reticulum het eiwit gevormd. Daar en in het Golgi-apparaat, een ander celorganel, wordt het nieuwe gevormde eiwit opgevouwen en gemodificeerd. Cellen beschikken over verschillende mechanismen om verkeerd opgevouwen eiwitten af te breken. In het geval van MPS III is de afbraak van verkeerd opgevouwen eiwitten jammer, want in een bepaalde groep patiënten zou het verkeerd gevormde eiwit namelijk toch zijn werk kunnen doen, mits het in het lysosoom zou aankomen. Chaperonnes zijn stoffen die het verkeerd opgevouwen enzym kunnen stabiliseren. Dat voorkomt afbraak en kan er in theorie voor zorgen dat het enzym toch naar het lysosoom wordt getransporteerd.

Om dichterbij een behandeling te komen voor MPS III is het van belang om nog meer inzicht te krijgen in de onderliggende ziektemechanismen. Het eerste deel van dit proefschrift richt zich daarom op de pathofysiologie van MPS III. Zoals eerder beschreven, kenmerkt MPS III zich door grote verschillen in het ziektebeloop (fenotype) dat varieert van een snel progressieve vorm tot een langzamer progressieve vorm. In het geval van MPS IIIA bestaat er een redelijk goede relatie tussen het type en de ernst van de onderliggende mutatie

en de ernst van het ziektebeloop. Door een grote verscheidenheid in onderliggende mutaties en het frequent voorkomen van nieuwe mutaties, is het in het geval van MPS IIIB moeilijker om het verwachte ziektebeloop te voorspellen. In **hoofdstuk 3** wordt een methode beschreven om MPS IIIB-patiënten met een snel progressief ziektebeloop van patiënten met een langzaam progressief ziektebeloop van elkaar te onderscheiden. Daarvoor worden huidcellen van patiënten in het laboratorium gekweekt onder normale omstandigheden, bij een temperatuur van 37°C, maar ook bij een temperatuur van 30°C. In huidcellen van patiënten met een langzaam progressief fenotype stijgt de enzymactiviteit van NAGLU bij het kweken bij 30°C tot 10% van de normaalwaarde (wat geassocieerd is met de afwezigheid van symptomen), in tegenstelling tot cellen van patiënten met een snel progressief fenotype waarin de enzymactiviteit in alle gevallen onmeetbaar laag blijft. In cellen van patiënten met een langzaam progressief fenotype wordt daarnaast minder stapeling van het heparansulfaat aangetroffen, wat aangeeft dat het enzym ook in de cel daadwerkelijk actief is. Ook lijkt de mate waarin cellen in staat zijn om onder kweekcondities van 30°C hun enzymactiviteit te verhogen, voorspellend te zijn voor de leeftijd waarop de patiënt bepaalde functies verliest. Op basis van deze resultaten blijkt enzymactiviteit na het kweken op 30°C een goede methode om ziekte-ernst te voorspellen. Dit is belangrijk voor het begeleiden en informeren van ouders van wie het kind net gediagnosticeerd is met MPS III, maar ook om het effect van een mogelijke behandeling te evalueren en te bepalen welk type behandeling voor welke groep patiënten geschikt is. Daarnaast zou het interessant zijn om het effect van het kweken van cellen bij 30°C met medicijnen na te kunnen bootsen. Dit zou als mogelijke behandeling kunnen dienen voor MPS IIIB-patiënten met een langzaam progressief fenotype.

Daarom worden in **hoofdstuk 4** de mechanismen die leiden tot een verhoogde enzymactiviteit na het kweken bij 30°C, verder onderzocht. Daaruit blijkt dat NAGLU in huidcellen van gezonde proefpersonen aanwezig is in twee vormen: als een precursor-eiwit en een matuur eiwit. In huidcellen van MPS IIIB-patiënten met een snel progressief fenotype is geen van beide vormen aanwezig. In huidcellen van patiënten met een langzaam progressief fenotype is bij 37°C alleen het precursor-eiwit aanwezig. Na kweken bij 30°C wordt in deze cellen ook het mature en enzymatisch actieve eiwit aangetroffen. Ten eerste lijkt dit het gevolg van een verhoogde aanmaak van NAGLU onder deze condities. Daarnaast lijkt het proces van eiwitmodificatie en -vouwing onder deze condities te zijn verbeterd. Dit blijkt uit het feit dat wanneer in huidcellen van MPS IIIB-patiënten met een langzaam progressief fenotype gekweekt bij 37°C, de hoeveelheid precursor-eiwit wordt verhoogd door de afbraak van het verkeerde NAGLU te remmen, dit niet leidt tot een verhoging van het mature eiwit. De verhoogde enzymactiviteit die in huidcellen van MPS IIIB-patiënten met een langzaam progressief fenotype wordt gemeten na kweken bij 30°C, lijkt dus het gevolg van een multifactorieel proces.

Niet alleen bij de ziekte MPS III, maar o.a. ook bij MPS I wordt heparansulfaat gestapeld zij het in het geval van MPS I in combinatie met een ander glycosaminoglycaan: dermatansulfaat. Net als bij patiënten met MPS III, bestaan er tussen MPS I-patiënten grote verschillen in de ernst van het ziektebeloop. Patiënten met de ernstige, snel progressieve vorm van de ziekte behoren tot de groep van MPS I-Hurler. MPS I-Scheie vertegenwoordigt de groep met een milder en langzamer progressief beloop. Daartussenin bevinden zich de patiënten met MPS I-Hurler/Scheie. Alleen in patiënten met MPS I-Hurler is ook het centraal zenuwstelsel in ernstige mate betrokken. Desondanks zijn er grote verschillen in het type neurologische symptomen van MPS I en MPS III en tussen patiënten met de verschillende MPS-subtypes onderling. In **hoofdstuk 5** wordt onderzocht of deze verschillen correleren met de totale hoeveelheid gestapeld heparansulfaat in het bloed van patiënten en de mate van sulfatering van de van heparansulfaat-afkomstige suikergroepen (disachariden). In de groep van MPS III-patiënten wordt, zoals al eerder bekend, een hoger totaal heparansulfaat gevonden in het bloed van patiënten met een snel progressief fenotype dan in patiënten met een langzaam progressief fenotype. Ook is er een hogere mate van sulfatering van de van heparansulfaat-afkomstige disachariden. Ook in MPS I-patiënten blijkt het totaal heparansulfaat verband te houden met de ernst van de ziekte. Daarnaast blijkt de mate van sulfatering van de van heparansulfaat-afkomstige disachariden te verschillen tussen MPS I en MPS III. De mate van sulfatering beïnvloedt in grote mate de functie van het heparansulfaat. Op basis van deze resultaten vermoeden wij dat de mate van sulfatering van het heparansulfaat bijdraagt aan de verschillen in de neurologische symptomen tussen MPS I- en MPS III-patiënten.

In het tweede deel van dit proefschrift komt de behandeling van MPS III aan bod. Op basis van de bevindingen in hoofdstuk 3 en hoofdstuk 4, wordt in **hoofdstuk 6** een groot aantal stoffen getest op hun vermogen om enzymactiviteit te verhogen in huidcellen van een MPS IIIB-patiënt met een langzaam progressief fenotype die goed reageert op het kweken bij een lagere temperatuur. Allereerst wordt daarvoor een methode ontwikkeld waarmee snel en nauwkeurig een groot aantal stoffen kon worden getest. De onderzochte stoffen zouden de eigenschappen kunnen hebben van de hiervoor beschreven chaperonnes en kunnen zowel een specifieke als aspecifieke werking hebben. Een deel van de onderzochte stoffen is effectief gebleken voor de behandeling van andere lysosomale stapelingsziekten. Geen van de door ons geteste stoffen blijkt echter in staat om de enzymactiviteit te verbeteren in huidcellen van een MPS IIIB-patiënt met een langzaam progressief fenotype. Daarom wordt ook een ‘bibliotheek’ onderzocht bestaande uit 1280 goedgekeurde middelen die al voor een andere indicatie zijn geregistreerd. Ook van deze verzameling stoffen blijkt helaas geen enkele in staat om de restactiviteit van NAGLU te verhogen.

Deel drie van dit proefschrift legt zich toe op de diagnose van MPS III en voornamelijk op het proces dat aan de diagnose voorafgaat. Het duurt vaak lang voordat de diagnose van zeldzame ziekten zoals MPS III, maar ook MPS I, wordt gesteld. In het geval van MPS III wordt dat mede veroorzaakt door de vaak aspecifieke symptomen waarmee kinderen zich presenteren en die ook goed zouden kunnen passen bij meer gangbare aandoeningen op de kinderleeftijd. Dit diagnostisch traject, waarin vaak meerdere medisch specialisten worden bezocht, wordt door ouders vaak als zeer belastend ervaren. Daarom is het stellen van een vroege diagnose belangrijk, zeker als er voor een ziekte een behandeling bestaat. Door eerdere onderzoeken, o.a. bij MPS I, is bekend dat een vroege start van de behandeling, voordat progressieve en onherstelbare schade is opgetreden, de grootste kans op succes geeft. De afgelopen jaren zijn er veel initiatieven gelanceerd om zeldzame ziekten onder de aandacht te brengen van artsen en andere zorgverleners, geïnitieerd door verschillende partijen waaronder patiëntverenigingen, maar ook overheden en farmaceutische bedrijven die bezig zijn met de ontwikkeling van een therapie of al een behandeling voor deze ziekten hebben ontwikkeld. **Hoofdstuk 7** beschrijft de resultaten van een onderzoek naar het diagnostisch traject van patiënten met MPS I en MPS III vanaf het moment dat ouders voor het eerst met hun kind de huisarts bezochten voor een MPS-gerelateerde klacht en de uiteindelijke diagnose. Ook wordt onderzocht of het diagnostisch traject in deze groep patiënten tussen 1988 en 2017 korter is geworden. Uit dit onderzoek blijkt dat kinderen met MPS I in het algemeen op jongere leeftijd worden gediagnosticeerd dan patiënten met MPS III. Dit heeft waarschijnlijk te maken met de meer uitgesproken symptomen van botten en bindweefsels in deze patiëntengroep waardoor eerder duidelijk is dat er sprake is van een (ernstige) ziekte. In zowel de groep MPS I- als MPS III-patiënten wordt over de afgelopen 3 decennia geen afname van de duur van het diagnostisch traject gezien. Uit deze studie blijkt wel dat MPS III-patiënten met een snel progressief ziektebeloop op jongere leeftijd (4 jaar en 6 maanden) worden gediagnosticeerd dan patiënten met een langzaam progressief fenotype (5 jaar en 11 maanden). Om in de toekomst echter een goed effect te behalen van een mogelijke behandeling voor MPS III, moet de diagnose bij voorkeur voor de leeftijd van 3 jaar worden gesteld. Dit is slechts in 9% van de kinderen met MPS III het geval.

Uit dit onderzoek blijkt dat er nog veel verbeteringen nodig zijn om de weg naar de diagnose voor MPS I en MPS III te verkorten. MPS I zal binnenkort worden opgenomen in de hielprikscreening. Zolang er geen behandeling bestaat voor MPS III, voldoet MPS III niet aan de criteria om ook aan de hielprikscreening te worden toegevoegd. Om MPS III-patiënten in een vroege fase van hun ziekte te diagnosticeren, moeten er betere landelijke richtlijnen komen waarin geadviseerd wordt om bij kinderen die zich presenteren met een ontwikkelingsachterstand of een taalspraakachterstand, laagdrempelig en in een vroege fase onderzoek te doen naar stofwisselingsziekten. Ondanks het feit dat er voor MPS III nog geen behandeling bestaat die het ziektebeloop kan beïnvloeden, kan een vroege

diagnose er wel voor zorgen dat patiënten op het goede moment de juiste ondersteunende behandelingen krijgen. Tegelijkertijd geeft dit deze kinderen de kans om mee te doen aan klinische studies naar mogelijke nieuwe behandelingen voor MPS III. Ook kunnen ouders met een kinderwens op deze manier beter begeleid worden.

Tot slot wordt in **hoofdstuk 8** een beschouwing gegeven op de bovenstaande hoofdstukken en de implicaties daarvan voor vervolgonderzoek in de komende jaren. Er is nog veel onderzoek nodig om de ziektemechanismen die aan MPS III ten grondslag liggen te begrijpen. Toch zijn de ontwikkelingen in dit veld hoopvol en worden er veelbelovende stappen gemaakt naar een mogelijke behandeling voor MPS III. Het vraagt om samenwerking tussen patiëntenverenigingen, artsen en onderzoekers, farmaceutische bedrijven, zorgverzekeraars en overheden om betrouwbaar en goed onderzoek naar deze behandelingen te kunnen doen en ze uiteindelijk voor een acceptabele prijs op de markt te brengen. Daarvoor is een consensus nodig over wanneer een behandeling voor MPS III effectief wordt geacht. Om het succes van deze behandelingen te vergroten zal de diagnose van MPS III in een vroegere fase moeten worden gesteld.
Bij de critici zal dit de vraag opwerpen waarom er zoveel onderzoek gaande is naar een ziekte die zo ontzettend zeldzaam is. Ten eerste, omdat MPS III een ziekte is met een zeer ernstig degeneratief beloop die de kwaliteit van leven van kinderen met deze aandoening, en daarmee ook die van hun ouders, broertjes en zusjes, ernstig beperkt. Daarnaast zijn alle zeldzame ziekten bij elkaar opgeteld, eigenlijk helemaal niet zo zeldzaam en hebben ze gezamenlijk een grote weerslag op onze maatschappij. Uiteindelijk zullen we ook door het bestuderen van dit soort zeldzame aandoeningen meer van de fascinerende en wonderlijke mechanismen kunnen ontrafelen die in het menselijk lichaam verborgen liggen en daarmee de mogelijkheden verkrijgen om deze en soortgelijke ziekten in de toekomst beter te behandelen.

Addendum

LIST OF CO-AUTHORS

P. van den Biggelaar
Laboratory of Genetic Metabolic Diseases, Department of Clinical Chemistry, Academic Medical Center, Amsterdam, The Netherlands

H. te Brinke
Laboratory of Genetic Metabolic Diseases, Department of Clinical Chemistry, Academic Medical Center, Amsterdam, The Netherlands

H.T. Brüggenwirth
Department of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands

L.H. Hoefsloot
Department of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands

L. IJlst
Laboratory of Genetic Metabolic Diseases, Department of Clinical Chemistry, Academic Medical Center, Amsterdam, The Netherlands

G. Kuiper
Department of Pediatric Metabolic Diseases, Emma Children's Hospital and Amsterdam Lysosome Center "Sphinx", Academic Medical Center, Amsterdam, The Netherlands

E.J. Langereis
Department of Pediatric Metabolic Diseases, Emma Children's Hospital and Amsterdam Lysosome Center "Sphinx", Academic Medical Center, Amsterdam, The Netherlands

H. van Lenthe
Laboratory of Genetic Metabolic Diseases, Department of Clinical Chemistry, Academic Medical Center, Amsterdam, The Netherlands

R. Ofman
Laboratory of Genetic Metabolic Diseases, Department of Clinical Chemistry, Academic Medical Center, Amsterdam, The Netherlands

A.T. van der Ploeg
Department of Pediatrics, Center for Lysosomal and Metabolic Diseases, Erasmus Medical Center, Rotterdam, The Netherlands

G.J. Ruijter
Department of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands

M.J. Valstar
Department of Pediatric Metabolic Diseases, Emma Children's Hospital and Amsterdam Lysosome Center "Sphinx", Academic Medical Center, Amsterdam, The Netherlands

N. van Vlies
Intravacc, Institute for Translational Vaccinology, Bilthoven, The Netherlands

T. Wagemans
Department of Biology, Medical and Biochemical Laboratory Research, Saxion University of Applied Sciences, Deventer, The Netherlands

L. Welling
Department of Pediatric Metabolic Diseases, Emma Children's Hospital and Amsterdam Lysosome Center "Sphinx", Academic Medical Center, Amsterdam, The Netherlands

F.A. Wijburg
Department of Pediatric Metabolic Diseases, Emma Children's Hospital and Amsterdam Lysosome Center "Sphinx", Academic Medical Center, Amsterdam, The Netherlands

LIST OF PUBLICATIONS

G. Kuiper, O.L.M. Meijer, E.J. Langereis, F.A. Wijburg. Failure to shorten the diagnostic delay in two ultra-orphan diseases (mucopolysaccharidosis types I and III): potential causes and implications. *Orphanet J Rare Dis*. 2018 [Accepted for publication].

O.L.M. Meijer, P. van den Biggelaar, R. Ofman, F.A. Wijburg, N. van Vlies. High-throughput screen fails to identify compounds that enhance residual enzyme activity of mutant *N*-acetyl-α-glucosaminidase in mucopolysaccharidosis type IIIB. *JIMD Rep*. 2017 [Epub ahead of print].

O.L.M. Meijer, H. te Brinke, R. Ofman, L. IJlst, F.A. Wijburg, N. van Vlies. Processing of mutant *N*-acetyl-α-glucosaminidase in mucopolysaccharidosis type IIIB fibroblasts cultured at low temperature. *Mol Genet Metab*. 2017;122(1-2):100-106.

O.L.M. Meijer, L. Welling, M.J. Valstar, L.H. Hoefsloot, HT Brüggenwirth, A.T. van der Ploeg, G.J. Ruijter, T. Wagemans, F.A. Wijburg, N. van Vlies. Residual *N*-acetyl-α-glucosaminidase activity in fibroblasts correlates with disease severity in patients with mucopolysaccharidosis type IIIB. *J Inherit Metab Dis.*2016;39(3):437-45.

O.L.M. Meijer, N. van Vlies and F.A. Wijburg. Treatment of mucopolysaccharidosis type III (Sanfilippo syndrome). Expert *Opin Orphan Drugs*. 2013;1(9):717-730.

D.G. van Vuurden, E. Hulleman, O.L.M. Meijer, L.E. Wedekind, M. Kool, H. Witt, P.W. Vandertop, T. Würdinger, D.P. Noske, G.J. Kaspers, J. Cloos. PARP inhibition sensitizes childhood high grade glioma, medulloblastoma and ependymoma to radiation. *Oncotarget*. 2011;2(12):984-96.

O.L.M. Meijer, R.K. Weersma, E.J. van der Jagt, H.M. van Dullemen. Endoscopic ultrasonography in suspected pancreatic malignancy and indecisive CT. *Neth J Med*. 2010;68(9):360-4.

Manuscript in preparation

O.L.M Meijer, T. Wagemans, H. van Lenthe, F.A. Wijburg and Naomi van Vlies. Quantity and structure of stored heparan sulfate may affect the nature and course of neuronopathic disease in mucopolysaccharidosis type I and type III.

PHD PORTFOLIO

General courses	**Year**
Laboratory Animals (Article 9)	2013
Mouse Morphology, Genetics & Function	2013
Crash Course - Basic Chemistry, Biochemistry and Molecular Biology for MDs	2013
Practical Biostatistics	2014
Introduction to Good Clinical Practice (GCP)	2014
Basic Course in Legislation and Organization for Clinical Researchers (BROK)	2014
Specific courses	
International Postgraduate course on Lysosomal Storage Disorders, Nierstein, Germany	2013
Lysosomal Biology, Bahia, Brazil	2014
Seminars, workshops and masterclasses	
Masterclass Amsterdam Kindersymposium, Amsterdam, The Netherlands	2014
ACM/MDL Meeting, Lunteren, The Netherlands	2014
Presentations	
Oral presentation Amsterdam Kindersymposium, Amsterdam, The Netherlands	2014
Poster presentation International Symposium on MPS and Related Diseases, Bahia, Brazil	2014
Poster presentation WORLD Symposium on Lysosomal Disorders, Orlando, USA	2015
Oral presentation Voorjaarsvergadering ESN, Rotterdam, The Netherlands	2015
Oral presentation Amsterdam Kindersymposium, Amsterdam, The Netherlands	2016
Poster presentation WORLD Symposium on Lysosomal Disorders, San Diego, USA	2016
Poster presentation International Symposium on MPS and Related Diseases, Bonn, Germany	2016
Poster presentation WORLD Symposium on Lysosomal Disorders, San Diego, USA	2017
(Inter)national conferences	
Amsterdam Kindersymposium, Amsterdam, The Netherlands	2013
Amsterdam Kindersymposium, Amsterdam, The Netherlands	2014
International Symposium on MPS and Related Diseases, Bahia, Brazil	2014
WORLD Symposium on Lysosomal Disorders, Orlando, USA	2015
Voorjaarsvergadering ESN, Rotterdam, The Netherlands	2015
International conference on Sanfilippo Syndrome and Related Lysosomal Storage Diseases,	2015
Geneva, Suisse	2016
Amsterdam Kindersymposium, Amsterdam, The Netherlands	2016
WORLD Symposium on Lysosomal Disorders, San Diego, USA	2016
International Symposium on MPS and Related Diseases, Bonn, Germany	2017
WORLD Symposium on Lysosomal Disorders, San Diego, USA	
Others	
Sub-investigator MOR-005 trial	2014-2015
Sub-investigator HGT-SAN-067-trial	2014-2017
Sub-investigator HGT-SAN-093-trial/SHP-610-201-trial	2014-2017
Teaching	
Supervising thesis of Peggy van den Biggelaar, master student	2014
Supervising thesis of Jeska Fritzsche, bachelor student	2015
Lecturing “pre-co onderwijs: lichamelijk onderzoek bij het kind”	2016
Parameters of esteem	
Selection for the masterclass of the Amsterdam Kindersymposium, Amsterdam, The Netherlands	2014
Group winner best oral presentation Amsterdam Kindersymposium, Amsterdam, The Netherlands	2016

CURRICULUM VITAE

Olga Laurentia Maria Meijer werd geboren in Groningen op 28 februari 1984. De eerste jaren van haar leven woonde ze in Brunei en groeide nadien op in de omgeving van Haarlem. Ze doorliep haar middelbare schooltijd op het Stedelijk Gymnasium te Haarlem. Tijdens de veldwerkweken in de Kennemerduinen onder leiding van haar biologiedocent mevr. Sevenster en gedurende het laboratoriumwerk in het kader van haar profielwerkstuk, waarin ze werd begeleid door kinderarts dr. R.H. Veenhoven, werd haar interesse voor de wetenschap gewekt. Na het behalen van haar eindexamendiploma in 2002 woonde en werkte zij gedurende 12 maanden in Santiago, Chili. De studie geneeskunde bracht haar in 2003 terug naar haar geboortestad. Haar coschappen doorliep zij in het Martiniziekenhuis in Groningen en via Das Klinikum in Oldenburg (Duitsland) en Wasso Designated District Hospital (Tanzania) keerde ze uiteindelijk terug naar de Randstad waar ze haar semi-artsstage kindergeneeskunde volgde in het Spaarne Gasthuis Hoofddorp. Tijdens haar studie deed zij onderzoek bij de afdeling gastro-enterologie van het Universitair Medisch Centrum Groningen en de afdeling kinderoncologie van het VU Medisch Centrum. Na het behalen van haar master geneeskunde in 2011 werkte zij als arts-assistent kindergeneeskunde in het Zaans Medisch Centrum en Sint Antonius Ziekenhuis in Nieuwegein. In 2013 kreeg zij de kans om terug te keren in de wetenschap en onderzoek te doen onder leiding van prof. dr. F.A. Wijburg en dr. N. van Vlies bij de afdeling metabole ziekten en het laboratorium genetisch metabole ziekten van het Emma Kinderziekenhuis / Academisch Medisch Centrum, wat resulteerde in dit proefschrift. Inmiddels werkt zij als arts-assistent kindergeneeskunde in het Wilhelmina Kinderziekenhuis / Universitair Medisch Centrum in Utrecht.

DANKWOORD

Op elke plek waar mensen samen leven en werken, bestaat er een bepaalde mate van afhankelijkheid. Dat laat zich door de wereld van de zorg en medische wetenschap dankbaar illustreren met een wederzijdse afhankelijkheid tussen patiënt, arts, onderzoeker en eenieder daaromheen. Dat alles om een ziekte beter te begrijpen, te kunnen diagnosticeren en waar mogelijk te behandelen. Daarin is '*de stroom der dingen niet veel anders dan de optelsom van alle acties en reacties van ieder betrokken individu*' [1], zo ervaarde ik als arts-onderzoeker in het laboratorium Genetisch Metabole Ziekten ('lab GMZ') van het AMC en voor de afdeling metabole ziekten van het Emma Kinderziekenhuis. Dit proefschrift heeft dan ook enkel tot stand kunnen komen dankzij de inhoudelijke inbreng, aanmoediging en het vertrouwen van al die betrokkenen.
Toch is het een afhankelijkheid die we in beginsel soms anders hopen of dromen. Dit proefschrift kan dan ook alleen worden opgedragen aan alle kinderen en (jong)volwassenen met mucopolysacharidose type III, hun ouders en iedereen die hen nabij is. Alleen door en voor jullie heb ik (we!) dit proefschrift kunnen schrijven. Waar dat voor mij allereerst een oefening in de medische wetenschap was, is het leven met een zeldzame, progressieve en onbehandelbare aandoening een oefening in aanvaarding, liefde en onvoorwaardelijkheid, tegen de klippen op. Ik heb diepe bewondering voor de wijze waarop jullie dat als families vormgeven en dragen. Naast basaal wetenschappelijk onderzoek, leerde ik tijdens dit promotietraject van jullie misschien wel de meest fundamentele lessen van het leven.

Ook binnen de wereld van de medische wetenschap zijn we in grote mate van elkaar afhankelijk, zeker een jonge arts-onderzoeker op weg naar haar doctorsgraad. Prof. dr. C.E.M. Hollak, prof. dr. B.T. Poll-The, prof. dr. A.J. Verhoeven, prof. dr. H.R. Waterham en prof. dr. R.A. Wevers, veel dank dat u allen bereid bent om zitting te nemen in mijn promotiecommissie. Geachte prof. dr. R.J.A. Wanders, beste Ronald, als hoofd van lab GMZ aan jou een bijzonder dank omdat ik vier jaar onder jouw vleugels heb mogen werken. Inhoudelijk zijdelings betrokken, maar moreel ben je een belangrijke factor geweest in het voltooien van dit proefschrift.

Beste Frits, 'niet menselijks is ons vreemd', grapten we ooit tijdens een gesprek in de wandelgang. Ik dank je voor die menselijkheid en het vertrouwen waarmee je mij als promotor in dit traject hebt begeleid. Ik bewonder het vuur, je blijvende nieuwsgierigheid en enorme vasthoudendheid waarmee je de metabole ziekten in Nederland en daarbuiten op de kaart hebt gezet en waardoor je perspectief blijft creëren voor je patiënten en hun families.

Naomi, als copromotor leerde je mij het evenwicht te vinden tussen volledigheid en perfectionisme. Een les om een leven lang te koesteren. Dank voor je geruststelling in tijden van tegenslag en je blijvende betrokkenheid, ook toen je al afscheid had genomen van het AMC. De wijze waarop jij fundamenteel onderzoek kunt vertalen in iets eenvoudigs en alledaags zal voor mij een voorbeeld blijven in hoe we de wetenschap en de maatschappij met elkaar kunnen verbinden.

Een arts in het lab blijft toch altijd een vreemde vogel. Daarom aan ieder in het lab GMZ: dank voor jullie antwoorden op mijn talloze vragen. Dankzij jullie heb ik zelfstandig leren vliegen.
Rob en Lodewijk, zonder jullie kennis, tijd, bereidheid en enthousiasme, geen proefschrift. Jullie zijn van onschatbare waarde geweest voor de opbouw, inhoud en kwaliteit van dit boek. Een staande ovatie, diepe buiging en een enorm groot dank. Tom, zonder jou geen begin van dit boek. Heleen, zonder jou geen einde: dank voor je eindsprint en al je punten op de i.
PhD-colleagues, you belong to the most colorful and authentic people I met during my journey through the lab. Thanks for all your help in finding my way around and for your stories from all over the world. Although typically Dutch, Sinterklaas will never be the same.

Collega's van de metabole ziekten Annet en Clara, Hidde en Charlotte (in mijn begin- en latere jaren), Evelien en Thessa. De wijze waarop jullie gezamenlijk zorg dragen (en droegen) voor kinderen en gezinnen met een metabole ziekte is uniek. Al hebben we als dokters soms niet meer te bieden dan een diagnose, jullie blijven met ieder kind en elk gezin een leven lang mee oplopen: toegewijd, betrokken, met een neus voor nieuwe ontwikkelingen en gericht op de stippen aan de horizon.
Mede-(metabole)onderzoekers Lindsey, Sandra, Gé-Ann, Stephanie, Jeannette en Suzan, mijn MPS III-voorgangers Marlies en Jessica, dank voor jullie loyaliteit. Een voorrecht en een groot plezier was het om samen verder te bouwen aan al het voorgaande onderzoek. Eveline, dank voor de bijzondere vriendschap die boven het werk uitsteeg. Daarmee dank aan alle collega (arts-)onderzoekers van de Rode Luifel en later H7, voor al jullie vrolijke noten, kamerrituelen en ontelbare goede en minder goede grappen waarin de grens tussen collegialiteit en vriendschap langzaam vervaagde. Werk was met enige regelmaat ook even feest.

Allerliefste vrienden, ver weg en dichtbij, het is mede dankzij jullie dat ik talent voor het leven kreeg. Want het ontcijferen van dat leven, het blootleggen van zijn wonderen en geheimen, al dat streven naar genezen, heeft geen waarde als we het onderwijl niet vieren,

koesteren, toezingen en -lachen, tussen de regels door de poëzie proberen te ontwaren en vreugdevuren bouwen. Paranimfen Job, Cato (en Mauk): jullie maken licht.
Lieve broer Rogier en Merel, waar ik ook heen waai, dank dat ik bij jullie altijd thuis mag komen. Lieve vader Robert en Dale-Anne, '*alles van waarde is weerloos* '[2]. In die wetenschap dit proefschrift, om het met glimmende wangen in de kast zetten. Lieve mammie Olga, '*voor*' maar vooral '*als wie ik liefheb wil ik heten*' [3]. Dank voor wie je bent, in alles, altijd, overal.

Tot slot,

Alles heeft een nieuw begin

1 Freek Vielen. Uit: *Afhankelijkheidsverklaring*. De Correspondent, 2016.
2 Lucebert. Uit: De *zeer oude zingt*. Verzamelde gedichten, 1974.
3 Vrij naar Neeltje Maria Min. Uit: *Mijn moeder is mijn naam vergeten*. Voor wie ik liefheb wil ik heten, 1966.

Een bijzonder dank aan alle donateurs die middels hun financiële ondersteuning het onderzoek in dit proefschrift mede mogelijk gemaakt hebben. Het partnerschap en de vaak grote persoonlijke betrokkenheid van de financiële donateurs bij de ziekte van Sanfilippo (mucopolysacharidose type III), is een belangrijke stimulans voor zowel ouders en kinderen, als voor onderzoekers en behandelaars.