Characterization of a murine model of mucopolysaccharidosis type IIID: A knockout model

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Characterization of a murine model of mucopolysaccharidosis type IIID:
A knockout model

by

Maryam Jamil

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Genetics

Program of Study Committee:
N. Matthew Ellinwood, Major Professor
Jodi Dee Smith
Anumantha Kanthasamy

Iowa State University
Ames, Iowa
2016

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The Mucopolysaccharidoses (MPS) are a class of lysosomal storage disorders, characterized by the primary lysosomal storage of either single or multiple species of glycosaminoglycan (GAG) which leads to cell, tissue, and organ dysfunction. This is manifested clinically by either bone/connective tissue disease, central nervous system (CNS) disease, or both, and often results in premature death. Mucopolysaccharidosis type III is a heterogeneous neuropathological disorder characterized by an accumulation of the heparan sulfate (HS). Of the four known types of MPS III in humans (A (OMIM: 252900), B (252920), C (252930), D (252940)), all have murine models except IIID. Additional to primary HS storage there is secondary lysosomal accumulation of multiple biological products including gangliosides. Mouse models are critical for advancing therapies for patients. Herein we describe the first mouse model of IIID.

The MPS IIID knockout lacks exons 2-13 of the 14 exons of N-Acetylglucosamine 6-Sulfatase (GNS) gene which encodes the enzyme (N-Acetylglucosamine 6-Sulfatase, EC 3.1.6.14) taking part in HS degradation pathway. Affected MPS IIID mice were compared to affected MPS IIIB and IIIA mice, as well as to wild type (WT) mice. Affected IIID animals had urine retention, hepatomegaly, and rough hair coat beginning at nine months, a phenotype similar to other forms of MPS III. Affected animals were further characterized by histology and biochemistry (at four, eight and twelve months), and behavior. At four months of age there was no detectable liver activity of GNS and an exponential increase of GAGs was seen. Mild vacuolation, particularly in CNS, was seen in hematoxylin and eosin (H&E) stained tissues of IIID affected mice. At twelve months H&E staining showed extensive vacuolation in hepatocytes, renal tubular cells, CNS, as well as cerebellar Purkinje cell loss. Luxol Fast Blue
(LFB) staining of CNS tissue showed positive staining for glycosphingolipids. The preliminary behavioral assessment data showed that IIID mice perform better on rotorod at least until 12 months of age compared to IIIB mice.

Our findings of IIID mouse are consistent with the histological and biochemical findings in humans. Furthermore, it has a similar phenotype and pathology to other MPS III murine models.
CHAPTER 1: LITERATURE REVIEW

Lysosomal storage disorders (LSDs) are a group of inborn metabolic disorders comprising approximately 50 inherited disorders caused by the deficiency of particular enzymes or other critical elements which leads to the accumulation of one or more compounds in lysosomes. The mucopolysaccharidoses (MPSs) represent a class of LSDs resulting in primary storage of glycosaminoglycans (GAGs). At this time treatments exist for some MPSs, but there are no definitive cures for these disorders, which increases the need for in depth analysis of LSDs.

Lysosomes
Lysosomes, first discovered by Christian de Duve, are organelles of eukaryotic cells containing degrading enzymes (2). Consisting of approximately 50 hydrolytic enzymes, lysosomes break down and recycle different biomolecules. Initially, lysosomes were only defined as terminal degradative compartment of the cell but over time this definition has been expanded as the involvement of the lysosomes in the endocytic, autophagic, and biosynthetic pathways became apparent.

Endosomal-Lysosomal Pathway
In high order organisms, cells send or receive signals from neighboring cells in response to environmental cues. Communication can be in the form of different transmitters, ligands, nutrients etc., recognized by specific receptors on the cell membrane. The receptors along with the signaling molecule are internalized by the cell making a membrane bound organelle called a sorting endosome. As the name suggests, sorting endosomes sort the molecules to be recycled (mostly receptors) and molecules to be degraded (mostly ligands) into the endocytic recycling compartment and late endosome respectively. In the endocytic recycling compartment, the
molecules and/or receptors are almost immediately recycled to the plasma membrane or the trans-Golgi network (TGN). Due to a comparatively lower pH and the influx of hydrolytic enzymes from the TGN, most of the degradation of ligands begins in late endosomes. Most of the hydrolytic enzymes are targeted to the late endosome via a mannose 6 phosphate (M6P) moiety; enzymes missing this signal are shunted to the extracellular space via vesicular transport to the plasma membrane. Endosomal components get into the lysosome via either ‘kiss and run’ mechanism and/or via direct fusion.

There are two M6P receptors (MPRs); the cation-dependent MPR (CD-MPR) and the cation-independent MPR (CI-MPR), which are similar but not identical. Both these receptors are involved in lysosomal enzyme sorting at the level of the TGN with CI-MPR being the dominant receptor. The CI-MPR is recycled back to TGN from the endosome before the degradation process continues on within the lysosome. Thus, late endosomes can be distinguished from lysosomes based on the presence of the CI-MPRs. Figure 1 shows a schematic representation of the endocytic pathway.

**Lysosomal Storage Disorders**

The primary concept of a lysosomal storage disorder (LSD) was articulated by H.-G. Hers while characterizing Pompe’s disease (3). He listed four points which each disorder must have to be considered an LSD.
1) It should be a storage disease
2) The substrate should be segregated in vacuoles related to lysosomes
3) Normal lysosome should be able to degrade the accumulated substrate
4) One of enzyme from the degradation pathway of the substrate should be inactive

In the 19th century, diseases such as Gaucher, Neimann-Pick, and Tay-Sachs were first identified, long before de Duve’s discovery of the lysosome. In the early 20th century more diseases were characterized which were similar to the previously described diseases. And, in mid-20th century, after the discovery of lysosome and the articulation by Hers of his concept of LSDs, these diseases were correctly categorized as lysosomal diseases. Roughly 50 lysosomal storage diseases have been identified to date. Amongst these LSDs, only a few have approved therapies available. The LSDs differ in that they may result from different genes being affected, and within a distinct disease caused by a specific gene defect, there can be clinical variability ranging from severe to attenuated disease depending on the functional consequences of specific mutations. But, all LSDs are similar in that they result in an accumulation of particular substrates in lysosome.

Classification
Lysosomal storage disorders comprise a large group of disorders, a group which grows as new LSDs are identified. Classification of LSDs can be according to various approaches. The early description of the diseases was based on clinical signs and pathology. Such diseases were mostly named eponymously after the clinician(s) who identified them such as Gaucher disease, Tay-Sachs disease, etc. Subsequently, these diseases were defined based on histological and biochemical characteristics, namely the type of stored substrate, e.g. the gangliosidoses include the diseases which accumulate gangliosides. Similarly the MPSs include the disorders which accumulate
GAGs (previously known as mucopolysaccharides). This form of classification combined with the early classification based on the clinical signs and pathology is the classification used to date.

An alternative, less commonly utilized, classification is based on molecular mechanism and the underlying type of defect that causes disease. Six major categories or mechanisms are encompassed by this classification system. The first category contains the disorders caused by a defect in a lysosomal hydrolase’s activity. Most of the disorders come under this category such as Niemann-Pick disease type A (NP-A), Gaucher, Tay-Sachs, the majority of MPSs, etc. The second category of disorders are caused by defects in post-translational processing of the lysosomal enzymes, e.g. multiple sulphatase deficiency (MSD). The disorders of a third category are caused by trafficking defects of lysosomal enzymes, e.g. mucolipidoses type II (ML-II, a.k.a. I-cell disease). The majority of lysosomal enzymes which are to be targeted to the lysosome contain an M6P moiety. In disorders like ML this signal is missing. Thus the enzymes are secreted from the cell instead of trafficking to the lysosome. Not all cells in this disease are affected which implies that there must be an alternative pathway through which certain enzymes reach the lysosomes. The fourth category is a defect in lysosomal enzyme protection, e.g. galactosialidosis. In such diseases the lysosomal enzymes are synthesized and trafficked normally but are degraded in the lysosomes due to intra-lysosomal proteolysis that proceeds to degrade an enzyme because of the lack of a protective protein. The fifth category of disorders are caused by defects in activator proteins. Lysosomes contain a number of soluble non-enzymatic glycoproteins which are essential for the degradation of substrates. These proteins are not hydrolytic but help lysosomal hydrolases to function, usually by “presenting” or binding substrates, thus acting as “activator” proteins. For example in a form of GM2 gangliosidosis, the GM2 activator protein, which binds GM2 and presents it to hexosaminidase, is deficient causing the disease (4). The last category consists of the
Disorders are classified according to traditional convention whereas different colors indicate the mechanism of the defective protein. Key: ■ Primary lysosomal hydrolase defect, □ Post-translation processing defect of lysosomal enzyme, ▲ Trafficking defect for lysosomal enzymes, ▲ Defect in lysosomal enzyme protection, ▲ Defect in soluble non enzymatic lysosomal proteins, ▲ Transmembrane (non-enzyme) protein defect. This chart has been inspired from (1) with modifications.
diseases caused by the defects in transmembrane proteins e.g. NPC-1 and MPS IIIC. Little is known about diseases like NPC-1 but it is suggested that either substrate shuttling, trafficking and/or fusion of the vesicles might be involved. This mechanistic form of classification, even though not frequently used, can aid in the conceptual development of therapies for LSDs. For example enzyme replacement therapies (ERTs) are proposed for MPS disorders but ERT is not predicted to be beneficial for MPS type IIIC which is caused by a defect in transmembrane protein. Both approaches to lysosomal storage disorder classification, a substrate approach, and a mechanistic approach can be seen in Figure 2.

Epidemiology

All lysosomal storage disorders follow Mendelian inheritance, all being autosomal recessive disorders with an exception of Fabry and MPS type II which are X-linked disorders. A study in Australia looking at the prevalence of LSDs from 1980 to 1996 estimated that, even though the prevalence of each LSD is low, they have aggregate prevalence of 1 per 7700 live births (5). Certain ethnic groups have high prevalence of specific LSDs for example the carrier frequency of Tay-Sachs disease among Ashkenazi Jewish populations was 1:25 in the Australian high school population and 1:33 in the Brazilian high school population (6, 7). Similarly there are certain regions and/or countries where inter-family and/or inter-caste marriages are a common practice. If studied, those regions will most likely have a higher incidence of lysosomal storage disorders.

Mucopolysaccharidoses

The MPSs are a class of lysosomal storage disorders characterized by the accumulation of one or more specific types of GAG. As mentioned previously the MPS disorders are autosomal recessive disorders with the exception of MPS II which is an X-linked syndrome. The MPSs are life threatening and progressive disorders, the severe forms of which may lead to death at an early age. There are a total of seven major MPS types, caused by the deficiency of eleven
different lysosomal enzymes. As multiple enzyme deficiencies may lead to the accumulation of a single species of GAG, some of the major types of MPSs have further subtypes for example MPS IIIA-IIIE.

**Classification**

The MPSs are classified on the basis of clinical disease and by the type of GAG accumulated. In human forms of MPS disorders eleven lysosomal enzymes consisting of five sulfatases, five glycosidases and one transferase take part in the catabolic pathways of five main classes of GAGs (8). Dermatan sulfate (DS), keratan sulfate (KS), heparan sulfate (HS), chondroitin sulfate (CS) and hyaluronan (HA) are stored singly or in combination, depending on the specific deficient activity of one of the mentioned eleven lysosomal enzymes. Table 1 lists all the major types and subtypes of MPS along with the corresponding accumulated GAG and the responsible enzyme deficiency.

**Glycosaminoglycans**

Glycosaminoglycans are complex carbohydrate conjugates (reviewed Jackson (9)), consisting of large linear repeating heteropolysaccharide units. These repeating units are disaccharides of an amino sugar (D-glucosamine or galactosamine) and a uronic acid residue (D-glucoronic acid or iduronic acid). There are six major GAGs known as HS, heparin, DS, KS, CS, and HA. The amino sugar of the polysaccharide chain can be sulfated on either carbon 4 or 6, or the non-acetylated nitrogen, while the overall backbone can be sulfated at multiple positions. Amongst six major types of GAG, only HA is non-sulfated. Glycosaminoglycans may also differ by the glycosidic linkage between sugar rings which can either be alpha or beta linked (α or β linkage) which further increases the conformational diversity of GAGs. The reducing ends of
most of the GAGs except heparin and HA are covalently attached to a core protein. This attachment of GAGs to the core protein is through a trisaccharide chain consisting of one xylose and two galactose. The linkage of GAG in most cases is via an O-glycosidic linkage to serine or threonine. These complex macromolecules attached to the core protein are called proteoglycans (PG). Figure 3 shows the basic structure of proteoglycans.

Glycosaminoglycans are produced by almost all the cells. GAGs, in the form of proteoglycans, can either be secreted into the extracellular matrix or reside in the plasma membrane. They have several functions which includes cell adhesion, growth, structure, signaling, differentiation, and anticoagulation, angiogenesis, metastasis, etc. As GAGs have diverse cellular roles, it is not surprising that when metabolism of GAGs is disrupted several different diseases may result.

Clinical Symptoms
In the MPSs, GAG accumulation leads to cell, tissue and multiorgan dysfunction, making it a multisystem disorder. Depending on the type of MPS pathology of the disease in certain organs is more predominant. The clinical signs may include soft tissue periarticular masses, dysmorphic facial features, short stature, organomegaly, CNS disease, etc. The clinical symptoms of all of the MPS types are related to the type of GAG accumulated, and the syndromes sharing clinical signs share a common profile of GAG accumulation. For example all...
the types accumulating HS manifest neurodegenerative symptoms and the types where dermatan sulfate (DS) is accumulated show skeletal disease. Those diseases sharing both HS and DS accumulation manifest both neurodegenerative skeletal disease.

Based on the clinical systems affected, MPS disorders can be divided into three major categories: 1) disease primarily affecting the CNS; 2) disease primarily affecting skeletal/connective tissue; and 3) affecting both systems. Roughly half of the MPSs show disease in both systems, with the others showing disease in one major system. Mucopolysaccharidosis type III primarily affects the CNS, and MPS IV, VI, and IX primarily affect skeletal/connective tissue whereas MPS I, II, and VII manifest disease of both systems. Clinically, MPS I and II are quite similar, both having cognitive and somatic impairments. MPS I is an autosomal recessive disorder whereas MPS II is X-linked syndrome. The clinical symptoms of severe forms of MPS I and II include skeletal deformities, hepatosplenomegaly, coarse facial features, ear, nose, and throat infections, short stature, and learning difficulties. Progressive neurodegenerative, cardiac, and respiratory disease is also observed. Corneal clouding has been seen in MPS I, VI, and VII patients but not in MPS II. MPS III also known as Sanfilippo syndrome is characterized by CNS degeneration and mild somatic impairment. Clinical symptoms of severe form of MPS III include hepatosplenomegaly, delayed development, aggression, cortical atrophy, dementia, hyperactivity, hearing loss, etc. which leads to a semi-vegetative state and an early death.

Patients with MPS IV and VI syndrome have predominantly skeletal dysplasia. Clinical signs include skeletal deformities, short stature, respiratory disease, cardiac impairments, hearing impairment, mild corneal clouding, decreased mobility, cervical spine instability, etc. MPS VII or Sly syndrome resemble MPS I and II in their clinical manifestations. Only a few cases of MPS IX have been described. Within most of MPS types a spectrum of clinical signs is observed
<table>
<thead>
<tr>
<th>ID</th>
<th>Eponym</th>
<th>OMIM</th>
<th>Enzyme deficiency</th>
<th>Gene/locus</th>
<th>Accumulated GAG</th>
<th>Primary disease manifestation</th>
</tr>
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<tbody>
<tr>
<td>I-H</td>
<td>Hurler</td>
<td>607014</td>
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<td>IDUA</td>
<td>Dermatan sulfate, heparan sulfate</td>
<td>Bone and CNS</td>
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<td>I-S</td>
<td>Scheie</td>
<td>607015</td>
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<td>I-H/S</td>
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<td>α-L-Iduronidase</td>
<td>IDUA</td>
<td>Bone and CNS</td>
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<td>Dermatan sulfate, heparan</td>
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<td>II</td>
<td>Hunter</td>
<td>309900</td>
<td>Iduronate sulfatase</td>
<td>IDS</td>
<td>Dermatan sulfate, heparan sulfate</td>
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<td>IIIB</td>
<td>Sanfilippo C</td>
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<td>HGSNAT</td>
<td>Heparan sulfate</td>
<td>CNS</td>
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<td>Morquio A</td>
<td>253000</td>
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<td>GALNS</td>
<td>Keratan sulfate, chondroitin 6-sulfate</td>
<td>Bone</td>
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<td>IVB</td>
<td>Morquio B</td>
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<td>β-Galactosidase</td>
<td>GLB1</td>
<td>Keratan sulfate</td>
<td>Bone</td>
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<td>V</td>
<td>No longer used (formerly Scheie syndrome)</td>
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<td>253200</td>
<td>Arylsulfatase B</td>
<td>ARSB</td>
<td>Dermatan sulfate</td>
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<td>VII</td>
<td>Sly</td>
<td>253220</td>
<td>β-glucuronidase</td>
<td>GUSB</td>
<td>Keratan sulfate, heparan sulfate</td>
<td>Bone and CNS</td>
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<tr>
<td>IX</td>
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<td>601492</td>
<td>Hyaluronidase</td>
<td>HYAL1</td>
<td>Hyaluronan</td>
<td>Bone and soft tissue</td>
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ranging from attenuated to severe symptoms. Thus a single class of MPS can be further sub
categorized on the basis of the severity of the clinical manifestations of the disease. For example
MPS I is sub-classified based on the severity of the clinical symptoms. A severe form of MPS I,
also known as Hurler syndrome, is named after Dr. Gertrud Hurler who first described the
disorder in 1919 (9). In 1962 Dr. Herold Scheie diagnosed a disorder in adults which resembled
Hurler’s syndrome but in attenuated form (initially classified as MPS V). This attenuated form of
MPS I is now known as Scheie syndrome (10). There is also an intermediate form of MPS I,
which is not as severe as Hurler syndrome and not as attenuated as Scheie, thus it is known as
Hurler-Scheie syndrome. Mucopolysaccharidosis type III also has phenotypic variability but to a
lesser degree than seen in MPS I, with MPS III patients still having substantially shortened
lifespan and neurodegenerative disease.

**Mucopolysaccharidosis type III**
As mentioned earlier, MPS III is an MPS disorders characterized by the exclusive
primary accumulation of HS. Mucopolysaccharidosis III is also known eponymously as
Sanfilippo syndrome after the pediatrician Sylvester Sanfilippo who first described MPS III
patients in the early 1960’s (11). Clinically patients of Sanfilippo syndrome are similar to other
MPS disorders with the exception that this syndrome is primarily characterized by severe
nervous system degeneration and mild somatic implications (reviewed in Valstar (16) and
Kakkis (8)). Like other type of MPS disorders clinical variation is observed in MPS III as well
but the attenuated forms of Sanfilippo syndrome are difficult to recognize and may be
misdiagnosed. Even in patients with severe forms of MPS III there is a delay in the diagnosis due
to mild somatic disease manifestations and the absence of coarse facial features.
Sanfilippo syndrome has four types in humans, type A through type D (type IIIE is only found in animals (12)). These four human MPS III types accumulate the same substrate (HS) in cells and tissues and show similar clinical symptoms but are categorized on the basis of the individual patient's enzyme deficiency. Due to the similar clinical signs it is impossible to distinguish patients with different subtypes of Sanfilippo syndrome on a clinical basis. The common clinical symptoms include delayed development, sleep disorders, hyperactivity, aggression, hearing loss, delayed speech articulation, neurodegeneration, progressive cortical atrophy, and dementia. Loss of attention span and social and adaptive skills have also been observed in most of the patients. Clinical onset of the disease is usually observed between the age of two to six, with severe neurological degeneration being observed by the age of six to ten in patients with severe Sanfilippo syndrome (13). Patients with MPS III have been known to show autistic behavior before the onset of severe neurodegeneration (14). This aspect of the disease may be useful to evaluate the effect of any future therapy, but currently there are no approved therapies.

One thing common between all the types of MPS III is the accumulation of HS. Exclusive primary accumulation of HS is the defining characteristic of MPS III but HS accumulation in itself is not unique to MPS III, as it is also accumulated in MPS I, II, and VII (Table 1). Heparan sulfate proteoglycans have numerous roles with most being localized on the cell surface (13). The normal function of HS as part of proteoglycans aside, the mechanism of neurodegeneration resulting from lysosomal HS storage is not clearly or completely understood.

**Heparan Sulfate**

Heparan sulfate is composed of repeating units of uronic acid with 1→4 linkages to glucosamine. The uronic acid residue consists of D-glucuronic acid or L-iduronic acid units
which are then attached to alternating units of \( \alpha \)-linked glucosamine. The uronic acid residues are sulfated and the glucosamine residues are either sulfated or acetylated on the amino group and sulfated on the 6-hydroxyl group. This makes heparan a compound which may vary by the degrees of sulfation and the ratio of glucuronic and iduronic acid residues. Such structural diversity is observed both among the individual disaccharide units within a HS chain, or between different chains overall. Heparan sulfate overall consists of three different repeated domains; NA-domains (N-Acetylated), S-domains (N-Sulfated), and mixed regions which consists of both NA and S-domains. The low acetylated regions like NA-domains are flexible segments between the S-domains giving structural and conformational variability to HS. This conformational variability is important for multi-functionality of HS. Heparan sulfate resembles heparin to a high degree but there are minor structural differences which cause the difference in the functions of HS as compared to heparin. Heparin in general resembles the S-domain of HS hence it is mostly composed of sulfated disaccharides. Also, the ratio of iduronic acid in heparin is higher compared to HS. Heparin is used pharmacologically as an anti-coagulant and has a highly conserved region. Whereas, due to the conformational flexibility and the structural diversity of HS is a part of cell-cell signaling and cell-protein interactions. Heparan sulfate is mostly found either in the plasma membrane or the extra cellular matrix (reviewed (15)).

Heparan sulfate is a structurally complex polymer hence different enzymes are a part of the degradation pathway of HS. A total of three glycosidases, five sulfatases and one acetyltransferase are known thus far to catalyze HS. Each of these enzyme deficiency is associated with a lysosomal storage disorder. Figure 4 shows the degradative pathway of HS along with the associated Sanfilippo disorders (8). Glycosidases are responsible for cleaving the disaccharide terminal units where \( \alpha \)-L-iduronidase cleaves L-iduronic acid and \( \beta \)-glucuronidase
cleaves glucuronic acid as the names suggest. Both L-iduronic acid and glucuronic acid disaccharide units are common amongst different types of GAGs thus deficiency of α-L-iduronidase causes accumulation of HS and DS (MPS I) whereas β-glucuronidase deficiency leads to accumulation of HS, DS, and chondroitin 6-sulfate (MPS VII). The third glycosidase is α-N-acetylglicosaminidase which cleaves N-acetylglicosamine unit of HS. The deficiency of this enzyme leads to MPS IIIB. There are five known sulfatases in the catabolic pathway of HS which includes iduronate sulfatase, glucuronate 2-sulfatase, heparan N-sulfatase, N-acetylglicosamine 6-sulfatase, and glucosamine-3-O-sulfatase. Iduronate sulfatase removes the sulfate from iduronic acid, the deficiency of which leads to accumulation of both HS and DS and causes MPS II. Glucuronate 2-sulfatase is required for the desulfation of sulfated glucuronic acid. This enzyme has not yet been associated with any lysosomal storage disease in humans. Heparan N-sulfatase removes sulfate from the amino group of glucosamine unit, it is also known as sulfamidase. The deficiency of this enzyme leads to MPS IIIA. N-acetylglicosamine 6-sulfatase desulfates the 6-sulfated N-acetylglicosamine in both α or β linkage. The deficiency of this enzyme leads to MPS IIID which is one of the rarest types of MPS III syndrome in humans. The last known sulfatase is arylsulfatase G (ARSG) which is a glucosamine-3-O-sulfatase which removes the 3-O-sulfate group from the glucosamine unit. The deficiency of this enzyme have not been observed in humans but recently an ARSG knock mouse model has been characterized. ARSG deficient mice showed HS accumulation and same phenotype as other MPS type IIIIs murine models. This new form of MPS III is named MPS IIIE. The acetyltransferase; enzyme for IIIC; is the only enzyme in the degradation pathway of HS which is not a hydrolase. It does not degrade HS directly but it is crucial for the catabolism. Acetyl-CoA: α-glucosaminide N-acetyltransferase is the enzyme responsible to the acetylation of the amino group of
Figure 4: Degradation pathway of HS

Heparan degradation pathway focused on Sanfilippo syndrome. Enzymes in purple represent glycosidases, orange represents sulfatases, and purple represents transferases. This pathway is inspired from (8) with modifications.
glucosamine. After heparan N-sulfatase removes the sulfate group from the glucosamine amino group N-acetyl transferase attaches the acetyl group to the same exposed amino group. It is a membrane bound protein which acetylates itself on the cytoplasmic side by the transfer of acetyl group from acetyl-CoA and on the luminal side due to the acidic pH this acetyl group is transferred to the glucosamine residue. The deficiency of this enzyme is responsible for causing MPS IIIC. This enzyme is membrane bound thus application of already existing therapies like enzyme replacement therapy is not possible and it also complicates development of new therapies targeting MPS IIIC.

**Epidemiology of Sanfilippo syndrome**
Sanfilippo syndrome comprises a group of rare autosomal recessive diseases. The prevalence of MPS III varies by population studied. Meikle et. al. while looking at the prevalence of LSDs in Australia also looked at some LSDs individually (5). The MPSs had a combined prevalence of 1 per 22,500 live births. Mucopolysaccharidosis type IIIA showed to have a prevalence of 1 in 114,000; MPS IIIB 1 in 211,000; MPS IIIC 1 in 1,407,000; and MPS IIID 1 in 1,056,000 live births. This data clearly suggests that MPS IIIC and IIID are rarest of the MPS III types (5). In the Czech Republic from 1975 to 2008 the combined prevalence of all types of MPS was 3.72 compared to MPS III which was 1.91 per 100,000 live births. The prevalence of MPS IIIA was 0.47, MPS IIIB was 0.02, MPS IIIC was 0.42, and for MPS IIID the prevalence was 0.00 per 100,000 live births (16). In a study carried out in Northern Portugal from 1982 to 2001, the prevalence of all types of MPS was 4.8, MPS III was 0.84, MPS IIIA 0.00, MPS IIIB was 0.72, and MPS IIIC was 0.12 in 100,000 births (17). In the Netherlands, the birth prevalence of all types of MPS was 4.5 per 100,000 births from 1970 to 1996. The prevalence of MPS III was 1.89 where the prevalence of MPS IIIA was 1.16, MPS IIIB 0.42, MPS IIIC 0.21, and MPS IIID was 0.10 per 100,000 births (18). Another study looked at the
prevalence of MPSs in Scandinavian countries which was calculated as the number of MPS patients alive as of December 31, 2007. MPS III prevalence in Norway was 4, in Sweden prevalence was 15, and in Denmark prevalence was 5 per 0.88, 15, and 0.92 million inhabitants respectively (19). In Germany the incident rate of MPS III was 1.57 per 100,000 births from 1980 to 1995 (20).

Table 2: Prevalence of Mucopolysaccharidoses in different countries.

<table>
<thead>
<tr>
<th>ID</th>
<th>Czech Republic</th>
<th>Netherland</th>
<th>Portugal</th>
<th>Denmark</th>
<th>Sweden</th>
<th>Norway</th>
<th>Australia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per 100,000 live births</td>
<td>Per million inhabitants</td>
<td>Per live births</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPSs</td>
<td>3.72</td>
<td>4.5</td>
<td>4.8</td>
<td>33/6.03</td>
<td>39/4.24</td>
<td>32/7.06</td>
<td>1/22,500</td>
</tr>
<tr>
<td>MPS III</td>
<td>0.191</td>
<td>0.84</td>
<td>0.75</td>
<td>5/0.91</td>
<td>15/1.63</td>
<td>4/0.88</td>
<td>--</td>
</tr>
<tr>
<td>MPS IIIA</td>
<td>0.47</td>
<td>1.16</td>
<td>0.00</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1/114,000</td>
</tr>
<tr>
<td>MPS IIIB</td>
<td>0.02</td>
<td>0.42</td>
<td>0.72</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1/211,000</td>
</tr>
<tr>
<td>MPS IIIC</td>
<td>0.42</td>
<td>0.21</td>
<td>0.12</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1/1,407,000</td>
</tr>
<tr>
<td>MPS IIID</td>
<td>0.00</td>
<td>0.10</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1/1,056,000</td>
</tr>
</tbody>
</table>

Looking at the prevalence of types of MPSs in different countries it is clear that MPS IIIC and MPS IIID are the rarest amongst all the types of MPS III. Due to the lack of patients it becomes hard to study the pathology of the disease and the development of new therapeutic strategies is also impeded. Thus animal models of these disorders are critical for further study of the disease at a cellular and molecular level, as well as to investigate the pathology of the disease and the effect of different therapies.

*Animal Models of Mucopolysaccharidosis III*

The clinical symptoms and pathology of genetic disease can be evaluated in human patients but in depth study of the pathology and of molecular mechanism of disease is difficult to study directly in the patients due to practical and ethical considerations. This situation is
compounded in rare hereditary diseases with few patients to study. The use of animal models throughout the history of medical sciences has accelerated our understanding of disease and our development of therapies. Both small (e.g. mice) and large (e.g. dog) have both benefits and limitations, but both have proven to be important for investigating rare genetic disorders. These animal models may result for genetic engineering (i.e. a knock out model), or may result from naturally occurring disease causing mutations.

The selection of an animal model for any particular metabolic disorder is dependent on many factors. For example to investigate the pathology of the disease the use of small animal models like murine models has advantages as they have a short life span, are highly inbred, have shorter gestation time, can generate large number of affected animals, are easily manageable, and cost effective. Murine models are also effective for studying the initial response to any new therapy, in so called proof of principle studies. Large animal models like feline and canine models are very useful as they exhibit similar neurological, pathological, clinical, and biochemical characteristics to human patients. Also, such models are crucial for studying the long term effects of any therapy. Not only do such models help evaluate how effective a therapy is in the long run but, but they allow long term evaluation of potential side effects of a therapy in ways that may not be achievable with murine models. For example while working on developing a therapy for Niemann-Pick C disease, the administration of the drug hydroxypropyl-beta-cyclodextrin (HPβCD) attenuated the effects of the disease in murine models. The same therapy when carried out in a feline model showed that after few dosages of 4000mg/kg or one dosage of 8000mg/kg in cats, there was a significant increase in auditory threshold (21). This side effect of using high dosage of HPβCD was not easy to notice in murine models.
The use of animal models to study MPS disorders has a rich and long history. Below I will only discuss the murine models of MPS type III.

**Murine Models of MPS III**

*MPS IIIA*

The murine model of MPS IIIA is a naturally occurring mouse model. It was discovered in 1999 by a group at Albert Einstein College of Medicine working with a colony of knockout mice with mannoside acetylglucosaminyltransferase 3 (*Mgat3*) deficiency (22). A fourteen month male mouse was found to have behavioral changes and scruffy appearance. This mouse was not the only one and others were also found among both *Mgat3*-/− and *Mgat3*+/+ mice, indicating the mutated line was separated from the *Mgat3* line. The life span of the mice was 7-10 months and mice at end stage had enlarged bladder, less active, rough hair coat, hepatosplenomegaly, etc. The clinical onset of the disease was observed between six to seven months of age and at approximately seven months of age corneal opacity was also noted. Upon further investigation the mutated mice showed zebra bodies and clear cytoplasmic accumulation in cells on ultrastructure from different organs including brain, liver, kidney, etc. The storage was within the lysosomes as revealed through LAMP1 antibodies staining. Urine and brain were tested for GAG accumulation demonstrating accumulation of HS. In total six hydrolase enzymes assays were carried out which showed that only glucosamine-N-sulfamidase had reduced activity (~3%) whereas the rest of the enzymes had elevated activity. This mouse model was characterized as MPS IIIA and soon afterwards a novel point mutation was identified in the sulfamidase gene in affected mice which was different from the mutations found in MPS IIIA patients. The MPS IIIA mice carried a missense mutation (c.G91A) which led to the change in corresponding amino acid from aspartate to asparagine (p.D31N) (23).
This mouse model has been used extensively by different groups to study pathology as well as therapeutic strategies. The MPS IIIA murine model was investigated for behavioral deficits which are observed in human MPS IIIA patients. The MPS IIIA mice showed deficits in motor function by 3 weeks of age in an open field test. Neuromuscular strength, gait, and negative geotaxis responses showed impairments beginning 15 weeks of age (24). In 2004 Hopwood’s group looked at the response to intraperitoneal and intravenous enzyme replacement therapy (ERT) in MPS IIIA murine models (25). There were two groups of mice, one group started ERT after birth (day 0) and the second group started ERT at six weeks of age. Animals were given the enzyme every week till 20 weeks of age. The MPS IIIA affected male mice showed aggression starting at 8-10 weeks, the onset of aggression was delayed in early ERT group till 17 weeks of age. Animals were also tested for spatial learning abilities at 20 weeks of age by Morris water maze; a common test for their ability to learn and their memory. Both normal and early ERT MPS IIIA mice showed decrease in search time on 3, 4, and 5th day of the test. The untreated IIIA and delayed ERT IIIA mice showed no improvement in the search time in Morris water maze test. Animals with early ERT had a significant reduction in accumulated GAG in brain as well as other tissues. Hence, early ERT attenuated cognitive deficits, behavioral aggression, and GAG accumulation in most tissues including brain before maturation of blood brain barrier. Intracerebral injection of recombinant human sulfamidase directly in mouse CNS at different age points showed that early administration was successful in attenuating the effect of the disease but was not able to reverse the already existing signs of neurodegeneration such as axonal spheroids which are postulated to interfere with retrograde axonal transport. Whereas, injection of a high dose of recombinant human sulfamidase in cerebral spinal fluid (CSF) via cisterna magna reduced GAG storage in brain by up to 62% and in spinal cord by up to 71%
Reduction in axonal spheroids was also observed which was not seen in previous intracerebral injection (25). Intraventricular administration of adeno-associated vector (AAV) containing the gene for sulfamidase (SGSH) and Sulfatase modifying factor 1 (SUMF1; activator of sulfatases) showed synergistic increase in sulfamidase activity which resulted in reduction of GAG and improvement of motor and cognitive impairments in IIIA mice (27). The MPS IIIA model has also been investigated for the effect of substrate reduction therapy (SDT). Substrate reduction therapy uses the strategy of reducing the synthesis of the accumulated substrate which results in the balance of synthesis and degradation of substrate. As a therapeutic approach, SDT is useful when there is at least some activity in the affected individuals, such that there is not enough to degrade all the substrate hence the resulition. As mentioned earlier IIIA mouse model has about 3% of sulfamidase activity (22). Thus this model has been used to evaluate SDT. Rhodamine B has been known to reduce GAG synthesis and weekly administration of rhodamine B for six months in MPS IIIA mice showed reduced levels of GAGs in multiple tissues as well as excreted urine, and improvement in spatial learning and memory (28, 29).

The MPS IIIA mouse model has been crucial in evaluating different therapies, with ERT and gene therapy being the most successful therapies, with bone marrow transplant and SDT as comparatively less successful (24-30). This model has also been useful for determining effective use of therapies like 1) determining most effective site of injection for ERT via CSF, e.g. ventricular route was most successful while lumbar was least successful (31), 2) Combination of multiple therapies, e.g. hematopoietic stem cell therapy (HSCT) in combination with gene therapy improved the pathology as well as the behavior in MPS IIIA animal whereas HSCT alone did not show effective results previously (32), 3) determining effective enzyme dose, e.g. continual low-dose of enzyme proved to be more effective than intermittent high-dose of enzyme
(33), 4) effects of targeting secondary substrate storage on the pathology of the disease, e.g. targeting ganglioside accumulation by N-butyledeoxynojirimycin (NB-DNJ); which reduces ganglioside synthesis; improves learning and fear response in IIIA mice (34).

**MPS IIIB**

In 1999 Li *et al.* generated an MPS IIIB mouse model by disruption of exon 6 of the Naglu gene resulting in α-N-acetylgalcosaminidase deficiency (35). This knock out MPS IIIB mouse model was studied in detail and appeared normal while young but showed urinary retention by the age of six months. The MPS IIIB mice survived from 8-12 months and at approximately 12 months the mice were euthanized due to extensive urine retention, skin ulcer around the genitalia, walking difficulties, and unkempt fur etc. Gross pathology of several tissues including the brain showed vacuolated macrophages. Tissues were examined at multiple ages which showed that the observed pathology became more prominent with age proving the progressive nature of the disease. Also, in an open field test older MPS IIIB mice (4.5 months old) showed more suppressed activity than the controls whereas the younger MPS IIIB mice (3.5 months old) did not have any significant difference in activity compared to controls. Enzyme activity of α-N-acetylgalcosaminidase was unmeasurable in affected mice and was half normal activity in heterozygous animals. The lowest α-N-acetylgalcosaminidase activity was observed in brain. The activity of several other hydrolytic enzymes was also tested most of which had elevated activity. There was a considerable increase in soluble GAGs (sGAGs) in multiple tissues in affected mice compared to controls. In brain tissues even controls showed a considerable amount of sGAGs and a slight increase was seen in affected mice. The accumulated sGAGs were treated with HS lyase I which removed almost all the accumulated sGAGs proving that the accumulated sGAG was HS. Along with GAG accumulation, a secondary accumulation of gangliosides (GM2, GM3) was also observed. This MPS IIIB murine model had phenotypical,
pathological, biochemical, and behavioral similarities with human form of MPS IIIB thus this model has been extensive used to explore different aspects of the disease.

Murine model of MPS IIIB has been extensively used to study the molecular and cellular basis of the disease as well as the pathology. In 2002, the same group which characterized the murine model also observed an increase in the relative density of reactive astrocytes in affected mice. These astrocytes showed limited response to injury unlike astrocytes in control mice (36). Also, inhibition of neurogenesis was seen in affected mice as early as one month of age. The FGFR-1 mRNA levels in MPS IIIB mice were also reduced, possibly due to the accumulation of HS (36). Activation of microglia was also observed (37). These changes in neuroplasticity, neural cell growth, and microglial activation in MPS IIIB mice may be crucial in determining the molecular and cellular basis of pathology in humans as well as for the development of new therapies to target these disease manifestations.

The generation of an MPS IIIB murine model allowed for development of therapies which could be tested efficiently and economically on a small scale. Yu et al in 2000 looked at the short term enzyme replacement therapy in MPS IIIB mice (38). The recombinant human Naglu (rhNaglu), prepared from Lec1 mutant Chinese hamster ovaries, was administered intravenously three times with two day intervals at three months of age. As substantial amounts of the enzyme were only seen in liver and spleen, the dose of the enzyme was increased and mice were administered enzyme five times instead of three times at three months of age. Still most of the enzyme activity was seen in the liver and spleen with lesser amounts were seen in other organs. This study showed that intravenous administration of the enzyme might not be an effective therapy for MPS IIIB as no activity was observed in brain. Similar results were seen in a gene therapy study of intravenously administered a lentiviral-Naglu bearing vector (39).
Transgene expression was observed in some organs but not in the brain. Thus intravenous administration of enzyme or gene therapy may not be useful for neurodegenerative diseases like MPS IIIB as enzyme or vector cannot pass the blood brain barrier. In 2002 Haiyan Fu et al., tested two adeno-associated viral (AAV) vectors with different promoters for gene therapy (40). The vectors contained human Naglu cDNA and either the cytomegalovirus (CMV) or neuron-specific enolase (NSE) promoter. These vectors were tested in cell culture before in vivo studies. Vectors were directly injected into different brain areas of adult MPS IIIB mice. For in vivo studies, the vectors also contained the marker gene enhanced green fluorescent protein (EGFP) to evaluate the distribution of the vector in the brain. After the microinjection of the vector (1 µl AAV) directly into the brain, transduction was seen only in the surrounding neurons of the site of injection. Nevertheless long-term correction was observed, with a decrease in lysosomal storage in a larger area surrounding the site of injection. Thus, secreted Naglu was able to defuse to the surrounding neurons. In another study, a single injection of vector (AAV type 2 or AAV type 5) directly into the brain at 6 weeks of age improved pathology which extended beyond the areas of the injection site (41). The accumulation of secondary substrates GM2 and GM3 was also reversed. Direct administration of the vector into the brain removes the difficulties and problems associated with the blood brain barrier but the practicality of such a therapy in humans is limited as multiple areas would need to be injected to treat the comparatively larger human brain. In 2004 Zheng et al., looked at the transplantation of gene-modified bone marrow in murine model of MPS IIIB (42). This strategy was particularly helpful in reducing the pathology of the disease in the brain. As IIIB is primarily a neurodegenerative disease this strategy could prove an effective treatment model, but due to the safety concerns of retroviral vectors, this therapy may present risks when applied to humans. Substrate reduction therapy has also been tested in this
murine model. Genistein, a soy isoflavon, and a protein tyrosine kinase inhibitor, was administered daily in affected and control mice. Improvement was seen in the total amount of GAG in liver but not in the brain, possibly due to the blood brain barrier (43). The blood brain barrier remains the greatest obstacle to developing effective therapies for neurodegenerative diseases including MPS IIIB. The murine model of IIIB has been used to assess therapeutic agents that cross the blood brain barrier. To target the blood brain barrier mannitol infusion has been used. Mannitol is an osmotic agent which is used in medicine to open the blood brain barrier for a short time. Using mannitol before intravenous administration of an AAV type 2 vector with the Naglu cDNA; helped the transduction of the CNS (44). As the blood brain barrier is open for only a short time period, the timely administration of the vector is critical to ensure effective results. McCarty et al., showed that administration of intravenous AAV eight minutes after mannitol infusion gives a 10 fold increase in vector transduction of CNS. This strategy addresses the blood brain barrier, but is also complicated by the detrimental effects of the regular infusion of mannitol. Recently AAV type 9 has been shown to cross the blood brain barrier. A single intravenous injection in adult mice improved the behavior and the life span of the affected animals (45). Thus far AAV9 has shown the most promising results, and pretreatment by mannitol further improves the outcome. Another strategy tested in an effort for developing a therapy for MPSs is targeting the previously proved cell traffic of nucleated maternal cells in to the fetus during pregnancy (46). Mononuclear cells from human umbilical cord blood intravenously administered to a pregnant heterozygous mouse at fifth day of gestation transmigrated into the embryos successfully, and the enzyme activity in all the embryos was similar to the heterozygous parents. In conclusion, thus far different strategies have been tested to treat MPS IIIB but all have advantages and limitations, and to date, none have been approved.
**MPS IIIC**

A murine model for MPS IIIC has been recently characterized by C. Martins et al. in 2015 (47). This murine model was created through gene trap technology (Zambrowicz et al. 2003) and produced in C57BL/N6 murine line in 2008 by Hansen et al. (48, 49). In the MPS IIIC murine model, a functional fusion of the β-galactosidase and neomycin resistance (aminoglycoside 3’-phosphotransferase) genes, was inserted after exon 7 of the Hgsnat gene the deficiency of which causes IIIC. The inserted sequence also contained a stop codon such that the exons downstream of the inserted sequence remain untranslated. Therefore the translated protein contains the Hgsnat amino acids encoded from exon 1 to 7 fused to β-galactosidase and aminoglycoside 3’-phosphotransferase. As expected the homozygous Hgsnat-Geo mice had reduced N-acetyltransferase activity but an elevated β-galactosidase activity. The activity of most of the other lysosomal enzymes was also elevated which is expected, as similar results were seen in the MPS IIIB and MPS IIIA murine models. The Hgsnat knockout mice conformed to Mendelian inheritance of an autosomal recessive disease. Affected animals were normal at birth and fertile as adults. No visible differences were observed between affected and normal animals until 11 months, after which mice showed abnormalities in gait. Affected mice didn’t differ from the wild type mice motor fuction assessments. Accelerating rotorod was used to test motor coordination and showed no significant difference between affected and normal mice. Whereas the affected mice did show hyperactivity and impaired learning which increased with age. Pathologically these mice were similar to other MPS murine models. Also, in addition to the storage of GAGs, secondary accumulation of gangliosides was observed, as was seen in MPS IIIA and IIIB mice. The MPS IIIC murine model also showed activation of microglia and astrocytes, also observed in MPS IIIB mice. Neuroinflammation as well as structural changes in mitochondria were also noted.
The MPS IIIC mouse model showed similarities with other MPS murine models in overall pathology, biochemistry, phenotype, and behavior, but it also differed from other murine models. Urine retention and bladder prolapse was not observed until around 15 months whereas this is observed at around 9 months in MPS IIIB and IIIA mice. The MPS IIIC mice survived up to 80 weeks of age. This longer life span had not been observed previously in MPS III mouse models. This model is unusual as this MPS disorder involves a membrane bound acetyltransferase. This model will prove important to study MPS IIIC and to address the considerable challenge of treating a membrane bound enzyme deficiency.

**MPS IIIE**

The MPS IIIE is an unusual MPS III type, in that it has not yet been diagnosed in humans. The murine model was created by Kowalewski et al. in 2012 by knocking out the arylsulfatase G (ARSG) gene (12). Arylsulfatase G is responsible for removing a 3-O-sulfate from glucosamine units. Such glucosamine units are found in relatively low abundance in HS. The accumulated substrate in these mice was found to be HS, and the non-reducing HS terminus were 3-O-sulfated N-sulfoglucosamine units, thus confirming the role of ARSG deficiency in murine MPS IIID. In mutant mice lysosomal storage was seen around 12 months of age in brain sections which is consistent with other murine MPS III subtypes. Activation of microglia and astrocytes was also seen in mutant mice, similar to the observations of other MPS III murine models. Arsg-deficient mice showed cognitive impairment which increased with age but were normal in other behavior tests like rotorod and grip strength etc. These mice were similar to other murine models of MPS III in pathology, biochemistry, and behavior. Thus this murine model was categorized as a new fifth subtype of Sanfilippo syndrome which was named MPS IIIE or Sanfilippo syndrome type E.
Murine model of all Sanfilippo types exist except Sanfilippo type D. In the next chapter we have characterized a first model of Sanfilippo syndrome type D or MPS IIID.
CHAPTER 2:
CHARACTERIZATION OF A MURINE MODEL OF MUCOPOLYSACCHARIDOSIS TYPE IIID

Introduction
The Mucopolysaccharidoses (MPSs) are a class of lysosomal storage disorders caused by the deficiency of one or more enzymes which lead to an accumulation of specific profiles of glycosaminoglycan (GAGs) species. Mucopolysaccharidosis type III or Sanfilippo syndrome is a type of MPS characterized by the accumulation of heparan sulfate (HS). Clinically MPS III patients have severe CNS degeneration with an accompanying wide range of mild somatic signs. Clinical symptoms include hyperactivity, aggression, insomnia, and loss of social skills, mental skills, speech etc. which progresses with age (8). There are four known types of MPS III in humans caused by the deficiency of four different enzymes: heparan N-sulfatase (SGSH, EC 3.10.1.1); α-N-acetylg glucosaminidase (NAGLU, EC 3.2.1.50); α-glucosamine-N-acetyletranferase (HGSNAT, EC 2.3.1.191); and N-acetyleglucoamine-6-sulfatase (GNS/G6S, EC 3.1.6.14). These deficiencies cause MPS IIIA to MPS IIID respectively. A fifth type of MPS III, MPS IIIE, only exists in a murine knockout model, deficient in arylsulfatase G (ARSG) activity (12).

All types of Sanfilippo syndrome share clinical, behavioral, biochemical, and histological findings in common. The first cases of Sanfilippo syndrome were characterized in early 1960’s by Sylvester Sanfilippo (11). Variablity in phenotype has been observed in all subtypes but comparatively to a lesser degree than other MPS types, perhaps because milder forms are difficult to recognize and either remain undiagnosed or are misdiagnosed. The first characterization of MPS IIID was done by Kresse et al. in 1980 who studied skin fibroblasts
from patients with a phenotype similar to Sanfilippo syndrome (50). Accumulation of HS was shown, with the accumulated HS having a N-acetylglucosamine 6-sulfate on the non-reducing end. As the activity of the enzymes whose deficiencies cause MPS IIIA, IIIB, and IIIC were normal, it was postulated that the enzyme missing in those patients was α-N-acetylg glucosamine 6-sulfatase which desulfates the N-acetylglucosamine 6 sulfate subunit with α linkage of HS but not N-acetylglucosamine 6 sulfate subunit in keratan sulfate which is in β linkage. With isolation and characterization of the GNS enzyme it was shown that GNS desulfates the N-acetylglucosamine 6 sulfate subunit in both α and β linkage. Patients with MPS IIID only store HS because of an alternative pathway exists for the degradation for keratan sulfate that is not degraded by GNS (51). In 1988 the cDNA of GNS gene was clone from human liver and showed homology with steroid sulfatase. The gene was was localized to chromosome 12 by in situ hybridization (52-54). More than two decades after the characterization of the index case of MPS IIID, the genomic DNA from the patient’s fibroblast cell line revealed a homozygous nonsense mutation in exon 9 of 14 (c.C1063T) which led to a premature termination of the translation (55). More mutations were identified as new IIID patients were diagnosed (56, 57). Not unexpectedly, MPS IIID patients showed phenotypic variation in disease severity.

The MPS IIID patients have similar clinical signs and symptoms, with CNS degeneration predominating, as is seen in the other MPS III types. This is not unexpected as all MPS III patients accumulate the same substrate, i.e. HS. The MPS IIID patients also have mild somatic symptoms which include hypertelorism, coarse facial features, ataxia, dyspraxia, hearing loss, heart enlargement, and developmental delay, which are symptoms, again, seen in other MPS III subtypes. Histological investigations have shown moderate to mild vacuolation in multiple organs including kidney, liver, bone, spleen, thymus, lymph nodes, pancreas, and skeletal muscle
In all MPS III patients, the enzyme deficiency is present in all cells, but some organs and cell types are affected more than others. This phenomenon likely depends on the differing rate and degree of HS presentation to the lysosome in different cell types.

Since the characterization of MPS IIID in 1980 multiple cases have been studied and it has been shown that MPS IIID is likely the rarest type of Sanfilippo syndrome (5). With the lack of MPS IIID patients, the use of animal models becomes crucial for studying the pathology of the disease as well as developing new therapeutic strategies. Thus far spontaneous emu and canine models of IIIB (61, 62), canine and murine models of IIIA (22, 63, 64), and a Nubian goat model of IIID have been characterized (65). Other than the natural models, transgenic murine models exist for MPS IIIB, IIIC, and IIIE (12, 22, 35). Animal models have proven significant in investigating the molecular and physiological mechanism of MPS III disease.

For MPS IIID, most of the data thus far collected has come from studying the Nubian goat model. The pathology and biochemistry of the Nubian goat model of IIID, characterized in 1992, resembles the human disease biochemically and histologically (65, 66). This model was shown to have a nonsense mutation (C → T) resulting in truncated protein and an introduction of AluI restriction site (67). Along with the primary storage of un-catabolized HS, secondary storage of glycosphingolipids (GSLs) was also observed in the brain (68). Storage of secondary compounds is not unique to this model and has been observed in lysosomal storage diseases in general (69). In the Nubian model activity of sialidase, a degradative enzyme for ganglioside, and GalNAcT, an enzyme for complex GSLs biosynthesis, were reduced in affected animals (68) possibly the cause for elevated gangliosides. Further in-vitro studies showed that HS leads to inhibition of both sialidase and GalNAcT. Hence it was postulated that accumulation of GSLs was due to the disruption by HS of a balance in the degradative and biosynthetic enzymes. Even
though this large animal model was used to evaluate the basic pathology of the disease and response to therapy (preliminary data on response to ERT), in depth investigation of disease manifestation and response to further therapy was not pursued, likely due to practical and logistical concerns.

Murine models of MPS disorders have proven to be the most practical first line model to study LSDs. Murine models are well studied, they are cost effective, experiments are less time consuming, and animals are easy to manage, making them an ideal model organism. Therefore, with the advent of new technologies, transgenic murine models of MPS IIIB, IIIC, and IIIE have been created (12, 35, 47). As discussed in chapter one, these murine models have been extensively studied. These studies have explored pathogenesis, therapy response, therapy optimization, and biomarker investigation. Murine models for all MPS III subtypes exist except for MPS IIID, leading us to acquire and characterize the murine model of MPS IIID described below. This new model was assessed for clinical signs, biochemistry, pathology, behavioral deficits, and life span. We have also compared this model with already existing MPS III subtypes, specifically murine MPS IIIA and IIIB.

Materials and Methods

Knockout model of MPS IIID

A knockout murine model of MPS IIID was created by Taconic Artemis laboratory. The murine Gns gene contains 14 exons and resides on chromosome 10. A targeting vector was generated using clones from C57BL/6J bacterial artificial chromosome (BAC) library. The positive selection cassette in the vector consisted of puromycin resistance gene flanked by Flp-FRT recombination sites. The deletion of the puromycin resistance gene was Flp mediated. Taconic Artemis C57BL/6N Tac embryonic stem (ES) cell lines were quality tested and grown
on a mitotically inactivated feeder layer. ES cells were transfected with linearized DNA targeting vector. Exon 2 to 13 were replaced with the positive selection cassette removing an approximately 22kb region through homologous recombination. This resulted in a predicted transcript missing 12 of 14 exons with a frameshift in the remaining last exon resulting in a premature stop codon. Puromycin resistant cells were isolated and analyzed by southern blotting and/or PCR. Recombinant cells were expanded, frozen and used after molecular validation. Ten to fifteen recombinant ES cells were injected in to a blastocyst from BALB/c female mated with a BALB/c male. Blastocysts for ES microinjection were removed from the uterine after 3.5 days post coitus. Each uterine horn of pseudo pregnant females were injected with eight injected blastocysts. Based on the hair coat highly chimeric mice from the progeny were bred with C57BL/6 females. The schematic representation of the targeting vector sequence and the homologous recombination in ES cell line is shown is figure 5.

Generation of experimental animals

Animal care and all the procedures related to animals were approved by the institutional animal care and use committee (IACUC) and institutional biosafety committee (IBC) of Iowa State University. Using MPS affected males bred with C57BL/6 Jax females, the line was expanded and heterozygous females from the progeny were bred to either the affected male parent or to additional progeny heterozygous males. At most four females were kept in one cage and three males in one with 24 hour food and water access. Approximately 82 experimental mice were generated, out of which at least three animals each were used for histology and biochemistry from each genotypic line at four month time point. At eight month time point only MPS IIID and control mice were investigated. Sixteen animals each from MPS IIIB, IIID and wild type lines were chosen for behavioral testing. Six animals from the sixteen animals were harvested at twelve month time point for biochemistry and histology and the remaining 10
Figure 5: Generation of MPS IIID mouse: Generation of MPS IIID C57BL/6NtTac-Gnstm3465Arte by targeted disruption of GNS gene. FRT-mediated removal of puromycin sequence.
animals from each genotypic line continued behavioral testing and are also a part of Kaplan Meier curve. Animals were euthanized at humane end points either by CO₂ asphyxiation or perfusion after anesthesia. All the animals for biochemistry were euthanized by CO₂ asphyxiation and animals for histology were perfused and fixed.

Genotyping Animals
Animals were identified based on toe clips taken between postnatal day 9-14, with the same toe clips used for DNA extraction. The samples were used for PCR reaction to genotype MPS IIIA, MPS IIIB, and MPS IIID animals. For MPS IIID a multiplex PCR was used for MPS IIID loci and Flp transgene. Five set of primers were used for the multiplex PCR. Two for Flp, 3’-GACAAGCGTTAGTAGGACAT-5’ and 5’-GCTGCCACTCCTCAATTGGAT-3’, and three primers to distinguish between the wild type and MPS IIID animals which consists of a common primer 3’-GTACTCATTTTCGAGAAGACC-5’, a wild type primer 5’-CTTCAGTGGCCTTCAAGACCC-3’ and a primer for the knocked out locus 5’-AGTGCCACCAAGCAGA-3’. The expected fragment for Flp is 488bp, for MPS IIID knock out is 363bp, and for the wild type is 275bp. Flp carrying animals were identified for the purpose of breeding only Flp negative animals in later stages to rid the colony of the Flp transgene. No obvious differences were found between the animals with and without Flp. The primers and the PCR conditions for MPS IIIB mice were kept the same as previously published by Ellinwood’s group in 2012 (70).

The mouse colony was monitored every other day for clinical signs including illness etc., body conditions, movement, and pregnancy. Animals were euthanized when they reached humane end points which included rectal prolapse, vaginal prolapse, severe hunched posture, low body condition, extensive urine retention, difficulty in walking, and/or hyperextension.
Some animals of the study were euthanized due to clinical signs such as malocclusion, head tilt etc. which have not been associated with the disease and are common to the inbred mouse strain used.

**Rotorod**

Animals were tested on rotorod every month starting from two months of age. Each animal was trained for two consecutive days prior to its first rotorod test which was done on the third day. The rotorod was set at a constant speed of ten revolutions per minute and the direction of the revolving rod changed after each revolution. Animals were given three trials each to stay on the rotorod for 300 seconds. If an animal completed the target in the first trial, then other trials were not carried out but if not, then only the longest performance time was used in the final results. Mice were weighed before each rotorod test. A total of 48 animals were enrolled in rotorod; 16 each from MPS IIID, MPS IIIB and wild type lines. Six out of those 16 animals per genotypic group were harvested at 12 months for the 12 month time point biochemistry and histology data and the remaining animals were used for life span data. The remaining animals were removed from the rotorod evaluation cohort as animals reached the humane end points mentioned above. The wild type mice will be removed from the rotorod two months after the humane end points of animals of the other genotypic lines. For the statistical analysis parameters including weight, age, sex and genotypic group were taken into consideration.

**Biochemistry**

A minimum of three animals at four and twelve month time point were analyzed from each genotypic line for biochemistry data. At eight months of age only MPS IIID animals were compared with the wild type animals. Kidney, liver, and brain tissues were homogenized in a triton-saline solution (0.2% triton and 0.9% saline), such that tissue was homogenized in a volume that corresponded to 3µl triton-saline solution/1mg tissue or 10µl triton-saline
solution/1mg for wild type and affected tissues respectively. Protein assays were carried out on the same day of homogenization using Thermo Scientific Pierce BCA protein assay kit. Quantification of GAGs was done through Alcian Blue Dye (ABD) which binds to sulfated GAGs. Soluble total sulfated GAGs were quantified indirectly by measuring the amount of ABD, read at 595nm absorbance after the dissociation of ABD-GAG complex by the addition of 4 M guanidium hydrochloride. 50 µl of each homogenate was used for the GAG assays and soluble sulfated GAGs were measured per mg of the protein. Standard curve was generated by dilution of heparin sulfate in 0.3% BSA previously standardized with HS. To all the samples BSA was added to make the final concentration same as standards i.e. 0.3% BSA.

Tissue homogenates were also used for enzyme assays which is a fluoro metric assay using 4-methylumbelliferyl (4-MU) containing substrates. Cleavage of such a substrate by the enzyme in the tissue homogenate releases a florescent 4-MU molecule which emits a light at a specific wavelength when excited. The florescent molecules were read using a flurometer. For a secondary enzyme assay of β-glucoronidase all homogenates were diluted 1:1000 with nanopure water because less diluted samples had shown activity above the range of the flurometer used. For the assay 25 ul of the sample with 25 ul of water and 50 ul of substrate (4-Methylumbelliferyl-β-D-glucuronide by CalBiochem) in 0.1 M sodium acetate buffer was incubated for 4 hours at 37° C. After incubation 2 ml glycine stopper (0.32M glycine, 0.2M Na carbonate, pH 10.5) was added to each tube and the enzyme activity was calculated from the fluorescence as nMoles 4-MU/ml/hr per mg protein. Standard curve was generated by dilution of 0.2mM 4-methylumbelliferone stock.

A subset of liver samples from MPS IIID-/-, +/- and +/- each were sent to Dr. Patricia Dickson’s lab at UCLA LABioMed for detection of GNS activity. Samples were homogenized in
reaction buffer consisting of 0.2 M sodium acetate (pH 5.6), 20 mM lead acetate, and 0.01% Triton X-100. A two-step fluorometric measurement of GNS activity was performed as described previously by He et al. (71) with modifications. Volume of 2.5 µl tissue homogenate was incubated with 2.5 µl of 10 mM 4-methylumbelliferyl alpha-N-acetylglucosaminide-6-sulfate (4-MUGNS; Toronto Research Chemicals, Toronto, Canada) in reaction buffer at 37°C for 1-4 hours, and the reaction was then stopped by addition of 10 µl of phosphate-citrate buffer (0.4 M Na2HPO4 / 0.2 M citric-acid buffer, pH 4.7). 5 µl of concentrated rhNAGLU-IGFII conditioned medium (specific activity > 100 nmol/hr/µl; (72)) media was supplied and incubated for a period equal to the GNS reaction at 37°C to release 4-MU fluorophore. Reactions were quenched by the addition of 200 µl of glycine carbonate buffer, pH 10.5. Fluorescence measurements were obtained using a microplate reader at excitation and emission wavelengths of 360 nm and 450 nm, respectively. One activity unit of GNS was defined as 1 nmol of 4-MU converted substrate per hour (first step) at 37°C. Protein concentration was determined using Bradford Reagent (Bio-Rad Laboratories).

Catalytic activity of NAGLU and β-hexosaminidase (combined A and B isoforms) liver tissues was determined by hydrolysis of the fluorogenic substrates, 1.0 mM 4-methylumbelliferyl-N-acetyl-α-glucosaminide, and 1.25 mM 4-methylumbelliferyl-N-acetyl-β-glucosaminide (EMD Millipore Chemicals) in the incubation mixture, respectively. For both enzymes, a unit of activity is defined as release of 1 nmol of 4-methylumbelliferone (4MU) per hour. Protein concentration was estimated by the Bradford method, using BSA as standard.

Histology
Animals were perfused; after anesthesia; intracardially with approximately 30 ml saline and 20 to 30 ml 4% paraformaldehyde. After perfusion animals were placed in 10% neutral
buffered formalin at 4° C. Tissues were dehydrated in ethanol and xylene series and embedded in paraffin blocks which were sectioned at 5µm with a microtome. Sectioned tissues were placed on slides, deparaffinized, stained, and analyzed. All tissues were stained with hematoxylin and eosin (H&E) and brain sections were additionally stained with luxol fast blue (LFB). Stained tissues were evaluated by an American College of Veterinary pathologist at the College of Veterinary Medicine, Iowa State University.

Statistical analysis

Biochemistry and rotorod data were analyzed using mixed procedure of SAS (version 9.4). Multiple comparisons of least square means were performed to determine if there were any statistical difference at P<0.05. Rotorod data before 9 months of age (till round 7) were not included in the statistical analysis because no variation was observed in the performance between different groups as almost all animals complete the 300 seconds target.

Results

A total of ~82 animals were evaluated with approximately 28 MPS IIID, 22 MPS IIIB, 4 MPS IIIA, and 28 wild type animals. Animals were genotyped by PCR and the frequency of the progeny followed Mendelian inheritance (Figure 6). Animals were checked frequently for clinical signs. The MPS IIIB mice started showing signs between the ages of 9-12 months which included enlarged bladder leading to rectal/vaginal prolapse at which time animals were euthanized due to humane consideration. The MPS IIID mice showed similar signs of bladder enlargement which was seen even at gross examination at 8 months of age. Rectal and vaginal prolapse was not seen until 12 months of age and these animals had a better body condition than age matched MPS IIIB animals. The MPS IIIA mice were used for the four month time point only hence there was no data for the 12 month time point. But previously MPS IIIA mice have been shown to have similar clinical signs and behavior as MPS IIIB mice (22, 35). At gross
examination both MPS IIIB and MPS IIID mice had an enlarged bladder with approximately 3ml urine collected at 12 month time point (Figure 7). Affected mice showed reduced abdominal fat compared to C57BL/6 aged matched mice which have been shown to get obese with age.

Between the ages of 9 to 12 months MPS IIID mice were only distinguished from the wild type mice by the rough hair coat and comparatively low body condition. MPS IIID mice did not show signs of ataxia till 12 months of age.

Animals performed equally well on rotorod from round 1 to round 7, corresponding to 2-9 months of age. No significant differences were found at 9 and 10 months of age (round 8 and 9
respectfully) between the least square means of performance of the animals between different genotypic groups (figure 8). The MPS IIIB mice performed significantly worse from MPS IIID and normal (p=0.0002, 0.0001 respectively) at 11 months of age (round 10) whereas performance of MPS IIID mice was not significantly different from normal mice (p=0.8577). No significant differences were found between all three genotypic lines at 12 months of age (round 11). Figure 8 shows the least square estimate of rotorod performance of animals from each genotype along with the number of animals (n) evaluated at each round.

Biochemistry

In liver samples of approximately 4 months old animals both wild type and IIDD+/- animals showed GNS activity. Tissue samples from wild type animals showed comparatively higher enzyme activity than tissues from GNS+/- animals. The IIDD affected animals had no detectable GNS activity in approximately 4 month old liver samples (table 3). Same liver homogenates were assayed for NAGLU and β-hexoaminidase, both of which had elevated enzyme activity in GNS-/- tissues.

Table 3: GNS activity in liver along with secondary enzyme assays

<table>
<thead>
<tr>
<th>Genotype</th>
<th>GNS nmol/hr/mg protein</th>
<th>NAGLU nmol/hr/mg protein</th>
<th>β-Hex nmol/hr/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNS +/-</td>
<td>2.4</td>
<td>0.6</td>
<td>378.9</td>
</tr>
<tr>
<td>GNS +/+</td>
<td>3.3</td>
<td>0.6</td>
<td>375.4</td>
</tr>
<tr>
<td>GNS +/-</td>
<td>2.6</td>
<td>0.6</td>
<td>283.9</td>
</tr>
<tr>
<td>GNS -/+</td>
<td>1.3</td>
<td>0.9</td>
<td>665.1</td>
</tr>
<tr>
<td>GNS -/+</td>
<td>1.6</td>
<td>2.0</td>
<td>1516.9</td>
</tr>
<tr>
<td>GNS -/-</td>
<td>No detectable activity</td>
<td>1.7</td>
<td>2486.8</td>
</tr>
<tr>
<td>GNS -/-</td>
<td>No detectable activity</td>
<td>1.6</td>
<td>2531.1</td>
</tr>
<tr>
<td>GNS -/-</td>
<td>No detectable activity</td>
<td>1.6</td>
<td>1645.0</td>
</tr>
</tbody>
</table>
Quantification of accumulated soluble sulfated GAGs was done in different tissues at multiple time points (Figures 9 a-d). In kidney there were significantly higher levels of s-GAGs in MPS IIID affected animals compared to controls at 4, 8, and 12 month time point (p= 0.0028, <0.0001, and <0.0001 respectively). In liver levels of s-GAGs at 4 months old MPS IIID tissues were not significantly high whereas at 8 months and 12 months there was a significant increase in accumulated s-GAGs (p= 0.125, <0.001, 0.033 respectively). Tissues of MPS IIID mice were also compared with IIIA and IIIB mice. As expected no significant differences in s-GAGs were found between different MPS IIIs affected groups. Figures 9 a & b show the comparison of MPS IIIA, IIIB, and IIID at different time points. The activity of β-glucuronidase was high in affected animals at all time points (Figure 10 a & b). Changes over time are represented by different alphabets in the graph, same alphabets on different bars represent no significant difference between the two timepoints. At four and eight month time points there was no significant difference in activity between GNS-/- and unaffected animals in whole kidney (p= 0.266 and 0.076 respectively). Whereas at 12 month time point activity levels were significantly high in affected animals compared to the controls (p= 0.0053). Enzyme activity in liver was significantly elevated at all time points i.e. 4, 8, and 12 months (p= 0.019, <0.0001, and 0.0004 respectively).
Figure 8: Rotorod data

Round 8 to 11 corresponds to 9-12 months of age. Different rounds were not compared with each other as the number of “n” was significantly different at each round giving false positive results.
Figure 9a: Kidney: Accumulated s-GAG comparison of IIID with IIIB and IIIA. Least square means of soluble sulfated GAGs/mg protein. Four stars represent p value less than 0.0001, three stars p<0.001, two stars p<0.01, and one star p<0.05. Numbers above the bars represents the sample size. Changes over time represented by different alphabets in the graph, same alphabets on different bars represent no significant difference between the two bars.

Figure 9b: Liver: Accumulated s-GAG comparison of IIID with IIIB and IIIA. Least square means of soluble sulfated GAGs/mg protein. Four stars represent p value less than 0.0001, three stars p<0.001, two stars p<0.01, and one star p<0.05. Numbers above the bars represents the sample size. Changes over time represented by different alphabets in the graph, same alphabets on different bars represent no significant difference between the two bars.
Figure 9c: Kidney: Accumulated s-GAG comparison of IIID with wild type. Least square means of soluble sulfated GAGs/mg protein. Four stars represent p value less than 0.0001, three stars p<0.001, two stars p<0.01, and one star p<0.05. Numbers above the bars represents the sample size. Changes over time represented by different alphabets in the graph, same alphabets on different bars represent no significant difference between the two bars.

Figure 9d: Liver: Accumulated s-GAG comparison of IIID with wild type. Least square means of soluble sulfated GAGs/mg protein. Four stars represent p value less than 0.0001, three stars p<0.001, two stars p<0.01, and one star p<0.05. Numbers above the bars represents the sample size. Changes over time represented by different alphabets in the graph, same alphabets on different bars represent no significant difference between the two bars.
Figure 10: Secondary enzyme assays

Figure 10a: \(\beta\)-glucoronidase activity in whole kidney. Least square means of the enzyme activity in nmol/hr/mg protein. Four stars represent p value less than 0.0001, three stars p<0.001, two stars p<0.01, and one star p<0.05. Numbers above the bars represents the sample size. Changes over time represented by different alphabets in the graph, same alphabets on different bars represent no significant difference between the two bars.

Figure 10b: \(\beta\)-glucoronidase activity in liver. Least square means of the enzyme activity in nmol/hr/mg protein. Four stars represent p value less than 0.0001, three stars p<0.001, two stars p<0.01, and one star p<0.05. Numbers above the bars represents the sample size. Changes over time represented by different alphabets in the graph, same alphabets on different bars represent no significant difference between the two bars.
Histology

Hematoxylin and eosin stained sections of liver, kidney, spleen, eye, and multiple areas of the brain of 4 month old MPS IIID-/- mice showed cytoplasmic vacuolation. Mild to moderate paranuclear expansions of the cytoplasm of neurons was observed in cerebellar nuclei (figure 11 part a). Similar results were seen in the neurons of medulla oblongata (figure 11 part b). In the cerebellum there was no evident loss of Purkinje cells whereas some of the cells were found to be vacuolated (figure not shown). Neuronal satellite cells were also moderately vacuolated in the cerebral cortex (figure 11 part c). Brain sections stained with LFB showed dark blue granular cytoplasmic staining in cerebellar nuclei as well as medulla oblongata (figure 12 part a & b). Furthermore moderate amounts of paranuclear granular dark blue cytoplasmic staining was observed in cerebral cortex, midbrain, thalamus, and brainstem (figures not shown). Liver sections had glycogen vacuolation but at high magnification hepatocytes had fine peri/paranuclear vacuolations (figure 11 part d). Tubular epithelial cells in kidney were also finely vacuolated whereas medullary tubules looked similar to the control sections (figure 11 part e). There were no obvious pathological changes in spleen (figure not shown). Control mice did not show any pathological changes other than the artifacts like occasional dark neurons, glycogen vacuoles, and mild clear cytoplasmic vacuolation (figure 11 part f, g, h, i, j and figure 12 part d, e). At 13 months of age the differences between the affected and wild type animals were more obvious. Five sections of the brain were evaluated consisting of caudal-middle and rostral-middle medulla oblongata with cerebellum, rostral cerebrum, rostral thalamus, and olfactory bulbs. Extensive loss of Purkinje cells in the cerebellum was observed in GNS-/- animals with frequent stretches of no Purkinje cells and some stretches with normal Purkinje cells (figure 11 part k). Some of the Purkinje cells also had cytoplasm vacuolation. Neurons in the medulla oblongata had mild to moderate paranuclear accumulation of finely vacuolated
cytoplasm (figure 11 part l). Cortical, thalamic neurons, and glial cells also had mild to moderate paranuclear storage of vacuolated and eosinophilic cytoplasm (glial cells figure 11 part m). Hepatocytes in liver sections were swollen and significantly vacuolated where some clear vacuoles were artifacts due to lipid storage (figure 11 part n). Small aggregates of the Kupffer cells were often crenated and also had pale yellow material thus the vacuoles were possibly due to lipofuscin. In kidney occasional lipid type vacuolation was observed just like in liver. Cortical tubular epithelial cells had a foamy appearance and were moderate to abundantly vacuolated (figure 11 part o). Urinary bladder was markedly distended with urothelium equivocally vacuolated (figure not shown). Spinal cord contained finely granular eosinophilic material (figure not shown). No specific pathologic findings were identified in the remaining tissues: stomach, pancreas, colon, duodenum, testis, salivary gland (mixed), jejenum, cecum, heart, lung, sciatic nerve (small portion), and pelvic limb. Control sections did not have any specific pathological findings other than the occasional artifacts similar to the ones seen in four month old control mice (figure 11 part p, q, r, s, t). At approximately 13 months LFB stained brain sections had frequent positive cytoplasmic dark spots in cerebellar nuclei and medulla oblongata (figure 12 part g and h). Purkinje cells had low to moderate LFB positive staining (figure 12 part e). Eye sections of both four month and twelve months old animals did not have any specific pathological changes and the affected animals looked similar to the unaffected controls (figure not shown).
Figure 11: H&E sections

Hematoxylin & Eosin (H&E) stained tissue sections of 4 month old GNS-/ (a-e), normal (f-j), and 13 month old GNS-/ (k-o), normal (p-t) animals. Panels a) 400x cerebellar nuclei w/ 600x inset b) 600x medulla oblongata c) 600x cerebral cortex d) 600x liver e) 600x kidney f) 400x cerebellar nuclei w/ 600x inset g) 600x medulla oblongata h) 600x cerebral cortex i) 600x liver j) 600x kidney k) 200x cerebellar w/ Purkinje loss with 400x inset l) 600x medulla oblongata m) 600x glia n) 200x with 600x inset liver o) 600x kidney p) 200x cerebellar cortex with 400x inset q) 600x medulla r) 600x glia s) 200x with 600x inset liver t) 600x kidney.
Luxol Fast Blue (LFB) stained brain sections of 4 month old GNS-/-(a-c), normal (d-f), and 13 month old GNS-/-(g-i), normal (j-l) animals. Panels a), d), g), j) 600x cerebellar nuclei, b), e), h), k) 600x medulla oblongata, and c), f), i), l) 600x Purkinje cells.
Discussion

Considered in the aggregate, Sanfilippo syndrome is amongst one of the most common types of MPS. However, as a single enzymopathy, MPS IIID is amongst the rarest of the MPS IIIIs. There is no approved therapy for MPS IIID, and, due to the paucity of patients, development of new therapies by evaluation in patient populations is not feasible. Mouse models of all MPS III subtypes have been previously characterized except MPS IIID. We have herein characterized a novel murine model of MPS IIID which was produced by TaconicArtemis GmbH. This knockout model was produced by knocking out exons 2 to 13 of the 14 exons of the murine Gns gene. This murine model of MPS IIID followed the same progressive disease pattern as seen in other MPS III murine models. The affected mice were healthy while young and began to show clinical signs from the age of 9 month onward. Affected animals had an enlarged bladder, and rough hair coat starting from 9 months of age. Rectal, preputial, and/or vaginal prolapse was not observed until 12 months on age. On gross examination, MPS IIID mice showed urine retention and low body fat compared to wild type mice. Affected IIID mice were also compared to MPS IIIA and IIIB murine mice at multiple various ages. Clinical signs of MPS IIIB mice, including low body condition, rough hair coat, urine retention, and rectal prolapse, were seen earlier compared to MPS IIID, and were evident starting at 9 months of age. Thus it may be concluded that MPS IIID mice may have a slightly diminished rate of disease progression, as note by the age of onset of signs in the MPS IIID mice compared to the MPS IIIB mice.

Rotorod data of young MPS IIID, IIIB, and wild type mice showed no significant differences between any of the groups. This was an expected finding, as murine MPS III progresses slowly over time and signs are not visible at a young age. According to previous studies the performance of MPS IIIB mice on a rocking rotorod started to decline at approximately 9 months of age (73). Our results show a decline in the MPS IIIB performance at
rotorod evaluation round 10, which corresponds to 11 months of age. The MPS IIID mice performed better than IIIB mice at 11 months of age, suggesting that MPS IIID mice start showing motor deficits at a comparatively later age. At 12 months of age (round 11) we see no significant difference between any of the affected groups, which contrasts with the round 10 results. We consider that this may result from a skewing of the data due to lower numbers of animals evaluated at the time as part of round 11. As this study progresses, we anticipate robust differences to become evident between the different genotypic groups. At this juncture, we postulate based on round 10 data that MPS IIID mice tend to perform better on rocking rotorod than MPS IIIB mice.

Biochemistry data showed no detectable activity of GNS in MPS IIID-/- mice whereas IID+/- and IID++/ mice had normal GNS activity in the tested liver samples. Previously in all murine models an increase of other lysosomal enzymes has been consistently observed with the deficiency of one enzyme (12, 22, 35, 47). A secondary enzyme assay of kidney and liver samples of all the MPS IIID mice showed an increased activity of β-glucuronidase. Secondary enzyme assays of NAGLU and β-hexoaminidase on a subset of liver samples also showed elevated levels in affected animals. The exact reason for the increase of lysosomal enzymes is not known but may be due to: an increase in synthesis of the enzyme; an increase in the stability of the enzyme in the presence of stored substrate; an increase in the lysosomal compartment; or may (probably) be an interaction of some of these potentialities. In previous murine models most of the lysosomal enzymes were shown to have elevated activities but this pattern was not same for all the enzymes (22, 35), suggesting that there is not a random mechanism involved. Further studies are necessary to evaluate the activity level of all lysosomal enzymes in relation to the stored substrate in multiple tissues.
The gene for α-N-acetylglucosamine-6-sulfatase is responsible for removal of sulfate group from α-linked glucosamine residue in HS. Hence, by knocking out the gene for GNS we expect storage of HS. Tissues of MPS IIID animals at different ages had a significant increase in the amount of accumulated sulfated GAGs (s-GAGs). The pattern of s-GAGs accumulation in IIID mice is similar to IIIA and IIIB animals which was expected as all MPS IIIs follow a comparatively similar biochemical pattern both to that seen in human patients and other animal models. What is striking is the statistically significant difference between MPS IIIB and MPS IIID liver s-GAG levels at 12 months which show MPS IIID mouse livers to have a lower s-GAG content than MPS IIIB mice. This statistically significant difference is supported by non-significant trends seen at 12 months in kidney (MPS IIID < MPS IIIB) and at 4 months, at which age MPS IIID has levels lower than both MPS IIIB and MPS IIIA. The cause of this could be: a lower level at the HS non-reducing end of the α-N-acetylglucosamine-6-sulfate moiety in murine HS, relative to the moieties recognized by the enzymes deficient in MPS IIIA and MPS IIIB; a salvage pathway that may reduce the levels of stored HS in MPS IIID, albeit incompletely; or an as yet unknown mechanism or interaction.

Gross pathology of the H&E stained MPS IIID mice at 4 months of age revealed no obvious specific changes in morphology in liver, kidney, spleen, and eye but multiple brain sections had marked differences compared to wild type animals. In the brains of IIID-affected mice, there was occasional fine paranuclear cytoplasmic vacuolation of neurons and infrequent perineuronal satellite cells within the cerebral cortex were moderately vacuolated. Positive cytoplasmic staining with LFB was detected in neurons of IIID-affected mice, likely demonstrating ganglioside accumulation. Secondary storage of glycosphingolipids like GM2, GM3, and GD3 has been seen in all MPS III models previously characterized (74).
of age a progressive increase in the pathology of the disease was observed. Both liver and kidney
at this age had mild to moderate vacuolation. In the cerebellum sporadic cytoplasmic
accumulation in some Purkinje cells was observed along with a significant decrease in Purkinje
cells which was not present at 4 months of age. Histological data at 13 months compared to 4
months show that MPS IIID is a progressive neurodegenerative disorder, in which somatic cells
are affected at later stages of the disease. Pathology of MPS IIID mice is not significantly
different from IIIB and MPS IIIA mice as expected.

Amongst other murine models of MPS IIIIs, MPS IIIA and IIIB have shown severe
phenotypes whereas IIIC and IIIE have been known to have milder phenotype. MPS IIIC mice
start urine retention at roughly 65 weeks, have a lifespan of up to approximately 80 weeks, and
accumulate less s-GAGs compared to MPS IIIB mice (47). Looking at the phenotype and the
preliminary data of the rotorod performance of MPS IIID mice we expect this new model to have
a comparatively mild phenotype as well. Murine models with milder disease progression like
MPS IIIC still have significant morphological changes at 12 months of age and significant
increase in GAGs thus significant histological and biochemical changes of MPS IIID mice at 13
months of age do not contradict with the comparatively milder phenotype of the model.

The current research of characterizing MPS IIID murine is an ongoing study with rotorod and
lifespan data yet to be completed. The murine model of MPS IIID phenotypically, histologically,
and biochemically matches well with other MPS III animal models. Our study suggests that this
model is a well suited model to investigate MPS IIID disorder.

Future Perspectives
The murine model of MPS IIID will aid investigations into yet unanswered questions
relevant to Sanfilippo syndrome as well as to lysosomal storage disorders in general. Murine
models are cost effective, easy to manage, and have a relative short lifespan which makes studying progressive diseases comparatively easy. As discussed previously MPS type IIID is amongst the rarest forms of MPS III disorders and lack of patients makes it difficult to research the pathology of the disease. These practical considerations play a crucial role in research, for example, the natural goat model of MPS IIID, is not suitable for much in depth investigation of the molecular and physiological manifestations of the disease or in proof of principle therapeutic evaluations. Such large animal models are usually reserved for pre-clinical studies only. Murine models are the practical animal models for preliminary research and for testing therapeutic approaches. Murine models of other MPS III subtype have already been extensively explored and characterized as discussed in chapter one. Gene therapy, enzyme replacement therapy, substrate deprivation therapy are some of the therapeutic strategies which are yet to be investigated in MPS IIID murine model. This model is also well suited for evaluation of the efficacy of novel therapies.

One potential drawback of this model is the relative slower progression of disease compared to other MPS III models. This may make comparative evaluations of different approaches to therapy less easy to conduct. However, in addition to use in therapy development, this model may also prove useful to explore so called windows of therapeutic opportunity, i.e. the age within which a therapy may be successful in establishing a significant treatment effect. In this respect a more slowly progressive model may actually be an asset. Finally, with respect to the slower onset of disease, there are potential approaches to increasing or accelerating the severity of disease, including, among other approaches, double knockout mice involving other lines of knockout mice combined with MPS IIIB.
Different MPS disorders result in an accumulation of one or multiple GAGs but only HS has been seen to associate with severe CNS disease. Investigating the role of HS in brain is one area that needs to be explored further as targeting therapy for CNS disorders is difficult and expensive due to the blood brain barrier. A better understanding of the specific HS associated disease, could help address these effects directly, and thereby reduce the level of enzyme necessary to an achievable level given current delivery methods that overcome the blood brain barrier. A murine model is ideally suited to studying the effects of primary accumulation of HS on intra- and inter-cellular pathology associated with MPS III neurodegeneration. The exact cause of and potential pathological effect of secondary accumulation products remains an unanswered question. In 2012 Mohammad et al., looked at GalNAc transferase (GalNAcT) and MPS IIIB double knock out mice (70). The GalNAcT enzyme is required for the synthesis of GM2 and other complex gangliosides. By knocking out the GalNAcT gene these GSLs are not synthesized. All gangliosides present are the simpler forms such as GM3 and GD3. The double knockout mice had a rapidly progressive disease relative to MPS IIIB (euthanized at approximately 5 months, versus approximately 11 months for the MPS IIIB mice) and performed worse than MPS IIIB mice on rotorod. This study showed that GM2, a secondary accumulation product of MPS III, may either positively modulate the neuropathology caused by HS, or the absence of GM2 and other elaborate gangliosides, or some associated perturbation of ganglioside homeostasis, may negatively modulate the neuropathology caused by HS.

Use of the MPS IIID mouse in the context of a GalNAcT/MPS IIID double knockout may help generate a model with accelerated disease, which could help in the testing of therapies, as well as be useful to study pathogenesis mechanisms. This approach is particularly attractive as both the Gns and the B4galnt1 genes in mice reside near the centromeric terminus of MmA10,
separated by 5.8 megabases. If these knockout alleles could be produced in cis, production of
double affected mice will be far more efficient, with production equivalent to that of a recessive
disease.

Other areas of investigation, addition to the question of secondary accumulation products,
include the basic neuropathology of MPSs with regard to neurodevelopment, neuroplasticity, and
innate immune and inflammatory responses, e.g. microglial activation and astrocytosis, among
other things.

In conclusion, lysosomal storage diseases are a large group of rare metabolic disorders
and have a combined prevalence of 1 in 7700 live births (5). Understanding the molecular and
biochemical basis of such diseases in humans has been advanced largely by studying animal
models. Previously the animal models used were spontaneous models (often large animal models
characterized by veterinarians). With the advancement of technology, transgenic animal models
have been generated, supplying a need for multiple and particularly rare diseases. These highly
inbred small models have an advantage over large models and have been extensively studied. An
ideal strategy for determining the efficacy of any therapeutic strategy is to study the effects of the
therapy in small and large animal model before moving forward with clinical trials in human
patients. With the characterization of the murine model of MPS IIID and the availability of an
already existing large animal model (Nubian goat), development of an effective therapy for
patients with MPS IIID disorder is an achievable goal.
REFERENCES
