DIAGNOSIS AND THERAPIES FOR MUCOPOLYSACCHARIDOSES

by

Francyne Kubaski

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences

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by

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"The most important thing… is not to win but to take part, just as the most important thing in life is not the triumph but the struggle. The essential thing is not to have conquered but to have fought well."

-Pierre de Coubertin

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LIST OF ABBREVIATIONS

$\%FEV_{TOT}$- predicted forced expiratory volume
3MSCT- 3-minute stair climbing test
6MWT- 6-minute walk test
ABD- abdominal
aBMD- areal bone mineral density
ADAMTSs- a disintegrin and metalloproteinase with thrombospondin motifs
ADL- activity of daily living
AF- amniotic fluid
AIDHC- Alfred I. DuPont Hospital for children
APCI- atmospheric-pressure chemical ionization
ATP- adenosine triphosphate
BBB- blood-brain barrier
BDNF- brain-derived neurotrophic factor
BIPAP- bilevel positive airway pressure
BM- bone marrow
BMD- bone mineral density
BMI- body mass index
BMP- bis[monoacylglycero]phosphate
BMP4- bone morphogenetic protein 4
BSA- bovine serum albumin
CB- cord blood
CDC- Centers for Disease Control and Prevention
CESD- cholesterol ester storage disease
CI- chemical ionization
CLEAR- coordinated lysosomal expression and regulation
CLN- Ceroid-lipofuscinosis neuronal
CNS- central nervous system
CO_2-carbon dioxide
CPAP-continuous positive airway pressure
CS- chondroitin sulfate
CSF- cerebrospinal fluid
CT- chaperone therapy
CV- chorionic villi
CV- coefficient of variation
DBS- dried blood spots
DDP-IV- dipeptidyl peptidase IV

DMMB- dimethylmethylene blue
DS- dermatan sulfate
DXA- dual x-ray absorptiometry
EBV- Epstein-Barr virus
ECM- extracellular matrix
EDTA- ethylenediaminetetraacetic acid
ELISA- enzyme-linked immunosorbent assay
ER- endoplasmic reticulum
ERAD- endoplasmic reticulum-associated degradation
ERT- enzyme replacement therapy
ESI- electrospray ionization
F- female
F- full-time
FAB- fast atom bombardment
FEV_1- forced expiratory volume in one second
FEV_{TOT}- forced expiratory total volume
FGF2- fibroblast growth factor 2
FIM- functional independence measure
fRES- resonant frequency
FTICR- Fourier transform ion cyclotron resonance
FVC- forced vital capacity
GAGs- glycosaminoglycans
Gal- galactose
GalNAc- N-acetylgalactosamine
GALNS- N-acetylgalactosamine-6-sulfate sulfatase
GlcA- glucuronic acid
GlcNAc- N-acetylglucosamine
GM- ganglioside monosialic
GT- gene therapy
GUSB- β-glucuronidase
GVHD- graft versus host disease
HA- hyaluronan
HCII-T- heparin cofactor II-thrombin complex
Heg1- heart of glass receptor 1
HEP- heparin
HGMD- Human Gene Mutation Database
HLA- human leukocyte antigen
HPLC- high-pressure liquid chromatography
HS- heparan sulfate
HS-FOCUS- Hunter syndrome-functional outcomes for clinical understanding scale
HSCT- hematopoietic stem cell transplantation
HSR- heat-shock response
HT/MS/MS- high-throughput tandem mass spectrometry

ID- intellectual disability
IdoA- iduronic acid
IDS- iduronate-2-sulfatase
IEM- inborn errors of metabolism
IGFBP7- insulin-like growth factor binding protein 7
IOS- impulse oscillometry system
IQ/DQ- intelligence quotient/ developmental quotient
IRB- Institutional Review Board
IS- internal standard
ISSD- infantile sialic acid storage disease
IT- ion trap
KS- keratan sulfate
L- left
L- lumbar
LAMP-2A- lysosome-associated membrane protein 2A
LDF- lateral distal femur
LIMP-2- lysosomal integral membrane protein 2
LLOQ- lower limit of quantitation
LOD- lower limit of detection
LS- lumbar spine
LSD- lysosomal storage disorders
M- male
m/z- mass-to-charge ratio
M6P- mannose-6-phosphate
M6PR- mannose-6-phosphate receptors
MAD- median absolute deviation
MALDI-matrix-assisted laser desorption/ionization
ML- mucolipidoses
MMPs- matrix metalloproteinases
MPS- mucopolysaccharidoses
MRI- magnetic resonance imaging
MRM- multiple reaction monitoring
MS- mass spectrometry
MS/MS- tandem mass spectrometry
mTORC1- mammalian target of rapamycin complex 1
MVA- motor vehicle accident
NBS- newborn screening
NHANES- National Health and Nutrition Examination Survey
NMR- nuclear magnetic resonance
NO- nitric oxide
NPC- Niemann-Pick
NRE- non-reducing end
Ø- phase angle

OMIM- Online Mendelian Inheritance in Man
P/N- part-time or non-ambulators
PBSC- peripheral blood stem cells
PC- pharmacological chaperones
PCT- pharmacological chaperone therapy
PEDI- pediatric evaluation of disability inventory
PFT- pulmonary function test
PGs- proteoglycans
PhRTB- phase relation during total breath
Phθ- phase angle
PKU- phenylketonuria
PNT- pneumotachography
PPS- pentosan polysulfate
PRs- proteostasis regulators
QC- quality control
R- region
R_{20}- central airway resistance
R_5- peripheral airway resistance
RC- rib cage
RIP- respiratory inductance plethysmography
Rrs-resistance
SD- standard deviation
SE- standard error
SpO_2- saturation of oxygen
SRT- substrate-reduction therapy
Std- standard
SubQ- subcutaneous
T-thoracic
TAA- Thoracoabdominal asynchrony
TAM- Thoracoabdominal motion
TFEB- transcription factor EB
TGF-β- transforming growth factor-beta
TIMP-1- tissue inhibitors of metalloproteinase 1
TLR4- Toll-like receptor 4
TNF-α- tumor necrosis factor alpha
TNFR1- tumor necrosis factor 1
TOF-time-of-flight
U-unit
UA- uronic acid
uGAGs- urinary glycosaminoglycans
UPR- unfolded protein response
VC- vital capacity
WB- whole body

WC- wheelchair
Xrs- reactance
Zrs- impedance

ABSTRACT

Mucopolysaccharidoses (MPS) are lysosomal storage disorders characterized by progressive accumulation of glycosaminoglycans (GAGs) due to deficiency of specific lysosomal enzymes. MPS are classified according to the enzyme deficiency and accumulated GAGs. GAGs are negatively charged polysaccharides composed of repeating disaccharides and are important components of extracellular matrix, having a range of functions in multiple tissues. In MPS, GAGs accumulate in lysosomes, disrupting cellular homeostasis and leading to irreversible and progressive cell and tissue damage. All types of MPS are chronic and progressive with an extensive range of clinical manifestations such as skeletal dysplasia, corneal clouding, coarse facial features, joint rigidity or laxity, hepatosplenomegaly, neurodegeneration, and cardiac and respiratory dysfunction. The combined incidence of all MPSs is estimated as 1:25,000 live births. Most patients are asymptomatic at birth, so are not usually diagnosed until signs and symptoms arise in the first few years of life. Although no cure exists for MPS, some treatment approaches are available, including hematopoietic stem cell transplantation (HSCT), enzyme replacement therapy (ERT), substrate reduction therapy and chaperone therapy. Methods to evaluate treatment success are limited, and there is an overall consensus that treatments should begin before signs and symptoms appear. Current techniques for assessment of disease

progression are invasive, and effort-dependent so cannot be used to evaluate young, wheelchair-bound and neurologically impaired patients. Thus, new diagnostic approaches (prenatal to newborn) and better techniques to evaluate disease progression are needed to improve outcomes for patients.

In this study, I have established the efficacy of two non-invasive assessments for measuring disease progression in all MPS IV patients (Chapter 3) and developed a diagnostic approach using GAG analysis by liquid chromatography tandem mass spectrometry to understand natural history of MPSs (Chapter 4), and measure efficacy of different treatments (Chapter 5).

Skeletal abnormalities in some MPS IV patients precluded examination of bone mineral density (BMD) from conventional body sites, but it was discovered that BMD analysis could be performed on the lateral distal femur (LDF) of all MPS IV patients, regardless of physical or mental ability. Using BMD of LDF it was possible to determine the positive impact of ambulation in preserving BMD. Data from this study provides guidance to patients and their families on the importance of physical exercise in reducing the onset of disability. The subset of non-invasive pulmonary function tests demonstrated that restrictive and obstructive lung diseases are not always present in MPS IV, in which patients have small but functioning lungs. These tests will be able to inform clinicians of disease progression and should be valuable in evaluating the efficacy of treatment, allowing for a much wider range of patients to take part in clinical trials.

The majority of established GAG detection protocols are based on colorimetric assays that are limited by false negative results and also cannot be applied to blood samples. The GAG detection protocol using tandem mass spectrometry is very specific and sensitive, discriminating most MPS patients from unaffected controls at all ages in a variety of samples (amniotic fluid, blood, cerebrospinal fluid, dried blood spots and urine). In my study, the focus was on samples from newborn patients, and results indicate that GAG measurements can be used for newborn screening. This will enable early diagnosis of MPS and thereby allow early treatment to significantly improve outcomes for MPS patients.

I used this tandem mass spectrometry method in a study that showed that HSCT results in better outcomes for MPS II patients than ERT. GAG levels were lower in HSCT treated patients, who also had higher scores in an activity of daily living survey and fewer brain anomalies revealed by MRI than ERT treated patients. These new findings indicate that MPS II patients should be treated with HSCT at an early age and that, contrary to earlier reports, HSCT for these patients can have a positive impact on reducing neurological defects.

The methodologies established here for GAG measurements and non-invasive techniques to assess disease progression have the potential to drastically improve the quality of life for MPS patients. The laboratory test enables both early diagnosis and evaluation of treatment efficacy and the non-invasive assessment protocols can be used to follow clinical efficacy. This highly innovative and state of the art study can be applied to the next generation of patient care in MPSs. The BMD and lung function

assessments are also likely to be useful in the management of other diseases unrelated to MPS (skeletal dysplasias or neurological conditions), broadening the impact of this work. Early detection will also impact the choice of the most adequate treatment as well as reduce mortality, morbidity, and public health costs.

Chapter 1

INTRODUCTION

1.1 Inborn Errors of Metabolism

The term *inborn errors of metabolism* (IEMs) was introduced by Sir Archibald Garrod at the Croonian Lectures in 1908 [1]. IEMs are monogenic disorders characterized by failure during specific steps of metabolism that lead to the accumulation of substrates and lack of products [2]. They have a heterogeneous clinical presentation, ranging from very mild to fatal, and can affect any organ or system in which the disrupted metabolic process is important [3]. In 1909, Sir Archibald Garrod published his first book about IEMs, which describes the first four IEMs: albinism, cystinuria, pentosuria, and alkaptonuria [4].

To date more than 600 different IEMs have been described and advances in diagnostic methodologies, such as mass spectrometry (MS) and next-generation sequencing are providing accurate molecular approaches to more clearly define these IEMs [5]. Signs and symptoms of IEMs can appear from prenatal or juvenile throughout adulthood and for some people, environmental factors, such as stress and diet, can act as triggers [6].

Several classification systems exist for IEMs, based on the affected organ, affected organelle, and/or age of onset [5]. IEMs can also be classified according to

pathophysiology in three main groups: 1) disorders of intermediate metabolism (progressive toxicity by accumulation of metabolite, accompanied by vomiting, lethargy, and/or coma, amongst other complications or chronic symptoms, such as ectopia lentis, cardiomyopathy and developmental delays); 2) disorders involving energy metabolism (also known as small molecule disorders - have clinical presentation due to deficiency in energy production or utilization caused by defect(s) in organs related to energy, such as the brain, heart, liver, or muscle; main symptoms are hypoglycemia, hyperlactatemia, hypotonia, and/or myopathy with further heart and circulatory system failure); 3) complex molecule disorders (disorders affecting anabolism or catabolism of complex molecules that are progressive due to continual accumulation; clinical presentation will depend on affected organelle as well as organs) [3, 7].

The overall incidence of IEMs is reported as 1 in 2,500 to 5,000 live births [8, 9]. The incidence of IEMs varies from country to country as some ethnic groups have higher incidence rates, mostly due to founder effects and consanguinity [5, 10-12]. Some IEMs are very rare, but combined groups of similar IEMs are more common. Studies have reported the incidence as high as 1:591 in Saudi Arabia [5] (to approximately 1:2,500 in Canada and Italy [13, 14].

Diagnosis is mainly achieved by metabolite analysis (either up or downstream) with confirmation by enzyme assay. Different approaches can be used to diagnose IEMs, depending on the phenotype. Advances in metabolomics using quantitative techniques, such as mass spectrometry (MS) and nuclear magnetic resonance

spectroscopy (NMR spectroscopy), have improved the diagnosis of heterogeneous diseases and also enabled screening for several IEMs from dried blood spots (DBS) in newborn screening programs [2].

Therapeutic management is very specific to each group of IEMs. Some disorders can benefit from dietary interventions (e.g. restriction of phenylalanine in phenylketonuria patients), also known as substrate deprivation, while other groups will only improve with enzyme restoration via infusions of the recombinant enzyme (enzyme replacement therapy or ERT), tissue cross-correction (hematopoietic stem cell transplantation or HSCT), or constant enzyme secretion by gene replacement with gene therapy-GT [2]. Alternative treatment options that have also been beneficial in the management of IEMs are substrate-reduction therapy (SRT), by inhibiting substrate synthesis, thereby reducing substrate accumulation, and chaperone therapy (CT) to stabilize and activate miss-folded mutant enzymes [15-18].

1.2 Lysosomal Storage Disorders

The lysosome was discovered by Christian de Duve and his colleagues in 1955 [19]. Lysosomal storage disorders (LSDs) are a sub-set of rare congenital inborn errors of metabolism caused by progressive accumulation of undigested or malfunctioning metabolites due to defective activity of soluble lysosomal hydrolases, membrane proteins and lysosome accessory proteins that lead to impaired turnover of complex macromolecules, such as glycosaminoglycans, proteins, and lipids [20-22].

de Duve identified lysosomes as ‘suicide bags’ with lytic properties that he named ‘lysosomes’ (Greek for ‘digestive body’) [19, 20, 23-26]. This lytic organelle is membrane-bound and contains approximately 60 different acidic hydrolases, such as glycosidases, sulfatases, peptidases, phosphatases, lipases, and nucleases [21, 27]. The lysosome has an acidic pH (4.5 to 5.5), maintained by the vacuolar H^+ ATPase (v-ATPase) and counter flux of ions (Cl^-, Na^+, and K^+) [28-31].

Lysosomal enzymes are synthesized in the endoplasmic reticulum (ER), and when they are folded into their native conformation, they are translocated to lysosomes via specialized pathways. The majority of the enzymes are targeted to lysosomes by the addition of mannose-6-phosphate (M6P) tags during transport through Golgi, which then bind to mannose-6-phosphate receptors (M6PR) for delivery to the lysosome [21, 32-37]. For some enzymes, targeting is independent of M6P. For example, β-glucocerebrosidase is targeted to lysosomes by lysosomal integral membrane protein 2 (LIMP-2) [38, 39].

Substrates can be delivered to the lysosome via several routes depending on the cargo’s nature; usually specialized endocytic mechanisms are involved, such as phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, and clathrin-and caveolin-independent endocytosis, while intracellular products are transported mostly by autophagy [21, 40]. Impairment in any of these specialized mechanisms will lead to LSDs.

The term *autophagy* (Greek for ‘eating of self’) was introduced by Russel L. Deter and de Duve in 1967 [41]. Autophagy is one of the most important mechanisms

for cellular recycling and the removal of damaged organelles that are destroyed by the fusion of autophagosome-lysosome, which is also required during cellular stress [40, 42].

Three main types of autophagy exist: 1) macroautophagy (cargo is delivered to the lysosome via autophagosomes that are fused with lysosomes, forming an autophagosome); 2) microautophagy (cytosolic components are digested by lysosomes via invagination of the lysosome membrane); and 3) chaperone-mediated autophagy (targeted proteins are translocated to the lysosome membrane via chaperone proteins that are recognized by lysosome-associated membrane protein 2A [LAMP-2A] receptors) [43-46].

Autophagy dysregulation has been highly associated with LSD pathology in that intralysosomal accumulation impairs the fusion of autophagosomes and lysosomes, leading to secondary storage of toxic proteins and aberrant mitochondria that will cause cell damage, inflammation and consequent cell death (Fig. 1.1).

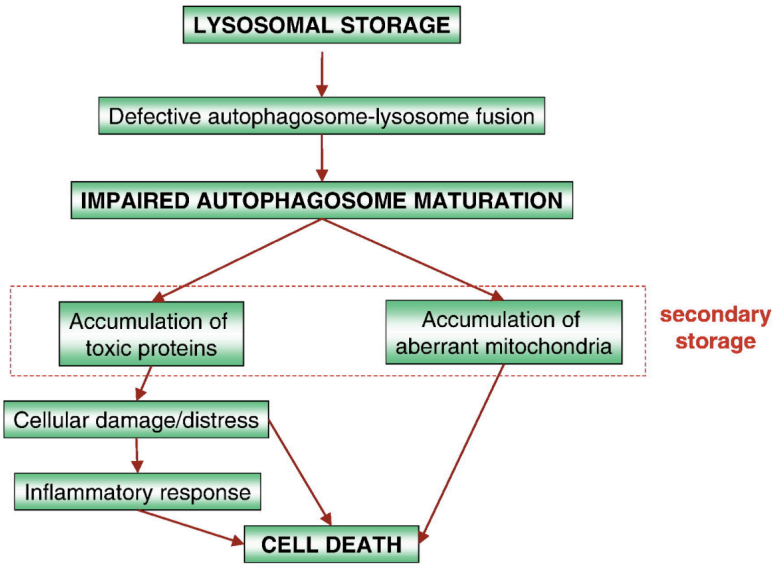

Figure 1.1: Pathogenesis of LSDs
Lysosomal storage impairs autophagy, promoting secondary accumulation of toxic proteins and autophagy substrates, which will lead to cell death.
Adapted from: Ballabio et al., 2009 with permission from Elsevier.

The concept of LSDs was introduced in 1963 after tissue fractionation showed that α-glucosidase is localized in lysosomes, allowing the classification of Pompe disease as LSD caused by lack of this enzyme activity [47-49]. Furthermore, the term *inborn lysosomal diseases* was proposed by Henri-Géry Hers in 1965 based on the previous description of inborn errors of metabolism by Sir Archibald Garrod [1, 49]. Approximately 70 different LSDs have been described [19, 22, 50]. The majority of LSDs are inherited in an autosomal recessive manner, with the exception of three X-linked disorders: Danon disease, Fabry disease, and mucopolysaccharidosis II [51-56].

Several LSDs are now recognized as ‘autophagy disorders. Furthermore, autophagy impairment has also been demonstrated in neurodegeneration [22, 31, 45, 50, 57-67].

Lysosomes are considered near-ubiquitous organelles that highlight the usual multisystemic compound of LSDs. Acidic lysosomal hydrolases act jointly to degrade key macromolecules that are catabolized in a stepwise fashion, but if a member of this machinery is deficient then the entire digestive orchestra is deprived [62].

The clinical presentation and prognosis of LSDs correlate with the residual function of the defective enzyme, in which null or very low levels of the enzyme will lead to early onset (fetal death, in utero symptoms or early childhood) and higher levels of enzyme give rise to milder forms with juvenile or adult onset [61].

Symptoms are highly heterogeneous with somatic (hepatosplenomegaly, bone, eye, heart, kidney, muscle impairment) and or neurological presentation (seizure, dementia and brainstem malfunction) according to the dysfunctional metabolite [50].

Normal lysosomal function seems to be vital for neuronal function and survival. Consequently two-thirds of LSDs have a neurological component [45, 68, 69]. Neurodegeneration has also been correlated with lysosomal dysfunction in diseases such as Alzheimer’s, Parkinson’s, Huntington’s and Creutzfeldt-Jakob [22, 50, 62, 70, 71].

In addition to catabolic and recycling properties, lysosomal enzymes are important for secretion, antigen processing, degradation of extracellular matrix,

plasma membrane repair, signaling, nutrient sensing, apoptosis, cellular growth and vesicle trafficking [21, 72-76].

Such complexity can be explained by the gene network required for lysosomal function; the coordinated lysosomal expression and regulation (*CLEAR*) network and transcription factor EB (*TFEB*) [68, 75, 77, 78].

TFEB-mediated regulation has also been demonstrated to play key roles in cell adaptation to homeostatic or non-homeostatic processes in which lysosome-to-nucleus signaling, by interaction with a mammalian target of rapamycin complex 1 (mTORC1) kinase complex with *TFEB*, can inhibit or prevent *TFEB* translocation into the nucleus [21, 78].

LSDs can affect signal transduction pathways in which stored compounds can act as ligands, modify receptor responses or alter receptor subcellular localization, and alter signal transduction cascades [60].

Thus not only autophagy, but also other cellular pathways and signaling mechanisms are impaired in LSDs, such as: calcium signaling (normal Ca^{2+} metabolism is required for trafficking, recycling and fusion events); iron metabolism (might play a role in neurodegeneration or brain pathology); inflammation and tissue damage (glycosaminoglycans activate Toll-like receptor 4 (TLR4); higher levels of proinflammatory cytokines lead to increased activity of metalloproteases with consequent tissue damage); fibroblast growth factor 2 (FGF2) signaling; bone morphogenetic protein 4 (BMP4) signaling (proper signaling is affected by extracellular accumulation of heparan sulfate); insulin signaling (due to changes in

plasma membrane fluidity by lipid storage; or insulin resistance due to accumulation of GM3 gangliosides) [50, 60, 62, 79-86].

Furthermore, the classical picture of undegraded substrate accumulation as a major cause of these disorders has shifted to describe a more heterogeneous and complex pathophysiology resulting from a cascade of events that leads not only to a primary accumulation but also to secondary accumulations that alter tissue physiology and gene expression which further activates tertiary biochemical pathways [20, 60, 69, 87, 88].

LSDs are usually classified according to the stored material and the defective enzyme (Table 1.1). Some LSDs have similar accumulations because most of the polymeric substrates are degraded in a stepwise fashion by the action of several lysosomal enzymes.

Table 1.1: Classification of Lysosomal Storage Disorders

Disease	Defective enzyme	Storage material
Sphingolipidoses		
Fabry	α-Galactosidase A	globotriaosylceramide
Farber	Ceraminidase	ceramide
Gaucher	β-glucosidase Saposin-C activator	glucosylceramide
Niemann-Pick A & B	Sphingomyelinase	sphingomyelin
Sphingolipid-activator deficiency	Sphingolipid activator	glycolipids
GM1 gangliosidosis	β-galactosidase	GM1 ganglioside
GM2 gangliosidosis (Tay-Sachs)	β-hexosaminidase A	GM2 ganglioside and other glycolipids
GM2 gangliosidosis (Sandhoff)	β-hexosaminidase A & B	GM2 ganglioside and other glycolipids
GM2 gangliosidosis (GM2-activator deficiency)	GM2-activator protein	GM2 ganglioside and other glycolipids
Krabbe	Galacto-cerebrosidase	galactosyl-ceramides
Mucopolysaccharidoses		
MPS I (Hurler, Scheie, Hurler/Scheie)	α-iduronidase	Dermatan and heparan sulfate
MPS II (Hunter)	Iduronate-2-sulfatase	Dermatan and heparan sulfate
MPS III A (Sanfilippo)	Heparan N-sulfatase	Heparan sulfate
MPS III B (Sanfilippo)	N-acetyl-α-glucosaminidase	Heparan sulfate
MPS III C (Sanfilippo)	Acetyl-CoA-α-glucosamide acetyltransferase	Heparan sulfate
MPS III D (Sanfilippo)	N-aceylglucosamine-6-sulfatase	Heparan sulfate
MPS IVA (Morquio)	N-acetylgalactosamine-6-sulfate sulfatase	Chondroitin-6- sulfate and keratan sulfate
MPS IVB (Morquio)	β-galactosidase	Keratan sulfate
MPS VI (Maroteaux-Lamy)	N-acetylgalactosamine-4-sulfatase	Dermatan sulfate
MPS VII (Sly)	β-glucuronidase	Chondroitin-4- and 6-sulfate, dermatan sulfate, heparan sulfate

Table 1.1 Continued

MPS IX (Natowicz)	Hyaluronidase	Hyaluronan
Mucolipidoses		
Sialidosis I (ML I)	Neuraminidase	sialic acid
I Cell and pseudo-Hurler (ML II & ML III)	UDP-N-acetylglucosamine- lysosomal enzyme N-acetylglucosaminyl-1-phosphotransferase	oligosaccharides, glycosaminoglycans and lipids
ML IIIC	Transferase-δ-subunit	oligosaccharides, glycosaminoglycans and lipids
ML IV	Mucolipin-1	lipids and glycosaminoglycans
Oligosaccharidoses and glycoproteinosis		
α-mannosidosis	α-mannosidase	α-mannosides
β-mannosidosis	β-mannosidase	β-mannosides
Pompe	α-glucosidase	glycogen
Fucosidosis	Fucosidase	fucosides glycolipids
Aspartylglucosaminuria	Aspartylglucosaminidase	aspartyl-glucosamine
Schindler (Kanzaki)	α-galactosidase B	glycosphingolipids, glycoproteins, oligosaccharides
Defects in integral membrane proteins		
Cystinosis	Cystinosin	cystine
Danon	LAMP2	cytoplasmic debris and glycogen
Infantile sialic-acid-storage disease and Salla disease	Sialin	sialic acid
Niemann-Pick C	NPC1 and 2	cholesterol and sphingolipids
Others		
Galactosialidosis	Cathepsin A	sialyloligosaccharides
Multiple sulfatase deficiency	Cα-formylglycine-generating enzyme	sulfatides

Table 1.1 Continued

Neuronal ceroid lipofuscinosis (Batten)		
CLN1 (infantile)	Palmitoyl protein thioesterase	saposins
CLN2 (late infantile)	Pepstatin insensitive carboxypeptidase	subunit c of the mitochondrial ATP synthase
CLN3 (juvenile)	Membrane protein	subunit c of the mitochondrial ATP synthase
CLN4 (Kuf's) (adult)	Unknown	subunit c of the mitochondrial ATP synthase
CLN5 (late infantile) (Finnish variant)	Membrane protein	subunit c of the mitochondrial ATP synthase
CLN6 (late infantile)	Unknown	subunit c of the mitochondrial ATP synthase
CLN7 (late infantile variant)	Unknown	Unknown
CLN8 (progressive epilepsy with mental retardation, EPMR)	Membrane protein	subunit c of the mitochondrial ATP synthase
Wolman and cholesterol ester storage disease (CESD)	Acid lipase	lipids
Infantile sialic acid storage disease (ISSD)	Sialic acid transporter	sialic acid and glucuronic acid
Salla	Sialic acid transporter	sialic acid and glucuronic acid
Cystine	Cystine transporter	cystine
Cobalamin F	Cobalamin transporter	cobalamin
Pycnodysostosis	Cathepsin K	bone proteins
Galactosialidosis	Neuraminidase and β-galactosidase protective protein	oligosaccharides, sialic acid

It is expected that patients with null or very low enzyme levels will have a severe phenotype, while moderate to higher levels of the residual enzyme will give rise to attenuated phenotypes. All LSDs are monogenic, but several mutations have been described for each of the coding genes responsible for LSDs, leading to high heterogeneity and challenging phenotype-genotype correlations [20, 89].

Although individually rare, the combined incidence of LSDs is between, 1:5,000 and 1:8,000 live births, depending on the genetic background of populations [21, 62, 90, 91].

As for other IEMs, diagnosis is done by analysis of stored material and confirmed by enzyme assay. Ultrastructural findings can aid prenatal diagnosis, which can be confirmed with a collection of amniotic fluid (AF) or chorionic villi (CV). Postnatally, DBS can be used to identify enzyme deficiency by newborn screening. Radiological findings can also aid diagnosis throughout the first years of life [89].

The elucidation of mechanisms of LSDs pathophysiology (Figure 1.1) has helped the development of better therapies. Until the early 1990s, treatment for LSDs was only palliative and supportive care. Advancements in the understanding of mannose-6-phosphate role and lysosomal uptake enabled the use of intravenous infusions of recombinant enzyme to help intralysosomal clearance (Figure 1.2) with great outcome in somatic pathology for several LSDs, such as Fabry, Gaucher, Mucopolysaccharidosis (MPS) I, MPS II, MPS VI, and Pompe, and to a lesser extent in MPS IVA [42, 92-96].

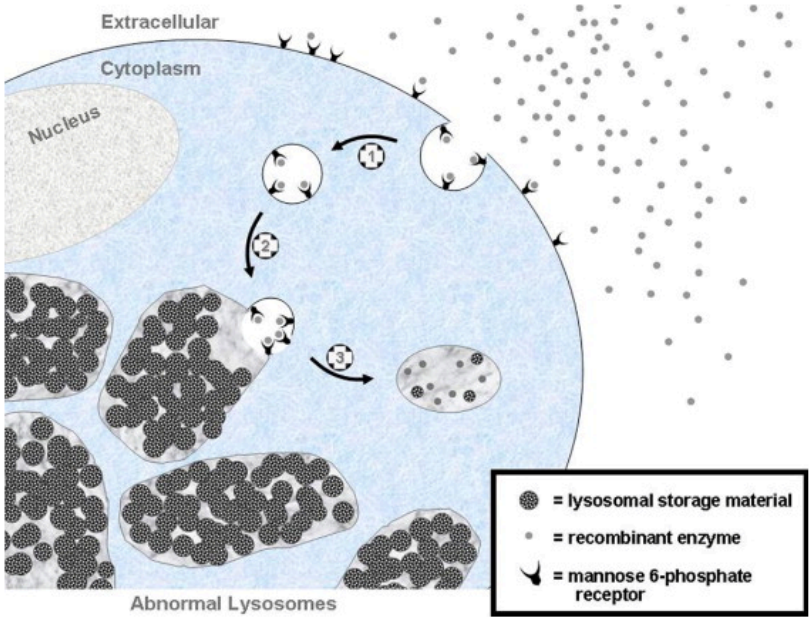

Figure 1.2: Schematic of Enzyme Replacement Therapy
1) Recombinant exogenous enzyme tagged with mannose-6-phosphate (M6P) is targeted to M6P receptors at the cell membrane and then endocytosed. 2) Endosome fuses with lysosome. 3) The recombinant enzyme aids in clearance of intralysosomal storage.
Adapted from: Heese BA, 2008 with permission from Elsevier.

ERT still has several limitations (antibody formation, the limited effect in bone and cartilage, inability to cross the blood-brain barrier [BBB], requiring weekly infusions) and a prohibitively high cost (approximately $300,000 per year per patient) [42, 97, 98].

HSCT provides tissue cross-correction and is recommended for young patients with MPS I, MPS VI, Krabbe disease and the attenuated form of metachromatic leukodystrophy [95, 99-104].

The use of pharmacological chaperones (PC) has been proposed for treatment of LSDs as they can improve folding of mutated proteins and/or increase their stability. Correct folding, enables mutant proteins to traffic from the endoplasmic reticulum (ER) to the Golgi, avoiding quality control (QC) mechanisms that trigger ER-associated degradation (ERAD) of misfolded proteins, leading to increased enzyme levels (Figure 1.3) [105-107].

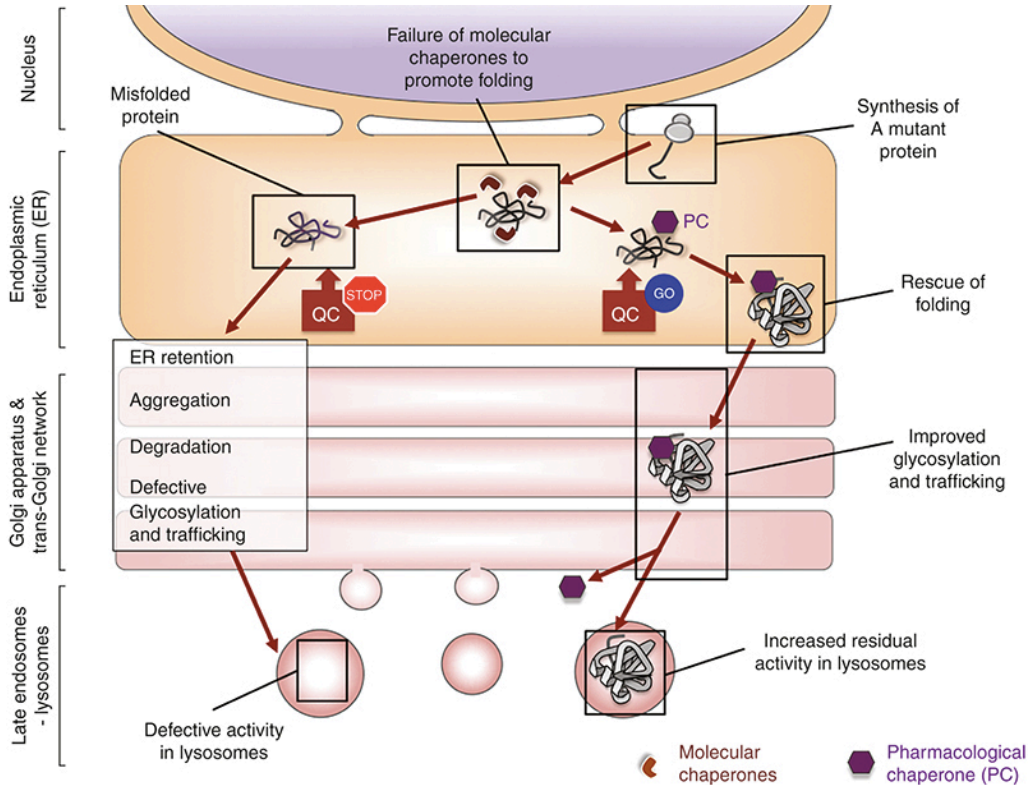

Figure 1.3: Schematic of Pharmacological Chaperone Therapy
Misfolded mutant proteins trigger endoplasmic reticulum (ER) quality control (QC) mechanisms and are degraded by ER-associated degradation (ERAD) mechanism. Pharmacological chaperones (PC) bind to the misfolded mutant protein and rescue folding avoiding ERAD and consequently increasing protein levels.
Adapted from: Parenti et al., 2015 with permission from Nature Publishing Group.

Pharmacological chaperone therapy (PCT) improves outcomes in Fabry disease and PCT is under investigation for Gaucher, Pompe, GM1 & GM2 gangliosidosis, Sandhoff, MPS IIIC, and Batten disease [95, 108-120]. PCT limitations include: only effective for missense mutations; chaperones can inhibit enzymes if they bind at the active site, and their binding affinity can be lower at an acidic pH compared to a neutral pH. The combination of PCT and ERT can result in higher levels of enzyme than either treatment alone [120-124].

Another therapy under investigation for LSDs is the use of proteostasis regulators (PRs). These regulators are important for protein synthesis, folding, trafficking, aggregation, and degradation, and thus have the ability to reestablish the balance between those mechanisms. PRs can alter signaling pathways such as unfolded protein response (UPR) and Ca^{2+} signaling proteostasis pathway (Mu et al., 2008). MG-132 and celastrol are two PRs that have been used for Gaucher and Tay-Sachs disease due to induction of heat-shock response (HSR) and UPR. Furthermore they also showed synergistic effect when combined with PCs enabling increased mutant enzyme concentration and activity [95, 125-127].

Substrate reduction therapy (SRT) has also shown to be successful in the treatment of LSDs as monotherapy or in combination with other therapeutic approaches, such as ERT, for Gaucher, Niemann-Pick type C, MPS III, Sandhoff, Fabry and Pompe. Typically, SRT is conducted using small-molecule inhibitors of substrate production and holds the advantage that usually such small molecules can

cross the BBB with good bioavailability to the central nervous system (CNS) [95, 128-138].

The combination of therapies is a common approach for treatment management in LSDs. Not only HSCT, PCT, and SRT can be combined with ERT, but also the manipulation of autophagic pathway seems to improve ERT outcomes [139]. As with autophagy, inflammation is a major component of LSDs pathophysiology and the use of anti-inflammatories has shown therapeutic benefits [42, 140-147].

Gene therapy (GT) is in the spotlight for the treatment of LSDs because they are all monogenic and they benefit from even small improvements in enzyme levels [148-150]. Several studies were conducted in animal models of LSDs and demonstrated clinical improvement and or reversal of disease pathology [149, 151-159].

Different GT vectors are currently available as adeno-associated virus, retroviruses and lentivirus with different targeted GT sites (e.g. liver or CNS) [148, 150, 160-163]. There is no current consensus of what vector or what delivery site is the most appropriate at present time, but clinical trials are planned or underway to demonstrate safety and efficacy of GT for LSDs (Gaucher and Fabry: NCT00001234; Metachromatic leukodystrophy: NCT01560182; Batten: NCT02725580; Tay-Sachs: NCT01869270; MPS II: NCT00004454; MPS IIA: NCT01474343).

1.3 Mucopolysaccharidoses

Mucopolysaccharidoses (MPSs) are one subgroup of inherited metabolic disorders caused by a deficiency of specific lysosomal enzymes required for the stepwise degradation of glycosaminoglycans (GAGs) [56]. The MPSs are classified according to the defective enzyme and accumulated substrate (Table 1.2) [20, 164].

Table 1.2: Classification of Mucopolysaccharidoses (MPSs)

MPS type	Defective enzyme	Primary storage
MPS I	α-l-iduronidase	DS+HS
MPS II	Iduronate-2-sulphatase	DS+HS
MPS IIIA	Glucosamine-N-sulphatase	HS
MPS IIIB	α-N-acetylglucosaminidase	HS
MPS IIIC	Acetyl-CoA: α-glucosamine N-acetyl transferase	HS
MPS IIID	N-acetylglucosamine-6-sulphatase	HS
MPS IVA	N-acetylgalactosamine-6-sulphatase	KS, C6S
MPS IVB	β-galactosidase	KS
MPS VI	N-acetylgalactosamine-4-sulphatase	DS
MPS VII	β-glucuronidase	CS, DS, HS
MPS IX	Hyaluronidase	Hyaluronan

CS: chondroitin sulfate; DS: dermatan sulfate; HS: heparan sulfate; KS: keratan sulfate.

1.3.1 Substrate GAGs

Glycosaminoglycans (GAGs) are negatively charged linear polysaccharides composed of repeating disaccharides with variable sulfation levels [165-168]. They are classified in four main families according to the repeating subunit: hyaluronan (HA) (glucuronic acid and N-acetylglucosamine); chondroitin sulfate (CS) /dermatan sulfate (DS) (iduronic acid/glucuronic acid and N-acetylgalactosamine); heparan sulfate (HS) (iduronic acid/glucuronic acid and N-acetylglucosamine); and keratan sulfate (KS) (galactose and N-acetylglucosamine) [169]. GAGs are one of the most important components of extracellular matrix (ECM) and are found in multiple tissues [170]. They can be attached to core proteins (proteoglycans-PGs) or exist as free polysaccharides [171, 172].

Studies have shown their importance in biological processes such as: cancer progression, angiogenesis, development, growth, microbial pathogenesis, cellular signaling (growth factors, cell surface receptors, cytokines, chemokines, enzymes, complement proteins), and anticoagulation [173-180].

In MPSs, these GAGs accumulate in lysosomes, disrupting their homeostasis and leading to lysosomal enlargement and deformation along with irreversible and progressive cell and tissue damage with further effects in other cellular pathways (e.g.: trafficking, metabolic pathways, other lysosomal pathways, and inflammation), consequently causing secondary and tertiary accumulation of substrates (Figure 1.4) [56, 181-184].

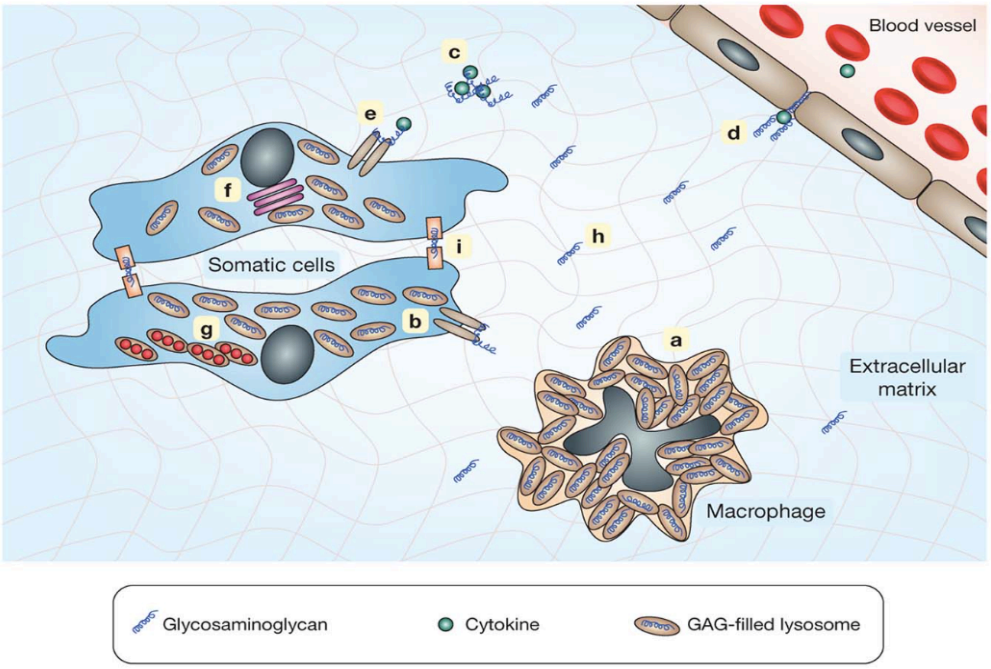

Figure 1.4: Impaired Physiological Processes in MPSs
Glycosaminoglycan accumulation will disrupt several processes and signaling mechanisms such as: a) impaired macrophage function due to intralysosomal storage; b) impaired assembly of plasma membrane receptors; c) defective sequestration of cytokines and growth factors; d) impaired recruitment of circulatory cytokines; e) impaired presentation of cytokines to signaling receptors; f) altered cellular trafficking; g) secondary accumulation due to defective function of hydrolases; h) altered extracellular matrix crosslinks; i) impaired cell attachment.
Adapted from: Clarke LA, 2008 with permission from Cambridge University Press.

1.3.2 Synthesis of GAGs

Synthesis of GAGs is a dynamic process that is regulated by the action of multiple enzymes, allowing high heterogeneity of products [180, 185]. GAG synthesis takes place in the late ER and/or cis-Golgi compartment by the addition of xylose to specific amino acids by xylosyltransferase. For CS, DS and HS, the tetrasaccharide linkage region is formed as follows: transfer of a galactose monosaccharide by

galactosyltransferase I to the xylose; further addition of a second galactose by galactosyltransferase II; followed by addition of a glucuronic acid by glucuronosyltransferase I [180, 186, 187]. The fate of CS/DS or HS/ heparin (HEP) is determined by the next saccharide added: addition of N-acetylglucosamine (GlcNAc) gives rise to HS, and the addition of N-acetylgalactosamine (GalNAc) gives rise to CS/DS [185]. HS sulfate is further modified by the addition of another GlcNAc and a glucuronic acid (GlcA) residue and extended by the action of two HS polymerases [188, 189].

Chondroitin sulfate chain extension involves the addition of GalNAc and GlcA by six enzymes; CS chains can be further modified by the addition of sulfate groups at the C-4 and/or C-6. Epimerization of GlcA to iduronic acid (IdoA) at C-5 results in DS formation [190-196].

Glycosyltransferases are responsible for KS synthesis, which is composed of repeating units of galactose (Gal) and GlcNAc without uronic acid (UA) [197]. KS proteoglycans can be formed by three unique linkage regions (KS-I: N-linked [asparagine]; KS-II: O-linked [serine or threonine]; and KSIII: O-linked [mannose O-linked serine] [198].

Hyaluronan (HA) is a non-sulfated GAG composed of GlcNAc and GlcA and is the only GAG synthesized at the cell membrane instead of ER by the action of three hyaluronan synthases [199, 200].

1.3.3 Catabolism of GAGs

GAGs are catabolized by the action of at least 14 lysosomal hydrolases, in a stepwise, directional manner, by removal or processing of terminal sugars at the non-reducing end (NRE) of GAG chains (Figure 1.5). Impairment in any of these lysosomal hydrolases will result in LSDs, in which deficiency of 12 enzymes will lead to MPS (Table 1.2) [201]. Hyaluronan is catabolized by the action of hyaluronidases, also known as endo-β-acetyl-hexosaminidases [202]. Aggrecan is degraded by matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase with thrombospondin motif (ADAMTSs), and other proteinases [172, 203, 204]. Digested protein fragments with bound GAGs are then endocytosed and degraded by lysosomal enzymes [172].

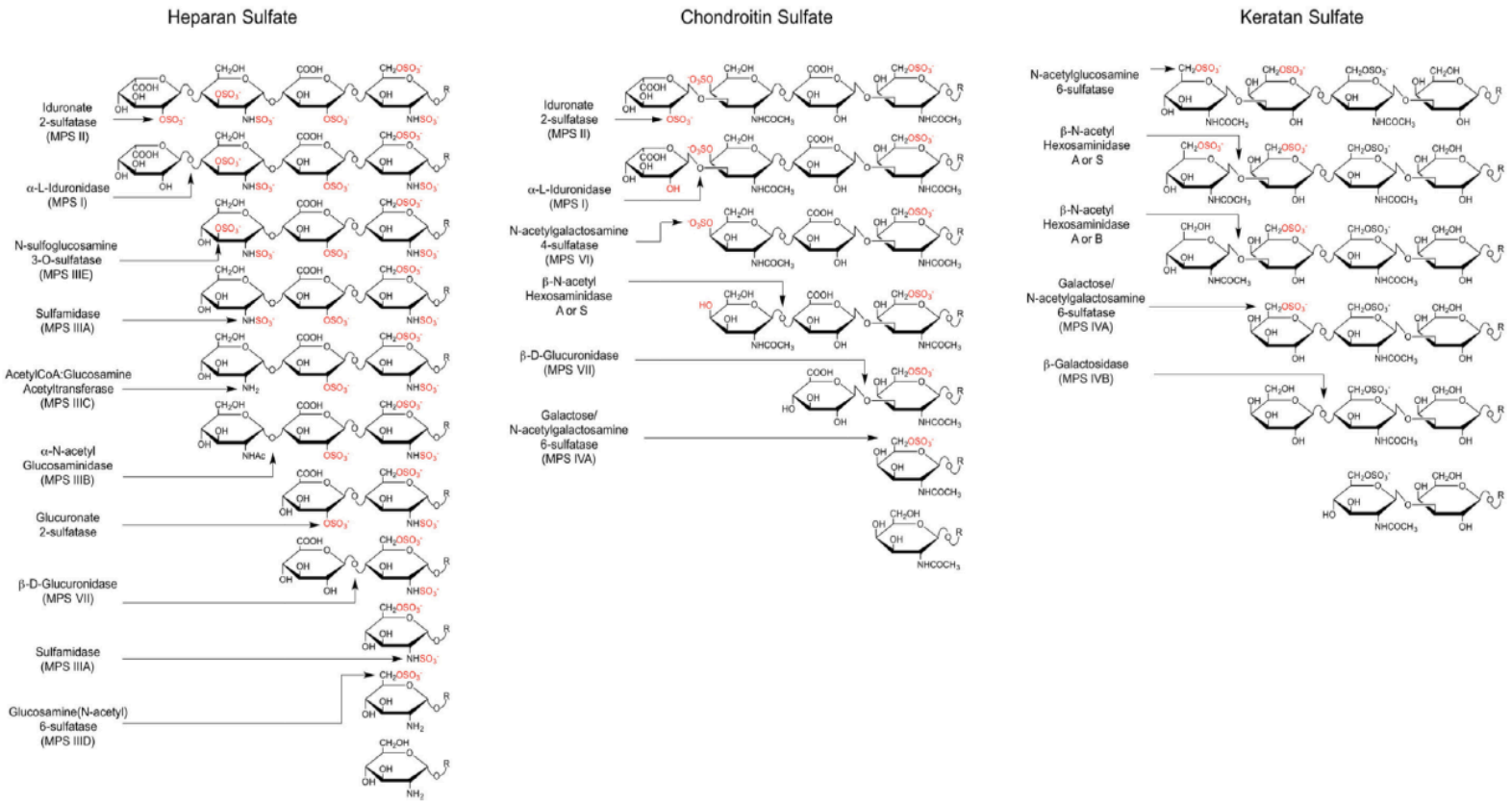

Figure 1.5: GAG Catabolism
GAGs (heparan sulfate [HS], chondroitin sulfate [CS], keratan sulfate [KS]) are sequentially degraded by the stepwise action of several lysosomal enzymes; deficiency in any of these lysosomal enzymes will lead to accumulation of undegraded or partially degraded GAGs giving rise to the different MPS subtypes.
Adapted from: Lawrence et al., 2014 with permission from Elsevier.

1.3.4 Measurement of GAGs

Due to their multiple roles in biological processes, many qualitative (toluidine blue, chromatography: paper and thin layer) and quantitative methods (alcian blue, 1,9-dimethylmethylene blue, chromatography: gas and high-pressure liquid chromatography-HPLC, capillary electrophoresis, enzyme-linked immunosorbent assay- ELISA, tandem mass spectrometry-MS/MS) have been developed to further elucidate their roles [205].

The most used methodologies to quantify GAGs are alcian blue and dimethylmethylene blue (DMMB) [206-208]. However, the use of urinary GAGs (uGAGs) as a long-term measurement is limited by lack of significant changes overtime, the age-dependency character of GAGs declining as a function of age, variation among anthropometric variables, and renal status [209-212]. The exact correlation between uGAG and somatic disease severity remains unclear [211, 213-215] and attenuated forms of the disease cannot be detected resulting in false negatives [216-218]. More importantly, urinary KS does not correlate with any clinical improvement; however it can be used for pharmacodynamic effect. Furthermore, such methodologies were established for use in urine and did not work with DBS, invalidating their use for newborn screening (NBS) [219].

More recently, mass spectrometry has been used to measure GAGs in biological tissues [220]. The principles that led to mass spectrometry were discovered over 100 years ago by the Nobel laureate Sir John Thomson, who discovered the

electron and was the first to demonstrate the separation of isotopes of a stable element [221, 222]. Mass spectrometry has now become one of the most useful analytical techniques due to its specificity, accuracy, and sensitivity [223-225] and is considered one of the most successful and useful techniques applied for NBS [226].

Tandem mass spectrometry (MS/MS) is a technique that measures compounds based on their mass-to-charge ratio (m/Q, m/q, m/Z, or m/z) [223, 224, 227]. Different ionization sources can be used, e.g.: electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI), fast atom bombardment (FAB), chemical ionization (CI), and matrix-assisted laser desorption/ionization (MALDI) [222, 228-232]. GAG analyses have been performed in different types of mass analyzers, such as time-of-flight (TOF) [233], ion trap (IT) [234, 235], fourier transform ion cyclotron resonance (FTICR) [236], and MS/MS [205, 237-239].

Mass spectrometry analysis can be full-spectrum, to detect all intact ions in mixtures of unknown compounds, ion monitoring, to measure levels of known intact ions, or multiple reaction monitoring (MRM), to measure different intact/product ion pairs that can distinguish compounds that have ions of identical mass/charge ratio but have different fragments [205, 225, 240, 241].

In 2001, Oguma et al. developed a method for disaccharide detection after digestion of heparan sulfate (HS) and keratan sulfate (KS) using mass spectrometry (MS) with further development of dermatan sulfate (DS) quantification in 2007 [238, 242-244]. Several groups have employed the same methodology or adapted protocols for biomarker analysis, treatment monitoring and newborn screening [245-254].

Another approach for GAG measurement based on chemical degradation by methanolysis was developed in 2011 for urine and cerebrospinal fluid (CSF) [255, 256]. However, chemical degradation protocols have not been developed for blood or DBS samples yet.

1.3.5 Clinical Features of MPSs

The natural histories of all types of MPSs are chronic and progressive, usually being asymptomatic at birth (sometimes with altered radiological findings) with progression through the first years of life, with an extensive range of clinical manifestations such as skeletal dysplasia, corneal clouding, coarse facial features, joint rigidity or laxity (Fig. 1.6), hepatosplenomegaly, cardiac and respiratory involvement, neurodegeneration and learning difficulties (exception of MPS IV and VI), and behavioral alterations (MPS III).

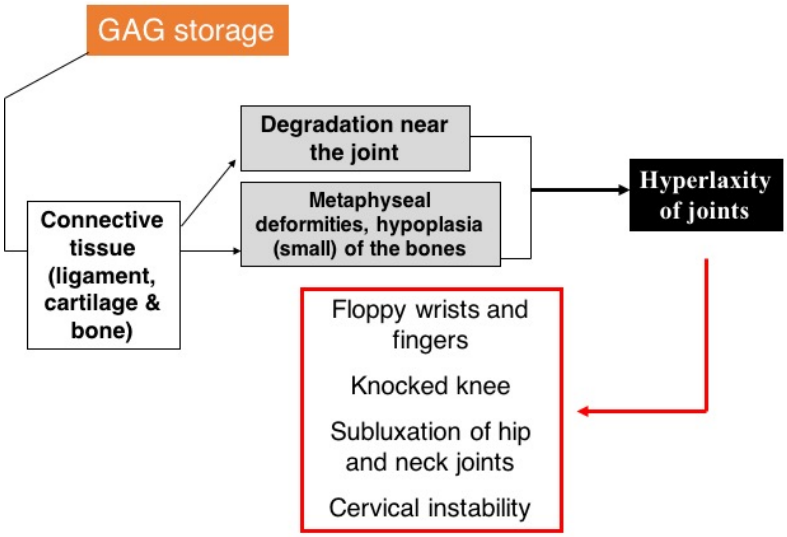

Figure 1.6 Pathophysiology of Hyperlaxity in MPS IVA
Proposed pathophysiology of hyperlaxity in MPS IVA is due to GAG accumulation in connective tissue and bone that leads to cartilage degradation and formation of smaller bones that could potentially lead to hypermobile joints.
Adapted from: Morquio Educational CD.

There is a wide spectrum of variation in the manifestations of these disorders, though typically two phenotypic forms of progression are presented; a severe form, with early-onset with rapid progression, and an attenuated form, with a later onset with slow progression [56, 257-260].

It is expected that severe phenotype patients will have no or lower enzyme levels when compared to attenuated phenotype patients, resulting in higher GAG accumulation. Correlation between genotype-phenotype is a challenge, as each MPS is caused by mutations in a different gene and such mutations are extremely heterogeneous. Some of those genes have more than 500 mutations described in the Human Gene Mutation Database (HMGD) to date. It is also known that the mutation

site will affect enzyme levels, in which mutations near the active site, buried in the hydrophobic core or on the surface, will affect the enzyme in different ways [261-273].

Although clinical manifestations usually do not appear at birth, GAG accumulation can be detected in a human fetus and placenta in some MPS subtypes, suggesting that GAGs can be detected before appearance of clinical manifestations [274-278]. The incidence of MPSs is variable among different subtypes and ethnicities, but the combined incidence is estimated as 1:25,000 live births [219].

At present, patients are mainly diagnosed after the appearance of clinical manifestations, followed by quantitative analysis of enzyme activity and GAG measurement. Molecular testing is also available and useful for prognosis, carrier testing, and prenatal analysis. All MPS patients must have their diagnosis confirmed by enzyme assay [259].

1.3.6 Biomarkers for MPSs

Biomarkers are useful for all diseases and treatments, but especially for orphan diseases in which patient cohorts are smaller [279]. The use of biomarkers for MPSs helps clinicians with diagnosis and also provides a tool for evaluation of disease progression, helping with prognosis and treatment monitoring [62, 280]. Biomarkers employed in the study of MPSs can be divided into two categories [279]; direct markers, or a storage molecule reflecting pathological storage material(s) (e.g. CS, DS, HS, KS and/or hyaluronan) as well as the deficient enzyme, and indirect markers,

which are molecules or changes in cell, tissue or organ function as a result of primary storage (e.g. FGF2, HCII-T, liver and spleen volume, etc).

Simple biomarkers have not yet been developed for MPSs. Total and urinary GAGs have been widely used as a biomarker for MPSs, and they are useful in diagnosis and treatment monitoring. The uGAG is the most used biomarker for MPSs and has been exploited as a surrogate marker in clinical trials for all approved ERT in MPSs (MPS I, II, and VI), respectively [281-284].

Indirect markers that have been reported as biomarkers for MPS include: fibroblast growth factor-2 (FGF2), heparin cofactor II-thrombin complex (HCII-T), dipeptidyl peptidase IV (DDP-IV), GM gangliosides, bis [monoacylglycero] phosphate (BMP), inflammatory and proinflammatory cytokines, insulin-like growth factor binding protein 7 (IGFBP7), heart of glass receptor 1 (Heg1), nitric oxide (NO), tumor necrosis factor alpha (TNF-α), tumor necrosis factor 1 (TNFR1), transforming growth factor-beta (TGF-β), matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinase 1 (TIMP-1), brain-derived neurotrophic factor (BDNF), tau proteins, cathepsin B, oxidative stress markers, and free radicals [87, 181, 285-310].

1.3.7 Treatment for MPSs

As for several other IEMs and LSDs, there is no current cure for MPSs but available treatments are ERT [311], HSCT [312], GT [313], SRT [130], PCT [104, 127], and anti-inflammatory therapy [145, 182].

The development of ERT was a breakthrough in the treatment for LSDs. It is based on the ability of cross-correction by targeting the recombinant enzyme with M6P [33, 314, 315]. ERT has shown significant therapeutic efficacy in resolving defects in visceral organs, but has several limitations: the large molecules cannot cross the blood-brain barrier (BBB); the enzymes cannot penetrate refractory tissues such as bone, cartilage and heart; enzymes have a short half-life requiring weekly or by-weekly infusions; they are immunogenic; and the procedure is very expensive ($300,000 to 400,000 per year per patient) [95, 316, 317].

The use of HSCT relies on the cross-correction of the enzyme deficiency using donor cells to secrete the deficient enzyme in a continuous way [318, 319]. A major advantage of HSCT is that this treatment yields microglial cells that can cross the blood brain barrier (BBB) and thereby deliver enzyme to the central nervous system (CNS) [104, 320]. However, its effects are limited by the patient's age, phenotype, immune reactions (allogeneic donors; regimens; risk of graft versus host disease-GVHD), higher mortality than other treatments, and limited effects in bone and cartilage [21, 312].

Due to the limitations of ERT and HSCT, gene therapy and alternative approaches are being pursued. At present, clinical trials for gene therapy have been approved for MPS II (USA), MPS IIIA (Europe), MPS IIIB (Europe). These studies will determine whether gene therapy will be a safe and feasible treatment approach for MPSs [21].

For SRT, a flavonoid called Genistein decreases GAG synthesis and reduces GAG concentrations both *in vitro* and *in vivo* and is being used in a clinical trial with MPS III patients in Europe [120, 321]. The biggest advantage offered by this SRT is that the small molecule can cross the BBB and thereby potentially treat neurological impairment [322].

PCT has been suggested as a future therapeutic approach for MPS due to their ability to protect unfolded proteins from ER degradation [323]. Another approach specific for nonsense mutations is based on the use of compounds that are able to promote translational read-through of premature stop codons restoring protein levels in some degree. Aminoglycoside antibiotics can restore expression of functional proteins; however severe-side effects are associated with its use. High toxicity levels lead to the development of new compounds with reduced toxicity and higher efficiency such as: Ataluren (PTC124) and NB54 [324, 325]. NB54 has been used in *in vitro* studies with MPS I constructs [325], and Ataluren is currently on phase 2 clinical trial for MPS I patients in Europe (EudraCT Number: 2014-002596-28) (https://www.clinicaltrialsregister.eu/ctr-search/search?query=2014-002596-28).

Another alternative treatment is anti-inflammatory therapy. Inflammation has been implicated as a major cascade that contributes to disease progression, with increased apoptosis and upregulation of cytokines [182, 326]. Schuchman et al. (2013) demonstrated positive effects of the anti-inflammatory compound pentosan polysulfate (PPS) in the treatment of MPS VI rats [143]. PPS caused reduced levels of GAGs and also showed chondrogenic properties. These findings led to pre-clinical trials in a MPS

I dog model (unpublished data) and clinical trials in MPS II patients in Japan (unpublished data).

There is no consensus on which treatment approach is the most appropriate for each MPS subtype. However, there is no doubt that early diagnosis followed by early treatment have a major beneficial impact on the outcome and prognosis [104, 327]. There are many clinical examples of better outcomes with earlier treatment [328-340].

Gabrielli et al. (2010) reported the effects of early ERT in two siblings with MPS I, in which the younger brother with an attenuated form of the disease had started treatment at five months of age, while his sister with a more severe form of the disease was untreated until five years of age. After five years of treatment, the brother had not developed any symptomatology, except corneal clouding, whereas the older sister had an improved phenotype but still displayed persistent symptoms [341]. The study was limited by having patients with different phenotypes; however, it was successful in showing the effects of early treatment regardless of phenotype. The patients were followed for the next 7 years, in which early treatment revealed substantial improvement in somatic features and very slow progression of bone, cartilage and cardiac involvement when compared to treatment after signs and symptoms had appeared [332].

McGill et al. (2010) have also compared the effect of early and late ERT for MPS VI in a sibling-controlled study. One sibling was diagnosed *in utero* and treated from birth, while the other did not start treatment until 3.6 years of age. The patient that was diagnosed *in utero* had a better clinical outcome and quality of life, whereas

pathological changes in the older sibling were not reversed, highlighting the benefits of early treatment [342].

ERT has been recently approved for MPS IVA [343]. However, the majority of patients are diagnosed after three years of the age after most of the clinical symptoms have arisen. ERT in MPS IVA mice has shown that ERT is therapeutically better if started at birth rather than later, although bone pathology was not completely cleared and heart valve defects were still refractory to early treatment [96].

1.3.8 Newborn Screening for MPSs

NBS provides early diagnosis, allowing genetic diseases to be treated as soon as possible to prevent irreversible disease manifestations. The first metabolic disorder tested with a NBS was phenylketonuria (PKU) in the early 1960s by Dr. Robert Guthrie [344, 345]. Since then, NBS has been growing as a priority in public health, and more conditions are targeted for identification at birth to provide early intervention [346]. In the 1990s, mass spectrometry was adopted for NBS and in the early 2000s; Chamoles et al. developed strategies for assays in lysosomal enzymes in DBS [347-350]. Both methodologies allowed a growing number of LSDs to be included in NBS programs [351-353].

It is evident that early therapy can modify the natural history of MPSs, and the urge for early treatment has lead to some MPSs to be included in newborn screening (NBS) programs. In 2011, The American College of Medical Genetics included MPS I, II and VI in a NBS guideline, recommending their inclusion in NBS testing [354].

Currently, MPS I is part of the mandatory NBS in Illinois and Missouri and MPS II in Illinois [355].

MPSs are strong candidates for NBS because patients usually lack symptoms during the newborn period but progression is rapid and irreversible during early development, and a number of treatment options are available [356].

Newborn screening programs for MPSs using DBS were proposed and are now under investigation [250, 253, 353, 355, 357-371]. The main methods currently under development are based on enzyme assays by MS/MS or fluorimetric assays [359-361, 367, 369, 370, 372-377] or GAG assay [170, 219, 244, 247, 250, 253, 297, 371].

The GAG assay relies on a two-tier project where GAG measurement will be followed by enzyme assay (Fig. 1.7) [371]. A major advantage of developing measurement of GAGs as a newborn screen for MPS is that levels of one or more GAGs should be elevated in any MPS, making a single initial screen more sensitive and cost-effective for these rare disorders, and they also allow prognosis and treatment monitoring rather than just diagnosis, as offered by enzyme assay methodologies [219]. A potential limitation is that GAGs can be elevated due to other conditions unrelated to MPS giving false positives [371].

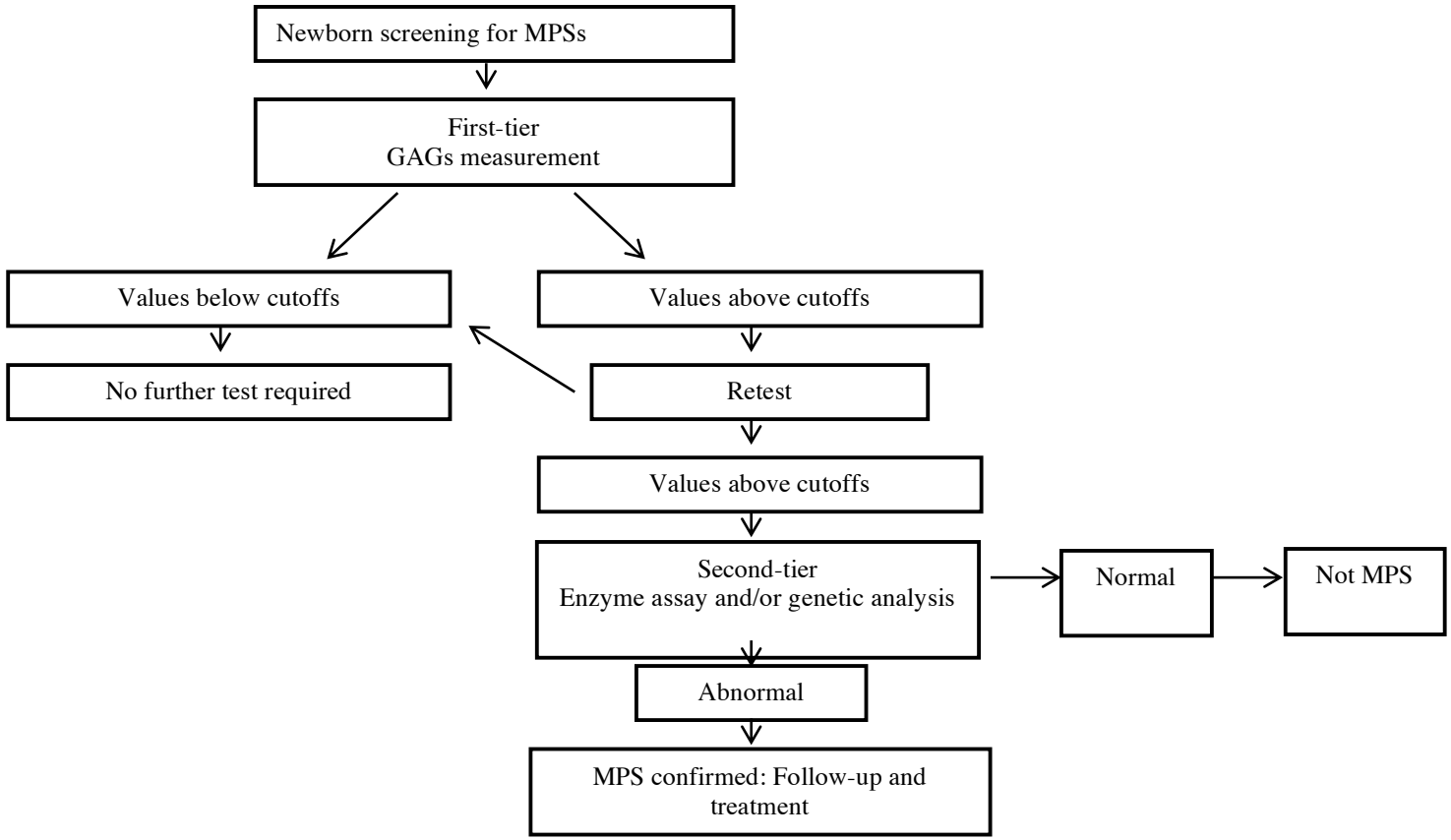

Figure 1.7: Algorithm for First and Second-tier Newborn Screening for MPSs
First-tier screening consists of quantification of GAG levels. Samples with GAG levels above cutoff values will be re-analyzed by GAG assay. In case elevated GAG levels are observed in the sample, enzyme measurement (second-tier) is performed to confirm MPS diagnosis.
Adapted from: Kubaski et al., 2016 with permission from Springer.

Chace et al (2010) and Turgeon et al (2010) suggested that a first-tier newborn screen could be performed with less selective methodologies (if they give minimal false negatives and not too many false positives) that could then be followed by a more selective second-tier screen (Fig 1.7). A better understanding of the cause of high levels of GAGs in the control population might help eliminate some false positives, but a first screen that eliminates 95% of the unaffected samples would be particularly valuable in reducing the costs of more expensive enzymes assays to define the specific metabolic defect [352, 378].

1.4 Significance and Innovation

According to the latest statistics from the Centers for Disease Control and Prevention (CDC), there were 3,932,181 newborns in the USA in 2013 [379]. Considering the incidence of MPSs, it is estimated that approximately 160 of those neonates will be affected with MPSs. Currently, most MPS patients are not diagnosed until clinical manifestations have arisen, and consequently treatment does not start until after many irreversible manifestations have begun. Delayed treatment can result in early death of MPS patients.

Development of a methodology that can diagnose MPS patients earlier will shift the current patient management to an entirely different perspective allowing early treatment and consequently slowing the progression of the disease leading to a better quality of life. Methods developed for NBS may also be able to evaluate treatment effect, creating the first available method for treatment monitoring for the MPSs.

Establishment of a single test for diagnosis and treatment efficacy will significantly improve current patient care, reducing morbidity, mortality, and public health costs.

Chapter 2

MATERIAL AND METHODS

2.1 Non-invasive Tests for MPS

Currently, assessments of disease progression and treatment outcomes are done by measurement of biomarkers (a measurable analyte that can be analyzed to indicate normal *vs*. pathologic processes are providing information about disease-progression) that can also be used as surrogate endpoints to reflect treatment effect [380].

Data on clinical examination findings, imaging, laboratory measurements, and patient reports of activity of daily living (ADL) are critical to understand how the disease will progress and how treatments are impacting or slowing disease progression [381].

Clinical trials for MPS usually include measurement of primary (enzyme assay and GAG quantification in blood and/or urine) and secondary (6-minute walk test [6MWT], 3-minute stair climbing test [3MSCT], imaging findings [echocardiographic and radiologic], pulmonary function test [spirometry]) endpoints [281, 283, 284, 343, 382, 383]. However, most of the secondary endpoints are effort dependent and cannot be used in young children or wheelchair-bound patients [384]. Thus, non-invasive tests for MPS should be further explored.

2.1.1 Bone Mineral Density[1]

Current medical status (functional abilities, anthropometrics, Tanner stage, medications) and medical history (fracture, physical development, birth events, diagnosis, medications, surgery) were ascertained through questionnaire and review of available medical records. Prior radiologic images, if available, were provided by subjects to help with the interpretation of dual x-ray absorptiometry (DXA) results. Available laboratory results were reviewed.

Bone mineral density (BMD) was assessed by DXA using a Hologic (Bedford, MA, USA) Discovery A model machine located in the Medical Imaging Department at Nemours/Alfred I. duPont Hospital for Children. All scans were acquired and analyzed by the same DXA technologist. The following body sites were scanned: whole body (WB), lumbar spine (LS), and lateral distal femur (LDF). In addition, on adult subjects, the proximal distal femur (hip) was scanned.

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[1] Published in Molecular Genetics and Metabolism

[455] H.H. Kecskemethy, F. Kubaski, H.T. Harcke, S. Tomatsu, Bone mineral density in MPS IV A (Morquio syndrome type A), Mol. Genet. Metab. 117 (2016) 144-149.

[456] F. Kubaski, H.H. Kecskemethy, H.T. Harcke, S. Tomatsu, Bone mineral density in mucopolysaccharidosis IVB, Mol. Genet. Metab. Reports 8 (2016) 80-84.

[†]The first two authors should be regarded as joint first authors.

The LDF scans were analyzed for three distinct regions of interest: region 1 (R1), region 2 (R2) and region 3 (R3) as previously described to assess bone density in different types of bone [385, 386]. R1 is predominantly trabecular bone, R2 is mixed trabecular and cortical bone and R3 is primarily cortical bone (Figure 2.1).

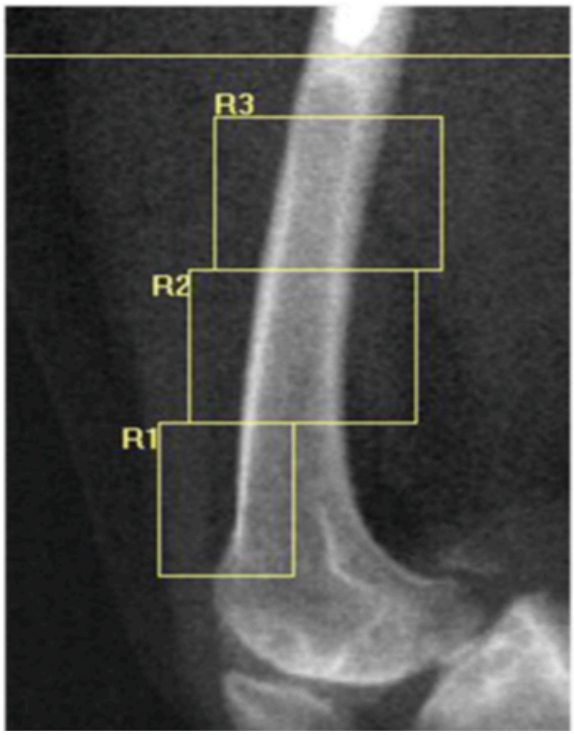

Figure 2.1: The Lateral Distal Femur (LDF) Scan is Analyzed for Three Regions of Interest
Region 1 (R1, anterior distal metaphysis) is essentially trabecular bone, region 2 (R2, metadiaphysis) is composed of both trabecular and cortical bone), region 3 (R3, diaphysis) is composed primarily of cortical bone.
Adapted from: Kubaski et al., 2016 with permission from Elsevier.

The LDF BMD was assessed bilaterally whenever possible (invalid in the presence of metallic hardware); left and right femur BMD values were averaged, and

Z-scores were calculated using published normative values). Radiologic images of the lateral spine, including radiographs and/or inter-vertebral assessment by DXA, were used to aid in correct region of interest placement on the LS DXA.

Bone mineral density results were compared with age and sex-matched norms to calculate Z-scores using manufacturer-provided normative values for the WB and LS and published norms for the LDF [387]. T-scores, used in adult BMD assessment, were not reported with our BMD MPS IV adult BMD results because no subject was post-menopausal or over age 50 [388]. For subjects older than 18 years of age, LDF Z-score was calculated using the oldest normative values available for each sex (18 years). The LDF values for patients younger than the lower age limit of the normative data available for the LDF were excluded from analysis. Data are evaluated based on age (adult over 18 years vs. children). The DXA values of BMD more than 2 standard deviations (SD) below the normal mean, expressed as Z-score ≤ -2, were considered abnormal. No height adjustment of BMD results was used because of the abnormal bone morphology seen in patients with MPS IV.

Because weight bearing is known to affect BMD, patients were grouped into two categories by ambulatory status: 1) full-time (F) ambulators (able to ambulate without the use of an assistive device [cane, crutches, or walker]) and 2) part-time or non-ambulators (P/N) (use of assistive device [walker or wheelchair] at school only or at the mall, or minimally able/unable to bear weight). The BMD results are examined for the entire group and relative to the amount of ambulation.

Height and weight measures were obtained at DXA scan acquisition, and height Z-scores were calculated using National Health and Nutrition Survey (NHANES) LMMS tables (accessed 9/5/15) [389]. The maximum age available (19.9 years) was used for patients over this age. The Nemours Institutional Review Board approved the study, and informed consent was obtained from all participants.

2.1.2 Pulmonary Function Tests[2]

2.1.2.1 Spirometry

All spirometry (Medgraphics Ultima PF; BreezeSuite Software; and PreVent Flow Sensor; St. Paul, MN) determinations were performed at our pulmonary function (PF) outpatient laboratory at Nemours/Alfred I. duPont Hospital for Children utilizing our standard operating procedures. As noted above, predicted values for an individual of normal stature were determined based on arm length [390] and evaluated in the BreezeSuite Software for the National Health and Nutrition Examination Survey (NHANES) III database [391]. Forced expiratory volume in one second (FEV_1), forced expiratory total volume (FEV_{TOT})/Forced Vital capacity (FVC)/Vital capacity

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[2] Published in Molecular Genetics and Metabolism

[384] F. Kubaski, S. Tomatsu, P. Patel, T. Shimada, L. Xie, E. Yasuda, R. Mason, W.G. Mackenzie, M. Theroux, M.B. Bober, H.M. Oldham, T. Orii, T.H. Shaffer, Non-invasive pulmonary function test on Morquio patients, Mol. Genet. Metab. 115 (2015) 186-192.

(VC), predicted forced expiratory total volume (%FEV_{TOT}), FEV_1/FEV_{TOT} (timed forced expiratory volume/forced expiratory total volume), and %Predicted (FEV_1/FEV_{TOT}) were determined from the best of 3 spirometric tracings.

2.1.2.2 Impulse Oscillometry System

Impulse oscillometry system (IOS) measures resistance (R) at different frequencies (R_5-peripheral airway resistance; R_{20}-central airway resistance) and reactance (movement of the air in the airways) [392]. The device used for the oscillatory measurements was the Master Screen-IOS device (E. Jaeger, Höchberg, Germany) [393-395]. For each impulse, 32 sample points were analyzed. Daily calibration using a calibration pump (3.0 ± 0.01 L SD, Jaeger; Höchberg, Germany) and a reference impedance of 0.2-2.5 kPa/1/s were performed.

Briefly, small mechanical impulses were superimposed on the spontaneous breathing pattern. Through the phase relationship, the impedance (Zrs) was portioned into resistance (Rrs) and reactance (Xrs). Rrs included the airway, lung tissue, and chest wall resistance, whereas Xrs represented the balance of two (an elastic and an inertial) components or the net effect of two opposite (a compliant and an inertial) components.

After a brief explanation, patients performed some practice trials. When the patient was feeling comfortable, study testing started. Subjects performed the test in a sitting position, breathing quietly for 15 to 30 seconds using an oval mouthpiece with

the head in a neutral position, nose clips in place, and supporting both cheeks with help by the staff.

The following parameters were measured: Rrs at 5, 10, 15, 20, 25, 35 Hz and resonant frequency (fRES) (i.e., the frequency at which the reactance crosses zero). Three replicate oscillatory values were used to calculate the mean and the coefficient of variation (CV) for Rrs_5. Coherence value was used as a quality control parameter, and the CV for Rrs5 is used as a reliability index. Measurements were discarded if the time-flow and volume pattern showed an interruption of the oscillatory signal. It should be noted that the Jaeger software has established predicted values as a function of age, height, and gender for individuals of typical stature [396].

2.1.2.3 Pneumotachography

Air flow, CO2 graphs, and oximetry signals were recorded using a pediatric/adult monitoring system (Novametrix Medical Systems, Wallingford, CT). Testing was performed to collect at least 10 uniform breaths, and the data was stored for analysis. The breaths were collected from a long, continuous period of breathing (2–4 min). Measurements were recorded over time at the airway opening using a pneumotachometer via face mask; mask integrity was monitored for leaks (less than 10% tidal volume change throughout each breath), and a constant tidal volume breathing frequency history was observed for at least 10 breaths [397-399].

Airflow was measured with a low dead space volume pneumotachometer and integrated pressure transducer. End-tidal CO_2 was calculated by algorithms

incorporated into the monitoring system unit. Flow and CO_2 graphs were analyzed in real time and after each measurement (Miller et al., 2004). CO_2 graphs were used to assess airway obstruction. As an additional safety measurement, the transcutaneous saturation of oxygen (SpO_2) was monitored simultaneously with a small sensor on the finger of the patient.

2.1.2.4 Respiratory Inductance Plethysmography

Respiratory inductance plethysmography (RIP) is a noninvasive respiratory evaluation approach, which determines pulmonary ventilation or breathing by using thoracoabdominal motion (TAM) analysis with markers of thoracoabdominal asynchrony (TAA).

Previous studies demonstrated how much degree of the chest and abdominal excursions are out of phase due to respiratory abnormalities such as chest wall dysfunction, lung compliance abnormalities, and/or airway obstruction [384, 400-402].

Changes in TAM correlated with changes in resistance and compliance in infants/children/adults with airflow obstruction and/or restrictive disorders. These changes in TAM and other TAM indices expressed the degree to which chest and abdominal (ABD) excursion are out of phase, or thoracoabdominal asynchrony (TAA). Measurements were made using elastic coil inductance bands that fitted comfortably and snug enough to obtain adequate recordings; any excess of band material was folded and pressed together using a plastic forceps (Devon,

tyco/Healthcare, Princeton, NJ) in each band to prevent any unintentional change in position. The elastic bands were used to provide an indication of the tidal volume proportion to the rib cage and abdominal compartments.

Real-time raw signals and Konno–Mead loops/ Lissajous loops (x-y plot of abdominal component vs. rib cage for each breath) were monitored during the test to ensure proper signal and adequate quality using RespiEvents software 5.2 g (Nims, Inc., Miami, FL) [403].

Phase angle (Phθ) is based on synchrony of breathing, when breathing in synchrony phase angle is defined as zero degrees, but if abdomen and rib cage are out of synchrony phase angle can be calculated. In the current study, Phθ was calculated as previously reported [398, 399] based on the differences in phase shift between the abdomen and the rib cage compartments.

The percent rib cage contribution (%RC) is defined as the rib cage compartment volume over the rib cage plus abdomen. PFT methods included RIP using the SomnoStarPT Unit (Sensormedics, Yorba Linda, CA) and inductance bands (RespiBands Plus; VIASYS Respiratory Care, Yorba Linda, CA).

Initially, patients underwent RIP, in which the relationship between thoracic and abdominal contributions to the respiratory effort was assessed [396, 397, 399, 404, 405]. Bands containing inductive coils were carefully placed around the rib cage at the level of the axillae and around the abdomen mid-way between the xiphisternal junction and the umbilicus. We used an uncalibrated RIP method for phase and synchrony evaluations. The Respitrace device was used to calculate phase angle, to

construct Konno-Mead/Lissajous loops and for calculation of phase angle between the rib cage and abdominal movement associated with respiration.

Reported phase angle measurements and other indices of asynchrony were based on the average of at least 10 uniform Lissajous loops. The signals from the RIP bands around the rib cage and the abdomen were treated mathematically as sine waves. The phase angle was calculated with RespiEvents software 5.2 (NIMS, Miami, FL). This parameter and other thoracoabdominal markers expressed the degree to which chest and the abdominal excursion were out of phase. Normally, the rib cage moves outward during inspiration completely in phase with the outward movement of the abdomen (in phase). With progressive increase in the work of breathing, like in airflow obstruction, the rib cage lagged behind abdominal movement (out of phase) becoming asynchronous [398].

Recordings were made with the patient in the sitting position. During the test, raw signals and Konno-Mead (Lissajous) loops were monitored to ensure adequate signal quality and to select suitable breathing sequences for analysis with RespiEvents software 5.2.

2.2 Glycosaminoglycans Analyses[3]

2.2.1 Materials

Chondroitinase B, heparitinase, keratanase II, chondrosine (internal standard-IS), and the unsaturated disaccharides: heparan ΔDi-0S [2-acetamido-2-deoxy-4-O-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose] (HS-0S), heparan ΔDi-NS [2-deoxy-2-sulfamino 4-O-(4- deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose] (HS-NS), heparan Δ Di-di [2-deoxy-2-sulfamino-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-glucose (HS-diS_1), heparan Δ Di-tri (2-dexoy-2-sulfamino-(4-deoxy-2-O-sulfo-α-L-threo-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-glucose) (HS-triS), chondro Δ Di-4S [2-acetamido-2-deoxy 3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-D-sulfo-galactose] (Di-4S), chondro Δ Di-6S [2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepryranosyluronic acid)-6-O-D-sulfo-galactose] (Di-6S), mono-sulfated KS [Galβ1-4GlcNAc(6S)], and di-sulfated KS [Gal(6S) Galβ1-4GlcNAc(6S) were all provided by Seikagaku Co (Tokyo, Japan).

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[3] Published in Journal of Inherited Metabolic Disease

[371] F. Kubaski, R.W. Mason, A. Nakatomi, H. Shintaku, L. Xie, N.N. van Vlies, H. Church, R. Giugliani, H. Kobayashi, S. Yamaguchi, Y. Suzuki, T. Orii, T. Fukao, A.M. Montaño, S. Tomatsu, Newborn screening for mucopolysaccharidoses: a pilot study of measurement of glycosaminoglycans by tandem mass spectrometry, J. Inherit. Metab. Dis. 40 (2017) 151-8.

2.2.2 Stock Solutions

Lyophilized solutions of HS, and DS were reconstituted in ddH_2O (Millipore Milli-Q Reference A + System) to make 100 μg/mL of HS-0S (add 758.6 μL of ddH_2O), HS-NS (add 834.6 μL of ddH_2O), HS-diS_1 (add 994.8 μL of ddH_2O), HS-triS (add 1,155 μL of ddH_2O); 250 μg/mL of Di-4S (add 1,000 μL of ddH_2O) and Di-6S (add 1,000 μL of ddH_2O) (Kubaski et al., 2016). Solutions of 1000 μg/mL of KS (mono and di-sulfated) were received from Seikagaku Co (Tokyo, Japan).

Lyophilized chondrosine (IS) (300 mg) was reconstituted in 1,000 μL of ddH_2O (0.3 mg/μL). At the begining of each month, stock IS was diluted (1:300) in which 3 μL of stock IS + 900 μL of ddH_2O were aliquoted (1000 μg/mL). For each reaction, this IS stock (1000 μg/mL) was further diluted (1:200) in which 3 μL of IS (1000 μg/mL) were mixed with 597 μL ddH_2O (1000 μg/mL).

1% of bovine serum albumin (BSA) (0.5g of BSA + 50 mL of ddH_2O), 0.1% BSA (0.05g of BSA + 50 mL of ddH_2O), 50 mM Tris HCL (3g Tris base + 500 mL of ddH_2O) pH 7, 148 mM Ammonia (996 mL of ddH_2O + 10 mL of ammnonium hydroxide) were used.

2.2.3 Enzyme Preparation

The following enzymes were used to generate disaccharides of specific GAGs: chondroitinase B for DS (DS was measured as Di-0S after digestion of Di-4S by a 4S-

sulfatase present in the preparation of chondroitinase B), chondroitinase ACII for C4S, chondroitinase ACB for C6S, heparitinase for HS, and keratanase II for KS.

Lyophilized heparitinase (0.1U) and keratanase II (0.1U) were diluted in 1000 µL of 1% BSA to make 1mU. Lyophilized chondrotinase B (0.1U), chondroitinase ACII (0.1U), chondroitinase ABC (0.1U) were diluted in 1,500 µL of 1% BSA to make 0.6mU.

2.2.4 Sample Preparation

2.2.4.1 Dried Blood Spots

Two Disks (3.3mm) were cut from DBS samples using a DBS puncher (PerkinElmer®; Waltham, MA) and placed into a 96 well Omega 10K filter plate with 100 µL of 0.1% BSA on a 96 well receiver plate. Samples were incubated for 15 min and then centrifuged for 15 min at 2500 g.

The filter plate was transferred to a new receiver plate, and a cocktail mixture of 90 µL of 50 mM Tris HCL (pH 7.0), 10 µL of 5 µg/mL IS, 10 µL of chondroitinase B, 10 µL of heparitinase, 10 µL of keratanase II was added to each DBS. For standard curves serial diluted standards (Std8 to Std1) were added to the cocktail mixture in blank wells. Samples were incubated at 37 °C water bath overnight. The next day, the samples were centrifuged for 15 min at 2,500 g. The filter plate was discarded and receiver plates stored at -20 °C until injection of samples into the LC/MS/MS.

2.2.4.2 Amniotic Fluid, Serum or Urine Samples

10 μL of sample (amniotic fluid, serum or urine) were placed into a 96 well Omega 10K filter plate with 90 μL of 50 mM Tris HCL (pH 7.0) on a 96 well receiver plate. Samples were incubated for 15 min and then centrifuged for 15 min at 2500 g.

The filter plate was transferred to a new receiver plate, and a cocktail mixture of 90 μL of 50 mM Tris HCL (pH 7.0), 10 μL of 5 μg/mL IS, 10 μL of chondroitinase B, 10 μL of heparitinase, 10 μL of keratanase II was added to each DBS. For standard curves serial diluted standards (Std8 to Std1) were added to the cocktail mixture in blank wells. Samples were incubated at 37 °C water bath overnight. The next day, the samples were centrifuged for 15 min at 2,500 g. The filter plate was discarded and receiver plates stored at -20 °C until injection of samples into the liquid chromatography tandem mass spectrometry (LC/MS/MS).

2.2.5 Standard Curves

Standard curves were prepared using dilutions of the stocks as follows: 10μL HS-0S, 10 μL HS-NS, 10 μL mono-KS, 10 μL di-KS and 4 μL Di-4S in 956 μL of H_2O (Std8). 500 μL of Std8 were serially diluted in 500 μL of H_2O seven times giving rise to Std7 through Std1. Final concentrations for standard curves were as follows: HS, Di-4S and Di-6S (1,000; 500; 250; 125; 62.50; 31.25; 15.62; 7.81 ng/mL) and for mono and di-sulfated KS (10,000; 5,000; 2,500; 1,250; 625; 312.50; 156.25; 78.13 ng/mL). 10 uL of each standard was mixed with the cocktail mixture (as described

above). Results were analyzed by MassHunter Workstation software quantitative analysis Version B.05.00/ Build 5.0.291.0.

2.2.6 LC/MS/MS

The apparatus consisted of a 1290 Infinity LC system with a 6460 triple quad mass spectrometer (Agilent Technologies, Palo Alto, CA). Disaccharides were separated on a Hypercarb column (2.0 mm i.d. 50 mm length; 5 μm particles; Thermo Scientific, USA), thermostated at 60 °C. The method was modified from that developed by Oguma et al. [244].

The mobile phase was a gradient elution of 148 mM Ammonia (solution A) to 100% acetonitrile (solution B). The flow rate was 0.7 mL/min, and the gradient was created by adjusting buffer B as follows: 0 min 0% solution B, 1 min 50% solution B, 2 min 50% solution B, 2.20 min 100% solution B, 2.60 min 100% solution B, 2.61 min 0% solution B, 5 min 0% solution B.

The mass spectrometer was operated with electrospray ionization in the negative ion mode (Agilent Jet Stream technology) with drying gas temperature 350 °C, drying gas flow 11 L/min, nebulizer pressure 58 psi, sheath gas temperature 400 °C, sheath gas flow 11 L/min, capillary voltage 4,000 V, nozzle voltage 2,000 V.

Specific precursor and product ions, *m/z,* were used to quantify each disaccharide respectively (IS, 354.3, 193.1; DS, 378.3, 175.1; C4S, 458.4, 300.2; C6S, 458.4, 282.1; 416, 138.1 for HS-NS; 496.3, 416.3 for HS-diS_1; 542, 461.9 for di-KS; 462, 97 for mono-KS [244, 406-409].

The injection volume was 5 μL with a running time of 5 min per sample. Ratio di-sulfated KS in total KS was calculated as di-sulfated KS divided by (mono-sulfated KS + di-sulfated KS) x 100%.

The lower limit of quantitation (LLOQ) was defined as the lowest level of the signal with an accuracy of better than 20% and the lower limit of detection (LOD) as a signal to noise ratio of <10 according to the Food and Drug Administration [410].

Chapter 3

NON-INVASIVE ASSESSMENTS ARE FEASIBLE AND PROVIDE CRITICAL INFORMATION ABOUT THE NATURAL HISTORY OF MUCOPOLYSACCHARIDOSES

Natural history studies are conducted to elucidate the natural course of diseases as well as to clarify disease progression with or without treatment. It is well known that due to continuous accumulation of undegraded GAGs, the natural course of all types of MPS is chronic and progressive with multisystemic impairment in the majority of MPS subtypes [258, 411-421]. Knowledge of natural history is essential for the development of new therapies and evaluation of long-term treatment outcomes [381, 412, 413].

In 1983, the US Congress passed the Orphan Drug Act (ODA) (law 97-414) to deliver incentives for biotech and pharmaceutical companies to develop drugs for rare diseases (1:200,000 live births) including grants, tax credits, exemption from fees and marketing rights for such diseases. After development, such drugs are submitted to the Food and Drug Administration (FDA), and if proven safe and effective they can be marketed [422, 423]. The FDA also recommends the use of biomarkers (a measurable analyte that can be analyzed to indicate normal *vs*. pathologic processes providing information about disease-progression) that can also be used as surrogate endpoints [380, 381].

Furthermore, it is critical to have feasible non-invasive assessments that will be able to accommodate a broad spectrum of patients (from attenuated to severe forms) from all ages and conditions (e.g. wheelchair-bound patients) to provide critical information about natural history in MPSs.

I proposed to determine whether bone mineral density (BMD) and non-invasive pulmonary function tests (PFT) in MPS IV are valuable tools for evaluation of natural history in MPSs.

3.1 Bone Mineral Density in MPS IVA[4]

Morquio syndrome type A (mucopolysaccharidosis type IV A, MPS IV A) is an autosomal recessive lysosomal storage disorder caused by a deficiency of N-acetylgalactosamine-6-sulfate sulfatase (GALNS) [56, 424, 425]. This deficiency leads to accumulation of excessive glycosaminoglycans (GAGs) keratan sulfate (KS) and chondroitin-6-sulfate (C6S) primarily in bone, cartilage, ligaments, and the

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[†]The first two authors should be regarded as joint first authors.

extracellular matrix (ECM) [56].

In patients with MPS IVA, even at birth, cartilage formation is disrupted, resulting in poor bone mineralization [145]. Systemic skeletal symptoms are caused by excessive KS storage and result in spondyloepiphyseal dysplasia, striking short trunk stature, cervical spinal cord compression, pectus carinatum, kyphoscoliosis, knock-knee, hypermobile joints, and an abnormal gait with an increased tendency to fall. Many patients become wheelchair dependent in their second decade and undergo multiple surgeries to alleviate serious medical complications [418, 426].

Three studies report bone mineral density (BMD) in MPS IV A, all with limited sample size (n = 2, 9, 2, respectively). Dual energy X-ray absorptiometry (DXA) was used to assess BMD in the studies. Both Rigante et al. and Koura et al. reported low BMD at several body sites measured. Lin et al. reported normal BMD results after applying the height-adjusted Z-score (HAZ) method [427] used to correct for the height deficits. Subsequently, the use of the HAZ method for correction of BMD has been brought into question in the presence of skeletal dysplasia [428].

Bone properties including shape, structure, and strength of bone are related to load and forces on the bone. The loss of BMD in medical conditions affecting the ability to bear weight such as cerebral palsy (CP), spina bifida, and muscular dystrophy has been described. Low BMD of the lower extremities as measured by the lateral distal femur (LDF) DXA is directly associated with lack of ambulation or weight bearing [429-433]. Henderson and colleagues described a strong association between low BMD at the LDF and fracture history [432].

We describe BMD, fracture history, and ambulation in 16 unrelated (18 total) MPS IV A patients to understand the natural course of BMD. We hypothesized a higher prevalence of low BMD in these patients compared with normal subjects. Because of the limitations of the whole body (WB) and lumbar spine (LS) measurements in this patient population, we also evaluated BMD by using another site, the LDF. This alternative measurement site applied to conditions affecting ambulation and spine abnormality is directly applicable to patients with MPS IVA. BMD was measured as described in section 2.1.1.

3.1.1 Subjects

In this prospective, cross-sectional study, we evaluated 18 patients (16 unrelated) with Morquio syndrome type A (13 females) ranging in age from 3.3 to 40.8 years (mean age 21.4 years) at our hospital. All patients were diagnosed by enzymatic assay.

3.1.2 Results

Phenotype analyses based on height were performed on all subjects [434]. For the 6 patients with available genotype analysis, phenotype/genotype correlations were performed (Table 3.1).

Table 3.1: Clinical Data for Mucopolysaccharidosis IVA Patients

ID	Height (cm)	Height Z-score	Weight (kg)	Age (yrs)	Sex	ERT	Genotype	Ambulatory status
1	88.9	−2.2	12.7	3.3	M	N	n/a	F
2	97	−2.2	16.2	4.9	F	N	n/a	F
3	99.1	−3.5	15.9	6.1	F	Y	p.G301C/G301C	F
4	86.4	−8.7	13.2	9.1	M	Y	n/a	P/N
5	106.7	−8.7	37.6	15.9	F	N	n/a	P/N
6	91.44	−8.7	28.6	16.3	F	N	P.ivs8+1G>c/ivs8+1G>c	P/N
7	114.3	−7.7	35.5	18	F	N	p.S287L/L352P	P/N
8	116.8	−8.1a	33.2	18.9	M	Y	n/a	P/N
9	113.8	−8.5a	34.7	21.0	M	Y	n/a	F
10	96.5	−10.1a	25.4	21.1	F	N	n/a	P/N
11	118	−8.0a	34.5	22.4	M	N	p.G42E/P125L	F
12	126	−5.7a	34.5	23.0	F	N	n/a	P/N
13	129.5	−5.2a	36.4	25.6	F	N	p.S287L/L352P	F
14	91.4	−10.8a	22.7	30.6	F	N	p.M41K/M41K	P/N
15	91.4	−10.8a	25.5	34.2	F	N	n/a	F
16	122	−6.3a	34.4	36.5	F	N	n/a	F
17	106.7	−8.6a	36.4	40.8	F	N	n/a	P/N
18	96.5	−10.1a	24	38.4	F	N	n/a	F

ERT: enzyme replacement therapy; F: full-time ambulators; P/N: part-time or non-ambulators; F: female; M: male; N: no; Y: yes;

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Four patients were undergoing enzyme replacement therapy (ERT). While every subject had the ability to bear weight, 9 were full-time ambulators. Of the 9 P/N ambulators, 7 were able to walk/bear weight but chose to use a power wheelchair full time for speed and convenience. Only 2 patients (both adults) were not able to ambulate without assistance and were unable to walk for over 50 yards with a walker. Only two fractures were reported and were due to trauma (hand slammed in door; femur from tripping while running with crutches) (Table 3.1).

3.1.2.1 DXA Utility by Body Site

The recommended body sites to measure by DXA differ between adults and children [435]. In children, WB and LS are the preferred sites along with LDF in certain conditions. In adults, the LS and proximal femur are recommended. The use of LDF in adults has been described by Henderson and colleagues [436]; because adult normative data are not available, the oldest available age for LDF Z-score calculations was used (age 18.9 years).

In the present study, we assessed the utility of DXA sites in patients with MPS IVA. Results for WB could be obtained on only 6 subjects (2 children, 4 adults) because of respiratory compromise caused by the position, the presence of hardware, or positioning difficulties. The WB was not well tolerated because of discomfort experienced by subjects when lying flat on their back; in a few cases, subjects were unable to breathe. The proximal femur was successfully acquired on only 1 of the 11 adults, but accurate analysis of the scan was impossible because of abnormal anatomy.

The LS scan was successfully acquired in all patients, but, because of the abnormal shape and position of vertebrae seen in Morquio syndrome type A, the technical validity of the LS DXA was compromised and limited to 10 (55.6%) of our subjects (Fig. 3.1).

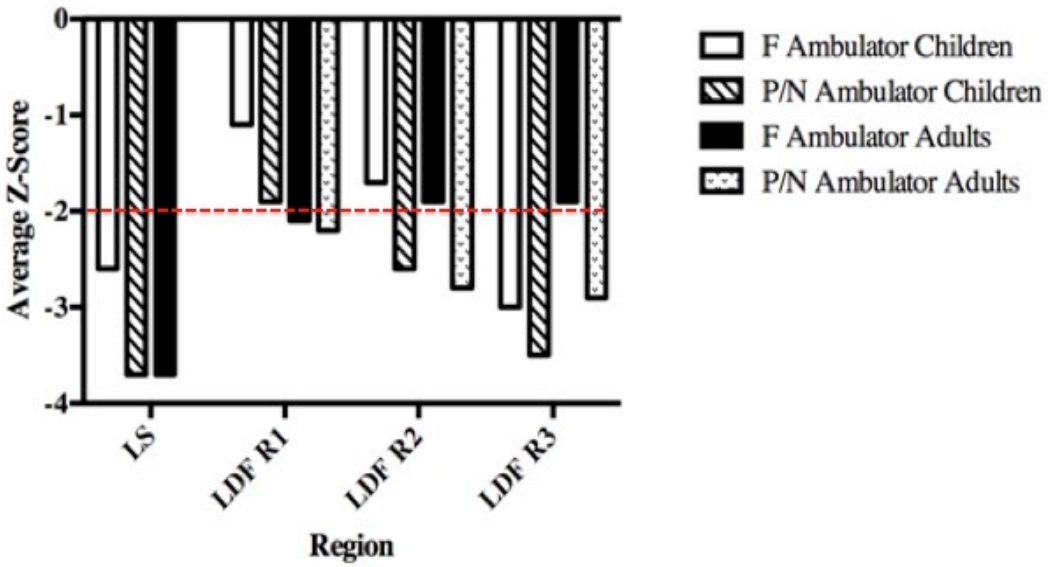

Figure 3.1: Lumbar Spine and Lateral Distal Femur Bone Mineral Density Z-scores in Children and Adults by Ambulatory Status
Overall Z-scores at all regions (lumbar spine [LS] and lateral distal femur [LDF]) were low regardless of age and ambulation. DXA values below ≤ -2 were considered abnormal (red line).
F: full-time ambulator; P/N: partial or non-ambulator;
Adapted from: Kecskemethy et al., 2016 with permission from Elsevier.

The LDF was the only site at which all patients could be scanned, showing technically valid results (Table 3.2).

Table 3.2: Utility of Dual-Energy X-Ray Absorptiometry in Children and Adults with Mucopolysaccharidosis IVA: Acquisition *vs.* Valid Results

Body site scanned	n	Number of scans acquired	Number of technically valid scans
WB	18	7 (38.9%)	6 (33.3%)
Proximal femur (hip)	11 adults	1 (9.1%)	0
LS	18	18 (100%)	10 (55.6%)
LDF	18	18 (100%)	18 (100%)

WB: whole body; LS: lumbar spine; LDF: lateral distal femur.
Adapted from: Kecskemethy et al., 2016 with permission from Elsevier.

3.1.2.2 DXA Results

We were able to obtain WB DXA results on 6 subjects (5 full-time ambulators). Average WB Z-score for the children was − 1.6 (range − 0.6 to − 2.5) and for the adults was − 2.2 (range − 0.3 to − 4.1). Average LS Z-score for the children was − 2.9 (range − 1.6 to − 4.4) and for the adults was − 4.0 (range − 2.6 to − 5.0). For the children, LDF Z-score results were as follows by region of interest: R1 = − 2.1 (range − 0.6 to − 3.3); R2 = − 2.6 (range − 1.4 to − 4.0); R3 = − 3.1 (range − 1.4 to − 5.2). For the adults, LDF Z-score results were as follows by region of interest: R1 = − 2.4 (range − 1.3 to − 4.3); R2 = − 2.3 (range − 0.8 to − 3.2); R3 = − 2.4 (range − 1.3 to − 3.7).

3.1.2.2.1 DXA by Ambulatory Status

Of the 18 patients, nine were F ambulators. Age did not predict full-time ambulatory status (Table 2.1). Of the 10 patients with valid LS BMD results, 8 were F ambulators. The LS BMD was low for both children and adults, regardless of

ambulatory status. At the LDF, BMD Z-scores were consistently lower in the P/N ambulation group at all regions and in children and adults (Fig 3.1).

3.1.2.2.2 DXA by Genotype and Ambulation Status

For patients with available genotype analysis (Table 2.1), 2 patients had the same genotype (p. S287L/L352P). Both were untreated females, aged 18 years (partial/non-ambulator) and 26 years (full-time ambulator). The partial/non-ambulator had consistently lower BMD across all body sites measured than the full-time ambulator (Fig. 3.2).

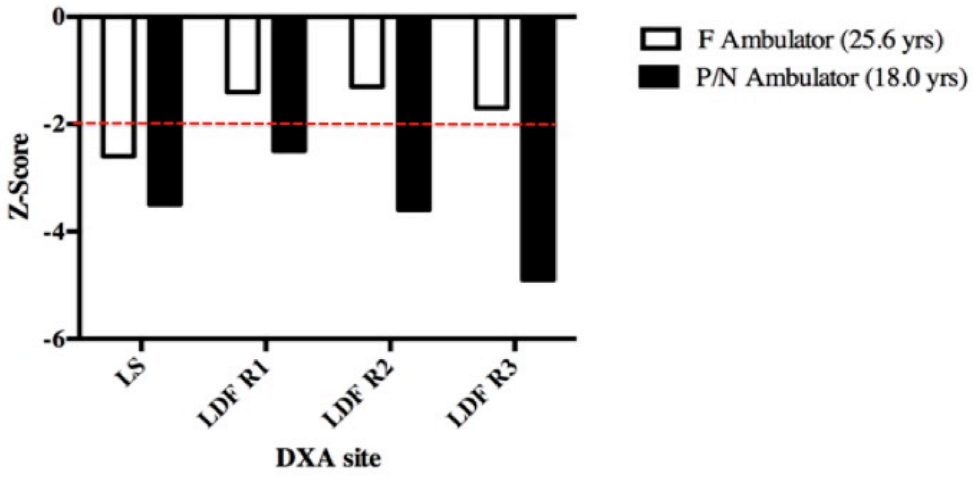

Figure 3.2: LS and LDF BMD Z-Scores by Ambulatory Status in Two Patients with p.S287L/L352P
Two unrelated untreated females with the same genotype were compared according to ambulatory status. Ambulation plays an important role in preserving bone mineral density in patients with same genotype at similar age range. The patient unable to ambulate had markedly lower Z-scores in comparison to the patient that could ambulate. DXA values below ≤ -2 were considered abnormal (red line).

F = full-time ambulator; P/N = partial or non-ambulator; DXA = dual energy X-ray absorptiometry; LS = lumbar spine; LDF R1 = lateral distal femur region 1; LDF R2 = lateral distal femur region 2; LDF R3 = lateral distal femur region 3.
Adapted from: Kecskemethy et al., 2016 with permission from Elsevier.

3.1.3 Discussion

In this study of patients with MPS IVA, we have described the technical considerations of measuring BMD (Table 2.2), reported BMD results (Fig. 3.1), and evaluated the influence of ambulation (Fig.3.2).

Undegraded C6S and KS accumulation leads to alterations in the connective tissue and cartilage ground substance, distorting bone mass acquisition and perturbing the regular microarchitecture of bone tissue [437]. Histopathological studies have indicated that patients with MPS IVA present impaired bone quality because of distortion of geometric shape, collagen disposition in ECM, and remodeling, resulting in poor bone mineralization [438, 439]. As patients with MPS IVA age, they frequently decide to use wheelchairs full time for convenience and comfort because of bone deformity and chronic pain.

While we expected to see older patients using wheelchairs because of progression of the disease, in the present study, only our oldest patient required the use of a wheelchair full time. The elementary school-age patients used assistive devices at school but still ambulated elsewhere. The high school-aged and older patients who were P/N ambulators opted to use the power chair for convenience (rather than need), foregoing weight bearing.

Impairment or lack of ambulation corresponds with low bone BMD of the LDF

in children with a variety of physical disabilities including CP, Duchenne muscular dystrophy (DMD), and spina bifida [429-433]. In this group of patients with MPS IVA, a trend of lower BMD in the P/N ambulators was observed in both adult and children and at all body sites measured (Fig. 3.1). The full-time ambulators had higher BMD at all ages. Overall, BMD values of the patients investigated in this study were below normal at most sites measured, but there was wide variation in BMD results in both age groups. Younger-aged patients had some normal BMD Z-scores regardless of ambulatory status. The P/N adults showed no normal BMD Z-scores.

There are more than 300 mutations already described in the *GALNS* gene illustrating the heterogeneity seen in this pathology (HGMD). In the present study, genotype analyses were available for 6 patients (Table 2.1). Within this subset of unrelated patients, two female adult patients shared the same genotype (p.S287L/L352P). In these two patients, one was a FT ambulator, and the other was a P/N ambulator. The BMD Z-scores were consistently higher at all body sites measured in the patient who walked full-time, despite the fact that she was older (26 years) than the P/N ambulator (18 years) (Fig. 3.2). This finding suggests that ambulation provides a protective effect on BMD.

Despite the low BMD values and commensurate Z-scores, just two fractures from trauma were reported. Similarly, in two studies of MPS patients (I, II, and VI; II, VI), only traumatic fractures were reported in 1 of 8 and 2 of 40 patients, respectively [428, 440]. In typical healthy adults, the correlation of low BMD DXA and fracture has been established. Only one study exists describing the relationship between LDF

BMD and fracture. In 2010, Henderson and colleagues described a strong association between low BMD at the LDF and fractured history in children with CP and DMD [432]. The relationship of low LDF BMD and fracture was not observed in our patients with MPS IVA.

In this study, height adjustments for DXA were not used for BMD because of the large height deficits seen in our patients with MPS IVA (Table 2.1). When using height adjustments, BMD results are frequently normalized. Lin and colleagues reported this normalization of BMD results after using the HAZ method in 9 children with MPS IVA [441]. Fung et al. also used the HAZ method in a group of children and adults with MPS II and VI and showed the same results overall normalization of WB and LS BMD after using the HAZ method [440]. It is important to recognize that the HAZ method was developed for children aged 6 to 18 years with height deficits, under the condition of normal bone morphometry. Zemel et al. describe that the shortest height used is − 2.6 SD below that of the age-matched controls [427]. Polgreen and colleagues [428] describe that the HAZ method is valid at the LS in patients with height deficits as low as − 6 SD, whereas WB BMD results are overestimated in the same population. The maximum height deficit acceptable for the HAZ method is unknown.

Polgreen et al. demonstrated that HAZ overestimated the WB BMD results, likely because of overall atypical bone geometry seen in MPS [428]. For the LS, they determined that the HAZ method yielded valid results. However, this is likely due to fewer spinal skeletal deformities and milder height deficits seen in MPS I and II.

While MPS VI patients have skeletal spinal abnormalities and height deficits more similar to those of patients with MPS IVA, it is important to note the average height Z-score in the MPS VI subjects was − 4.3 (± 0.6), whereas the average height Z-score in our study was − 7.4 (± 2.8).

The skeletal abnormalities seen in our patients were significant, particularly at the LS. While we were able to acquire LS DXA scans, the atypical morphometry observed in patients with MPS IVA precluded the valid use of the LS DXA in nearly half of our patients, and correct analysis required review of radiographic images (Fig. 3.3 & 3.4).

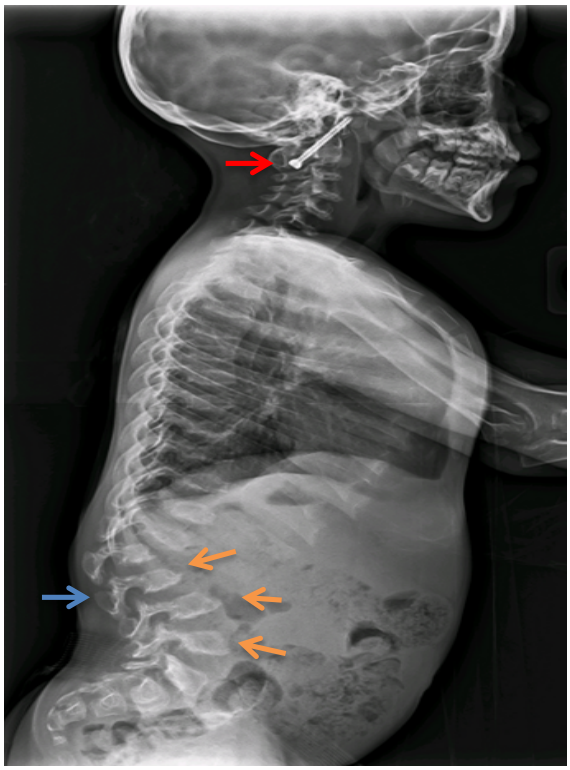

Figure 3.3: Morphologic Changes in the Spine in Mucopolysaccharidosis IVA Influence DXA Assessment

The lateral spine radiograph shows sharp angle kyphosis centered on L2 with a hypoplastic vertebral body (blue arrow). Anterior wedging and beaking are noted at multiple levels (orange arrows). There has been cervical fusion with fixation (red arrow).

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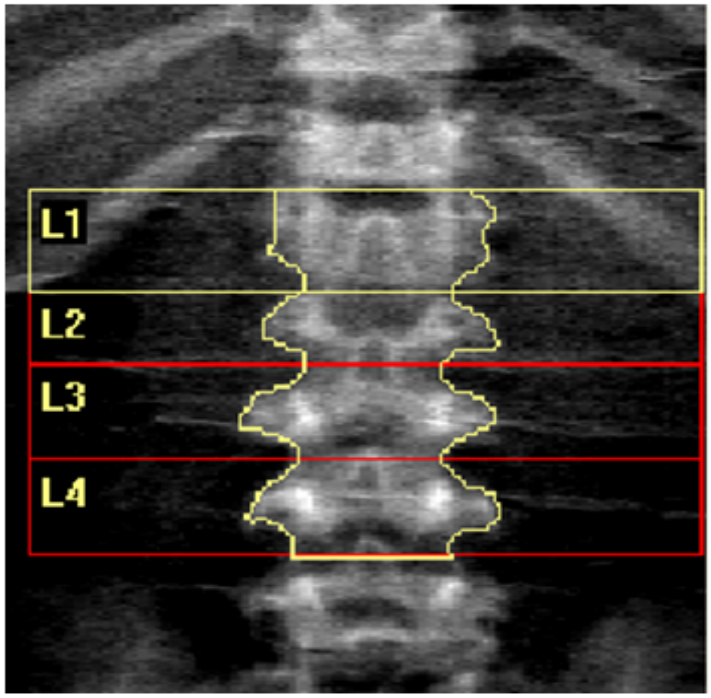

DXA Results Summary:

Region	Area (cm^2)	BMC (g)	BMD (g/cm^2)	T - score	Z - score
L1	6.34	2.00	0.315		
L2	4.49	1.76	0.392		
L3	5.81	2.67	0.459		
L4	6.22	3.27	0.525		
Total	**22.87**	**9.69**	**0.424**		**-1.6**

Total BMD CV 1.0%

Figure 3.4: Lumbar Spine DXA Requires Careful Delineation of Vertebral Body Levels and Margins
The DXA results summary notes bone mineral content is low in L1 and L2 with differences in the area resulting in BMD variation. Total BMD Z-score (-1.6) is in the normal range for age and sex.
Adapted from: Kecskemethy et al., 2016 with permission from Elsevier.

The WB DXA scan was not well tolerated, as described earlier (Table 3.2).

Only the LDF scan was feasible and well tolerated in all patients investigated in our

study. The LDF analysis takes into consideration size of the bone and adjusts the three regions of interest to the bone size. The HAZ method has been validated for the WB, LS, forearm, and proximal femur, but not the LDF.

This study has several limitations including a limited sample size with a wide range of ages (3.1–40.8 years). By splitting the groups by age and then by ambulation, the results are limited by even smaller sample sizes within the categories. While this was a prospective study, we were limited by a convenience sample of patients seen at our hospital who were willing to enroll in the study-even being an autosomal recessive condition; our group was composed of 77% females.

Genotype information was not available for every patient, limiting our ability to perform genotype/phenotype correlations or evaluating BMD relative to genotype. Our evaluation of bone was limited to DXA, which is an areal (2-dimensional) measurement that has limitations when assessing a 3-dimensional object (bone). Using DXA to assess bone with atypical bone morphometry presents technical problems that must be considered when interpreting results. Use of other volumetric assessment techniques would add value to the study of BMD in MPS IVA. While normative values are available for WB and LS within the age range in this study group, we were limited to the age ranges of 6 to 18 years that are currently available for the LDF. The age of peak bone mass for lower extremities is unknown and may exceed 18 years of age.

Despite limitations, this study has value in that we present BMD results for both adults and children in the largest cohort of MPS IVA patients to date.

Furthermore, we report BMD DXA results at a novel body site for patients with MPS —the lateral distal femur. All images were evaluated and interpreted by a pediatric radiologist ensuring the technical validity of our findings.

3.1.4 Conclusion

Measuring BMD by DXA in patients with MPS IVA presents unique and significant challenges when using standard body sites. The WB DXA was not well tolerated or feasible. Anatomical abnormalities of the spine and technical limitations of DXA made use of the LS challenging. The application of height adjustment Z-score correction to BMD results in patients with significant height deficits and atypical morphology and should be performed with caution. The Z-score LDF DXA was the most feasible BMD DXA measurement in patients with MPS IVA. On average, BMD was low at all body sites measured in children and adults, although there was broad variation. Full-time ambulation consistently was associated with higher BMD values at all ages. Fracture did not appear to be a major concern in this group of patients with MPS IVA.

3.2 Bone Mineral Density in MPS IVB[5]

Mucopolysaccharidosis IVB (MPS IVB, Morquio syndrome type B) (OMIM#253010) is an autosomal recessive inherited metabolic disorder caused by a deficiency of β-galactosidase (GLB1) [442]. This hydrolase is responsible for the catabolism of terminal β-galactose residues as keratan sulfate (KS) and GM1 ganglioside [443, 444]. Keratan sulfate accumulation in patients with MPS IVB causes skeletal dysplasia, growth retardation, keratansulfaturia, corneal clouding, and impaired cardiac function [443, 445].

The incidence of MPS IV is variable among different populations (1 per 75,000 in Northern Ireland to 1 per 640,000 in Western Australia) [446, 447]. To date more than 180 mutations have been described on *GLB1* (HGMD) [448], but fewer mutations are associated with the clinical phenotype of MPS IVB [443, 445, 449, 450]. There is no cure or established treatment for MPS IVB. Bone and cartilage are the main tissues affected in patients with MPS IVB, resulting in skeletal dysplasia. However, skeletal and cartilage involvement are not only caused by the primary GAG

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[†]The first two authors should be regarded as joint first authors.

accumulation but also by disruption of several secondary mechanisms and pathways as: signaling transduction pathways, regulation of humoral factors (chemokines and cytokines), endocytosis, autophagy, apoptosis, oxidative stress, innate and adaptive immune responses [114].

The growth deficits and bone deformities seen in MPS IVB are less severe than those observed in MPS IVA, resulting in a milder phenotype with greater functional abilities. Lack of ambulation is known to negatively impact BMD of the lateral distal femur (LDF) in patients with other medical conditions including cerebral palsy, Duchenne muscular dystrophy, and spina bifida [429-431, 433, 451].

Several reports have demonstrated low BMD in MPS IVA [452-455]. To date, no reports exist describing BMD for MPS IVB; this is the first report of BMD in MPS IVB. We describe BMD measured by DXA at standard body sites and the LDF [385, 386], and examine clinical correlates (anthropometric measures, medical and fracture history, and ambulation). Investigation of bone mineral density (BMD) in patients with MPS IVB contributes to understanding of disease pathology [456]. BMD was measured as described in section 2.1.1.

3.2.1 Subjects

This cross-sectional study prospectively evaluated three patients (2 females) with MPS IVB who were enrolled in this study at the Nemours/Alfred I. duPont Hospital for Children (AIDHC). The mean age was 26.9 years: 17.7 years (female), 31.4 years (male), 31.7 years (female). Patients were diagnosed biochemically by enzyme assay.

Informed consent was applied and the study was approved by the Institutional Review Board of the Institution (338578).

3.2.2 Results

Three Caucasian patients (two females) with MPS IVB were evaluated; aged 17.7, 31.4 and 31.7 years. Mean height was 131.2 cm (average Z-score − 5.4), and mean weight was 39.9 kg (average Z-score − 4.0) (Table 3.3). All patients were ambulatory: two walked independently without any aids and one used a walker and occasionally (once per month) used a wheelchair. One patient sustained two fractures (arm and femur) due to trauma (fall and motor vehicle accident, respectively). All three subjects were post-pubescent.

Table 3.3: Clinical Data for MPS IVB Patients

Patient ID	1	2	3
Age (years)	17.7	31.4	31.7
Gender	F	M	F
Height (cm)	129.5	137.2	127
Height Z-score	− 5.2	− 5.4	− 5.5
Weight (kg)	33.2	50	36.4
Weight Z-score	− 5.3	− 2.5	− 4.2
LS BMD (gm/cm^2)	0.915	Invalid	Invalid
LS BMD Z-score	− 0.8	n/a	n/a
Technical note	Wedging L3	L1–T12 overlap	L1–T12 overlap
LDF R1 BMD (gm/cm^2)	0.605	0.738	0.6775
LDF R1 Z-score	− 3.6	− 2.9	− 2.9
LDF R2 BMD (gm/cm^2)	0.886	0.986	0.883
LDF R2 Z-score	− 2.4	− 2.0	− 2.5
LDF R3 BMD (gm/cm^2)	0.990	1.024	0.961
LDF R3 Z-score	− 2.0	− 2.1	− 2.3
Fracture history? Y/N	N	Y × 2 (trauma)	N
Fracture details	n/a	L arm from fall; L femur from car accident	n/a
Ambulation details	Uses walker; manual WC 1 ×/month	Independent walker - no assistive devices	Independent walker - no assistive devices

LS: lumbar spine; LDF: lateral distal femur; R1: region 1; R2: region 2; R3: region 3; F: female; WC: wheelchair; M: male; L: left; MVA: motor vehicle accident.
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The presence of metallic artifact from prosthetic hips on every WB scan precluded valid assessment of the results (Fig. 3.5).

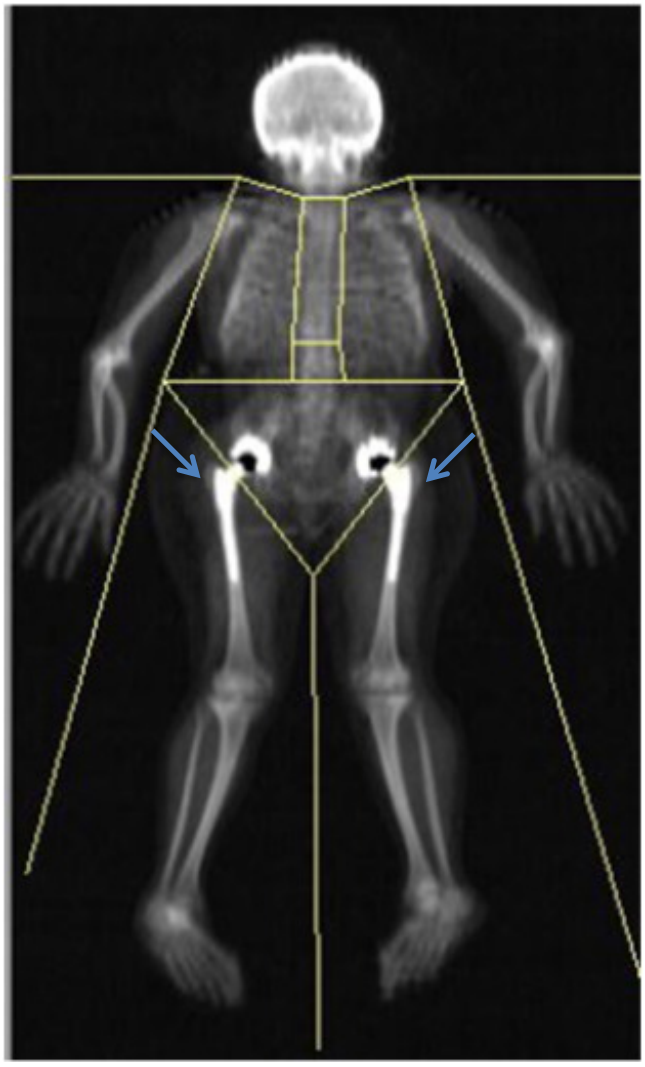

Figure 3.5: Interference on WB DXA BMD by Metallic Prostheses
All three patients had artificial hips (blue arrows), invalidating WB DXA results. Metallic prostheses invalidate the use of WB DXA BMD due to an artificial elevation of Z-scores. BMD: bone mineral density; WB DXA: whole body dual energy X-ray absorptiometry.
Adapted from: Kubaski et al., 2016 with permission from Elsevier.

Metal is interpreted as bone on DXA and therefore the presence of metal artificially elevates BMD. Two of the three patients had vertebral overlap at T12 and L1, invalidating LS scan results. The one technically valid LS scan resulted in a normal BMD Z-score of − 0.8, but wedging of L3, which can elevate LS BMD DXA results, was noted [457] (Fig.3.6).

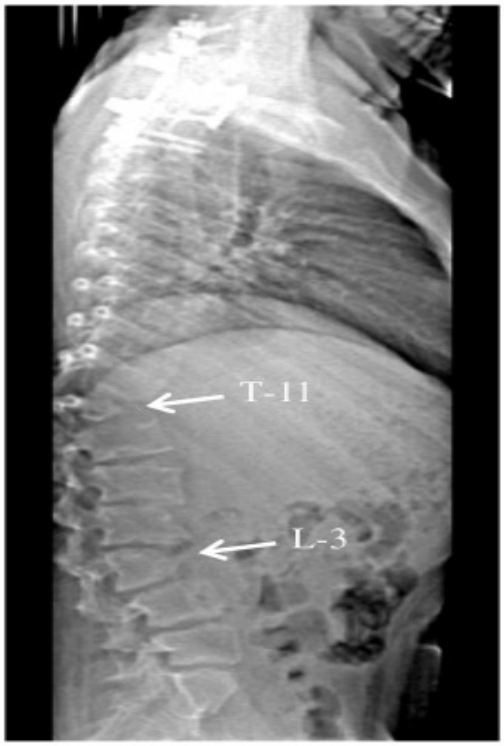

Figure 3.6: Lateral Spine Radiograph Used for Correct Identification of Lumbar Vertebrae for LS DXA
Note the dysmorphic vertebral bodies with anterior wedging of L-3 and a hypoplastic, wedge-shaped body at T-11. These result in focal areas of kyphosis. There has been spinal fusion in the cervico-thoracic region using metallic fixation. L: lumbar vertebrae; T: thoracic vertebrae.
Adapted from: Kubaski et al., 2016 with permission from Elsevier.

The LDF yielded technically valid results for all patients, and Z-scores were low in all three regions of interest with average Z-scores of − 3.1, − 2.3, and − 2.1 at R1–R3, respectively (Fig. 3.7). Every region of interest for all measurements (both femurs) was consistently below normal.

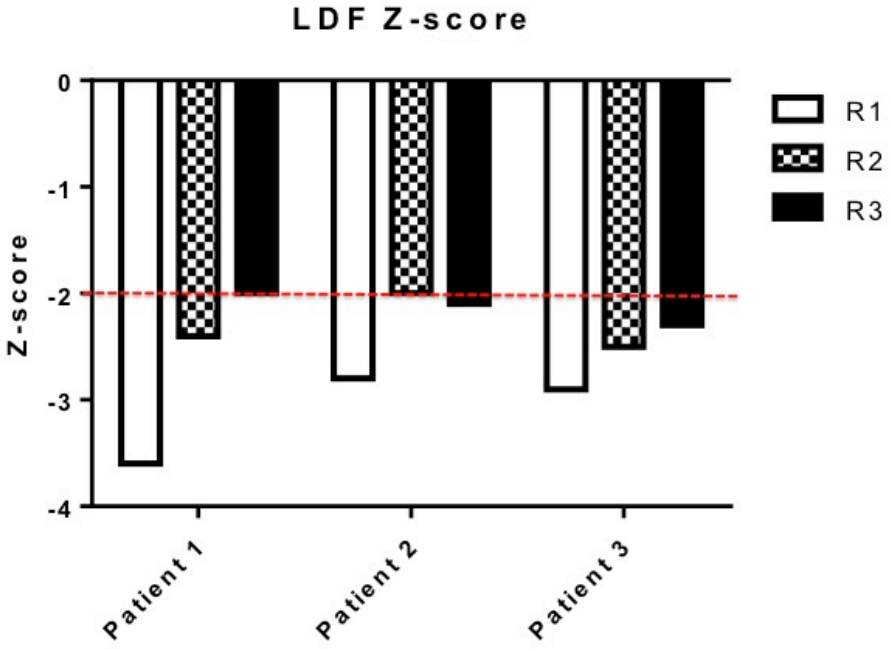

Figure 3.7: Lateral Distal Femur DXA BMD Z-scores in MPS IVB
Overall, all three patients had low Z-scores in all regions (R1, 2, and 3) indicating low BMD. DXA values below ≤ -2 were considered abnormal (red line). Adapted from: Kubaski et al., 2016 with permission from Elsevier.

3.2.3 Discussion

In this study, we evaluated and reported the BMD of three patients with MPS IVB. The skeletal abnormalities seen in patients with MPS IVB are primarily caused by the accumulation of KS. The exact mechanism of low BMD in MPS IVB is still

unknown, although as undegraded substrate accumulates, normal bone and cartilage formation is disrupted leading to impaired homeostasis, which could affect BMD [114, 458]. Low BMD has also been reported in other lysosomal disorders [458] (e.g. Gaucher's) and skeletal dysplasias [459] (e.g. achondroplasia and hypochondroplasia).

In general, patients with MPS IVB exhibit a less severe phenotype than those with MPS IVA. This fact is evidenced by greater functional ability (all patients were ambulatory) and less severe growth deficits in height resulting in an average height Z-score of − 5.4, compared with a group of patients with MPS IVA where nine of 18 patients were fully ambulatory and had an average height Z-score of − 7.4 [455].

All of our patients were essentially full-time ambulators (one used a walker and a wheelchair once per month). Despite this ambulation, the LDF BMD was uniformly below normal in all three patients. There was no history of non-traumatic fracture, often seen in patients with low BMD of the lower extremities. It is impossible to examine the relationship between BMD and fracture in this limited number of patients.

Abnormally shaped vertebrae that are wedge-shaped or beaked are common in MPS IVB (Fig. 3.6). Utilizing DXA to measure atypically shaped vertebrae can yield variable aBMD results [457]. This is a limitation of using the LS DXA to assess BMD in patients with MPS IV (A and B). Careful review of LS radiographs should be made to determine both the technical validity and correct identification of vertebrae on the LS DXA scan when assessing BMD in MPS IVB. Only one of three LS DXA scans in this study was valid, and still an elevation in overall LS BMD from wedging noted at

L-3 may have resulted.

While WB DXA scans could reliably be acquired, their validity was compromised by the presence of metallic artifact present with hip replacements (Fig. 3.7). The LDF measurement was established as an alternative measurement site for patients with joint contractures, hip dislocation, positioning limitations, or metallic hardware [386]. Only the LDF DXA consistently offered technically valid DXA results. This scan was easily obtained and well tolerated by patents with MPS IVB.

We do not adjust our one valid LS result by using the HAZ method because of the large height deficits seen in our patients with MPS IVB (Table 3.3). The HAZ method of correcting for height deficit has not been validated for the LDF DXA. The HAZ method was developed for children with height deficits, aged 6–18 years, who have normal skeletal morphometry. The HAZ method applied to one technically valid LS in this study resulted in a possible overcorrection: the unadjusted Z-score was − 0.8 and after adjustment, became +2.1. It would appear that using the HAZ adjustment might have resulted in an overcorrection of results. The maximum height deficit acceptable for the HAZ method is unknown. For patients with severe skeletal conditions affecting bone morphometry and severe height deficits, height-based adjustments of DXA results should be used with caution.

This study has several limitations including a limited sample size of three patients. MPS IVB is a rare condition and BMD in MPS IVB has not been described in the literature. Another limitation of this study is that our evaluation of bone density was limited to DXA, which is an areal measure. The oldest age available for the LDF

DXA normative values is 18 years. The mean age of our study population was 26.9 years (range, 17.7–31.7 years). However, peak bone mass in the typical population is thought to be acquired between 20 and 30 years of age depending on gender and body site [460]. We know neither the age of peak bone mass accrual in MPS IVB, nor the age at which peak bone is acquired at the distal femur.

3.2.4 Conclusion

In conclusion, despite these limitations, we have presented novel findings of BMD in patients with MPS IVB. We evaluate the technical validity of the DXA scans acquired at different body sites and present BMD findings at an alternative DXA site – the LDF. Images were evaluated and interpreted by a pediatric radiologist ensuring the accuracy of our findings in the presence of potentially confusing skeletal anatomy.

3.3 Non-invasive Pulmonary Function Tests on MPS IV Patients[6]

MPS IV patients can have cardiac and pulmonary complications [426, 439, 461-465]. Autopsied trachea showed tracheomalacia but little evidence of a narrow

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[6] Published in Molecular Genetics and Metabolism

[384] F. Kubaski, S. Tomatsu, P. Patel, T. Shimada, L. Xie, E. Yasuda, R. Mason, W.G. Mackenzie, M. Theroux, M.B. Bober, H.M. Oldham, T. Orii, T.H. Shaffer, Non-invasive pulmonary function test on Morquio patients, Mol. Genet. Metab. 115 (2015) 186-192.

airway due to storage materials [439]. The difficulty of intubation and extubation was observed during the surgical procedure, which could be associated with tracheomalacia. Tracheomalacia can cause a twisting, tortuous trachea, leading to a high risk during anesthesia.

It has been reported that respiratory issues in patients with Morquio syndrome are associated with two conditions: 1) restrictive lung disease (inability to inspire) due to short stature and thoracic cage deformity [415, 463, 466-468], and 2) obstructive lung disease (inability to expire) due to tracheobronchial abnormalities, large tongue, and adenoidal, tonsillar, and vocal cord hypertrophy. These respiratory issues lead to a high mortality rate or high risk during anesthesia [415, 467, 469].

The functional signs of restrictive and obstructive lung disease are that the patients cannot breathe synchronously and cannot effectively maintain normal gas exchange even at rest. However, we do not know whether the flows are less or greater than normal individuals when normalized for smaller volumes and stature.

Spirometry clearly evaluates several assessments of static and dynamic volume measurements; however, this approach is considered an effort dependent test requiring cooperation between subject and examiner [470]. Young patients and wheelchair-bound, and/or post-operative patients with severe muscle weakness cannot be given such physical assessments.

In the last two decades, pulmonary function tests (PFTs) have been revised to analyze tidal breathing in patients who are minimally cooperative due to age or clinical condition. These include impulse oscillometry (IOS), pneumotachography

(PNT), and respiratory inductance plethysmography (RIP), which have been used extensively in the minimally cooperative neonate and pediatric populations [397-399, 470-473]. In addition, IOS provides more information about total respiratory system resistance [474].

A number of studies have shown the significance of using measurements of airway resistance and reactance at different oscillation frequencies such as IOS in various clinical settings, for detecting chest wall abnormalities, lung compliance disorders, airway obstruction, assessment of chronic obstructive pulmonary disease and asthma [474-477]. Furthermore, the use of a non-invasive PFT would accommodate a broader spectrum of patients (young and or/wheelchair-bound).

In contrast, to our knowledge, no systemic study has been performed to evaluate age-dependent oscillometry measurements for patients with Morquio syndrome or other skeletal dysplasias in comparison with conventional spirometry studies.

In the present study, we sought to elucidate age-dependent changes in pulmonary function utilizing non-invasive PFTs correlated with conventional spirometry (when possible) in patients with Morquio syndrome. In addition, we hypothesized that the age-dependent alterations in lung function can be correlated with the “small lungs” (compared to age-matched controls from a database group with normal stature) as measured with spirometry. PFTs were measured as described in section 2.1.2.

3.3.1 Subjects

This was an open, non-controlled, single center, assessment study of patients diagnosed as Morquio A and B who were pulmonary asymptomatic at the time of the study. This study assessed key thoracopulmonary features. Patients with Morquio syndrome had one visit for clinical evaluation of lung function. All tests were clinically indicated and conducted on an outpatient basis at Nemours/Alfred I. duPont Hospital for Children. This study was conducted in accordance with the amended Declaration of Helsinki, and it was approved by the Institutional Review Board of the institution (750932). Informed consent was obtained from all patients and/or their guardians.

Twenty-two subjects with Morquio (18 Morquio A and 4 Morquio B), from 3 to 40 years of age were tested (mean = 16.5 years). Part of the physical examination included measuring height, weight, and systemic skeletal examinations. We used Morquio A growth charts [478] as a reference for height, weight, and body mass index (BMI).

Measurement of standing height in patients was difficult due to skeletal deformities, which limited patients' ability to stand erect. To obtain consistent data, we performed several established measurements at least twice per patient. The patient lay on a flat surface with knees flattened to extend the legs fully. Standing height was also measured. For pulmonary function, arm length conversion to height was performed to determine predicted values for pulmonary standards, often used in our outpatient PFT laboratory for patients with skeletal deformities [390]. Finally, of the 22 patients, 17

(77.3%) were able to perform spirometry, leading to a limited profile of spirometry (3 patients were too young to perform the test, one was physically unable to perform and one was non-cooperative). Data for control subjects were derived from previous studies (National Health and Nutrition Examination Survey—NHANES III).

3.3.2 Inclusion and Exclusion Criteria

The subjects were diagnosed as Morquio A and B by enzyme analysis. There were no exclusion criteria related to sex, age, ethnic background, scheduled operation, or physical ability.

3.3.3 Statistical Analysis

The influence of age on pulmonary resistance (Rrs5 and Rrs20) was described using the Pearson product-moment correlation coefficient. To specifically assess the effect of puberty on each of pulmonary resistance, phase angle and %rib cage, we categorized patients into pre- and post-puberty groups and assessed the mean pulmonary resistance between these two age groups for each of the three aforementioned outcomes using the two sample unpaired Student *t* test assuming unequal variance at two time points: Rrs5 and Rrs20.

The same hypothesis test was also used to contrast the difference between all Morquio patients' performance at Rrs5 and Rrs20, regardless of age. To simultaneously examine the relationship between resistance at Rrs5 and Rrs20 between the two age groups, we performed a two-way analysis of variance (ANOVA).

All tests were two-sided. The statistical significance for the individual tests was set at 5%. The relationship between the resistance (at each of Rrs5 and Rrs20) and % predicted vital capacity was modeled using the exponential model. The analyses were made using R software [479].

3.3.4 Results

Twenty-two patients participated in this study ranging in age from 3 to 40 years old (7 males). Of these patients, 22 (100%) were compliant for RIP and PNT, 18 (81.8%) for IOS and 17 (77.3%) were compliant with spirometry testing.

3.3.4.1 Vital Signs

Subjects had normal vital signs at rest including > 95% oxygen saturation, end tidal CO_2 (38–44 mm Hg), and age-appropriate heart rates (mean = 98.3, standard deviation = 19) (although two patients older than 18 years of age had elevated rates of 121 and 131). Normal predicted values (NHANES III) were matched for age, gender, and height (as converted from arm length due to the skeletal dysplasia of the patients) as compared to individuals of normal stature [384, 390].

3.3.4.2 Spirometry

Predicted forced expiratory volume total (%FEV_{TOT}) was normal until 10 years of age; however, %FEV_{TOT} decreased with age, showing a negative correlation

between %FEV_{TOT} and age (FEV_{TOT} = − 33.16 ln(Age) + 161.88; R^2 = 0.475) (Fig. 3.8).

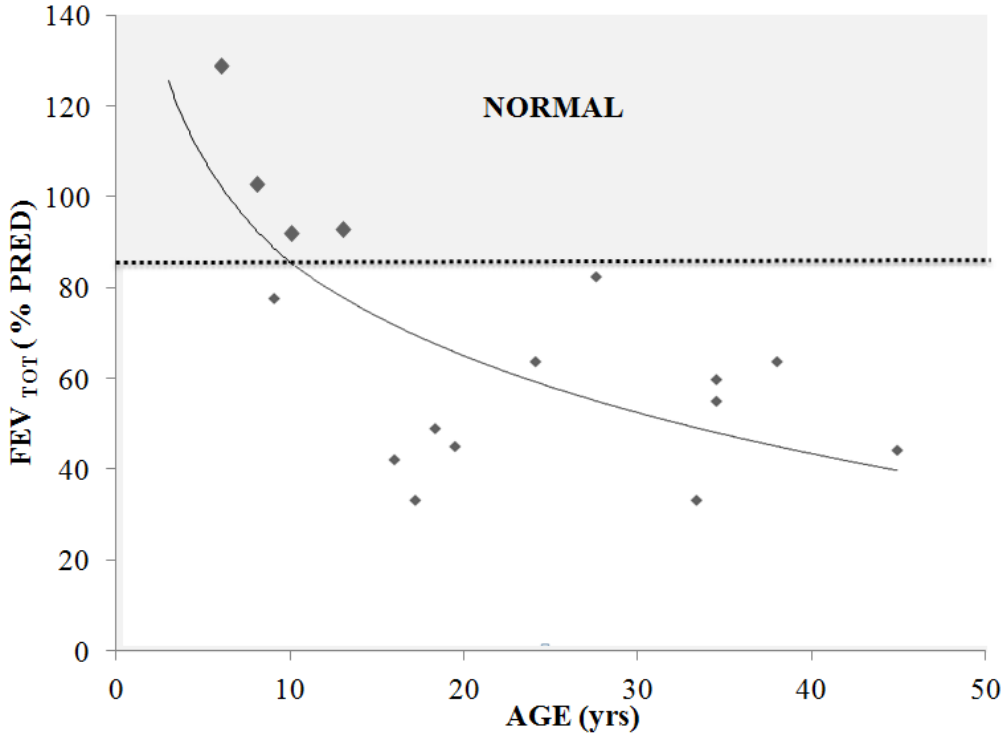


Figure 3.8: % Predicted FEV_{TOT} Plotted as a Function of Patient Age
Spirometry tracings were available for 16 patients. % predicted FEV_{TOT} was low in comparison with normative data in the majority of the patients (n=12 out of 16). %FEV_{TOT} decreased with age. Not all patients were compliant for spirometry. Adapted from: Kubaski et al., 2015 with permission from Elsevier.

FEV_1/FEV_{TOT} ratio did not correlate with age (p = 0.354386). Furthermore, %FEV_{TOT} was negatively correlated with body weight, indicating that the higher BMIs in patients with Morquio syndrome provide a detrimental impact on overall respiratory function. However, in all ages and both genders, the patients with Morquio syndrome

had similar values of $FEV_1/FEV_{TOT,}$ compared with those of age- and gender-matched controls (Fig. 3.9). When all patients with Morquio syndrome were compared with controls as a group (all ages and both genders), $\%FEV_{TOT}$ was reduced (72.8 ± 6.9%) but FEV_1/FEV_{TOT} was normal (110.0 ± 3.2%).

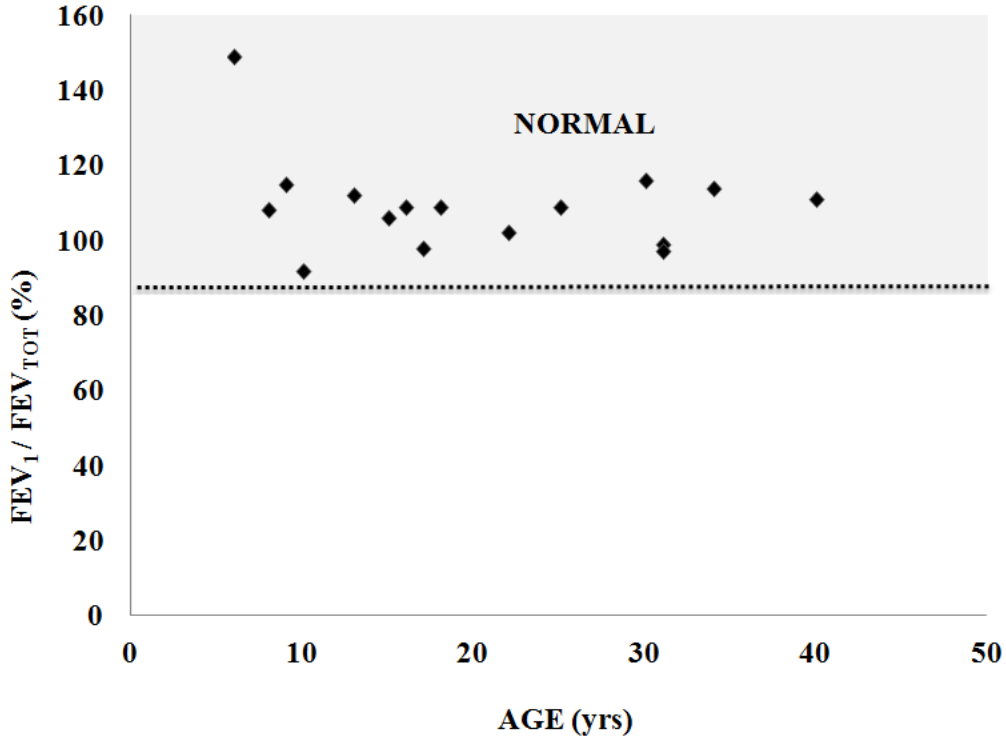


Figure 3.9: % Predicted (FEV_1/FEV_{TOT}) Plotted as a Function of Patient Age
FEV_1/FEV_{TOT} ratio was normal in all patients (n=16) and did not correlate with age.
*Not all patients were compliant for spirometry.
Adapted from: Kubaski et al., 2015 with permission from Elsevier.

3.3.4.3 Impulse Oscillometry System

18 patients were combined, independent of gender, the results of IOS resistance demonstrated a frequency dependence (change in resistance with aging such

that resistance at higher frequencies is less than resistance at lower frequencies) in that R_{20} was less ($p < 0.001$) than R_5, where R_{20} is 2.2 fold and R_5 is 2.5 fold higher than predicted control values, respectively (Fig. 3.10).

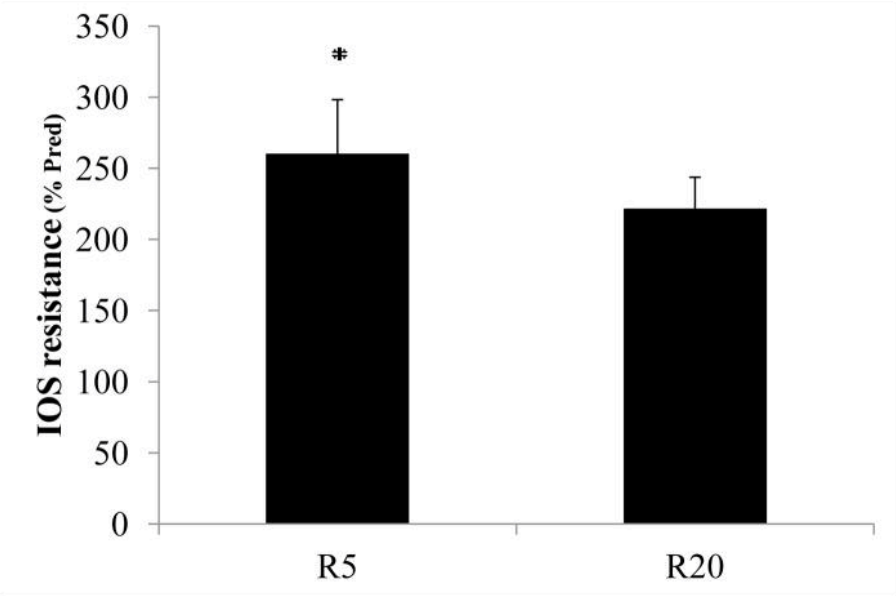
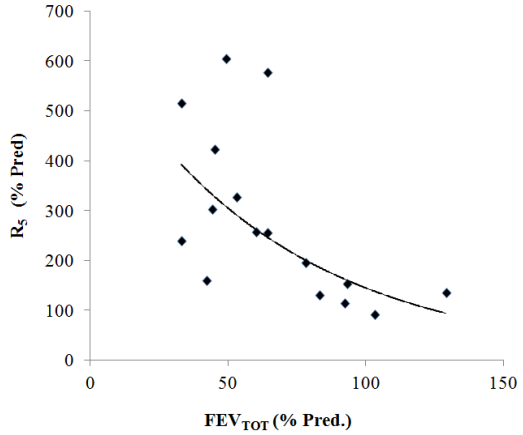


Figure 3.10: IOS Resistance Plotted for 5Hz and 20Hz Representing Peripheral and Central Resistance
18 patients were compliant with IOS measurements. Resistances are frequency dependent in which younger patients have higher resistance at low frequencies (R5) and older patients have higher resistance at higher frequencies (R20). Normal range: less than 200% predicated. Adapted from: Kubaski et al., 2015 with permission from Elsevier.

It should be noted that predicted resistance standards for IOS values, were generated for individuals of average stature, age, and gender for each patient; as such there was a clear relationship between IOS R_5 and R_{20} with $\%FEV_{TOT}$ (Fig. 3.11 A, B).

A



B

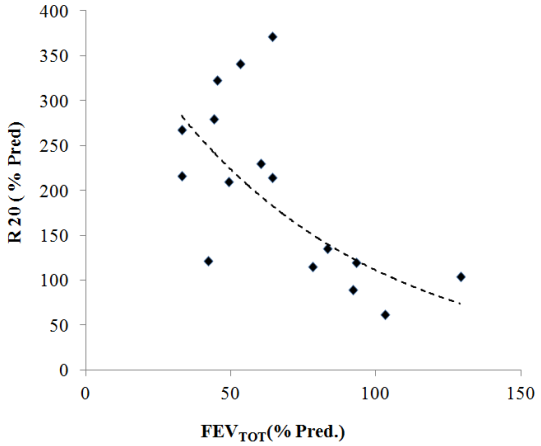


Figure 3.11: IOS Resistance as a Function of FEV_{TOT}
Figure 3.11 A: R_5 as a Function of FEV_{TOT}
Figure 3.11 B: R_{20} as a Function of FEV_{TOT}
16 patients had IOS measurements compared to predicted values. As lung volume increases, resistance decreases (R^2=0.57). R_5 ($y = 777.92e^{-0.018x}$; $R^2 = 0.57$), R_{20} ($y = 493.14e - 0.015x$; $R^2 = 0.57$) (exponential regression).
IOS: impulse oscillometry system; R5: peripheral airway resistance; R20: central airway resistance; FEV_{TOT} (% Pred.): predicted forced expiratory volume total.
Adapted from: Kubaski et al., 2015 with permission from Elsevier.

As lung volume increased, both R_5 and R_{20} decreased exponentially, $R_5 = 777.92e^{-0.018Age}$ ($R^2 = 0.57$) and $R_{20} = 493.14e^{-0.015Age}$ ($R^2 = 0.57$).

3.3.4.4 Pneumotachography

All parameters analyzed by PNT (air flow, CO_2 and oximetry) were normal in comparison with age and gender matched controls. As noted above, oxygen saturations measured by pulse oximetry was > 95% oxygen saturation, end tidal CO_2 was in the range of 38–44 mm Hg, and patients had age-appropriate heart rates (mean = 98.3, standard deviation = 19) although were elevated in two patients (121 and 131).

3.3.4.5 Respiratory Inductance Plethysmography

Mean ± SE results were as follows: phase angle = 32 ± 16°, %RC = 42 ± 13%, average f_{RES} = 25 ± 7. The resonant frequency, phase angle (Ø) and %RC were within normal limits when compared with normal healthy values (corrected with average stature, gender, and age) (Fig. 3.12).

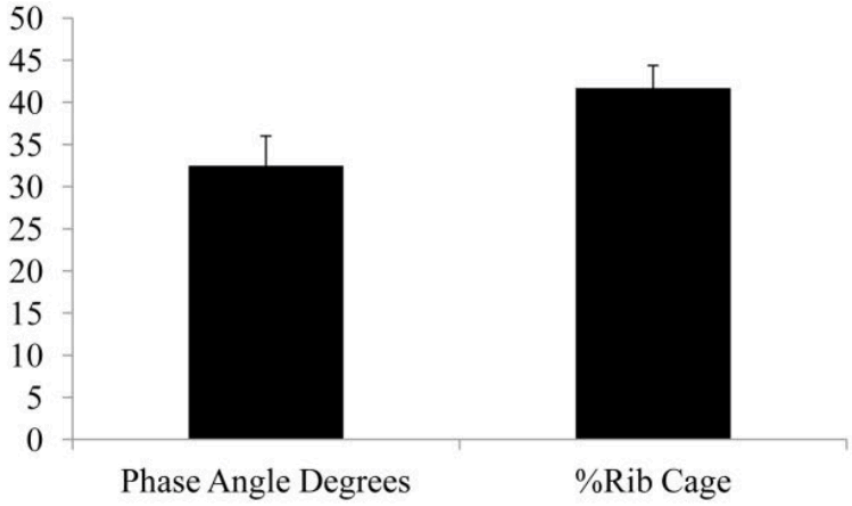


Figure 3.12: RIP Summary
Phase angle and %Rib cage demonstrated normal breathing synchrony. No normative data exist for patients with small stature and skeletal dysplasia.
Adapted from: Kubaski et al., 2015 with permission from Elsevier.

3.3.5 Discussion

Conventional spirometry may not be an appropriate approach to assess lung function in young children and children with disabilities [474, 480]. Also, spirometry is considered and effort-dependent test. In the Morcap study 66.3% of the patients were compliant with spirometry (FVC) [481]. Further, the predicted values are based on age-matched controls for normal height and normal chest wall. These data do not include unusual chest development, which makes the interpretation of spirometry even harder in Morquio patients.

Herein we have performed non-invasive and conventional PFTs in patients with Morquio syndrome, which provide a unique "experimental model" associated with severe skeletal dysplasia. We have demonstrated the following: 1) all 22 Morquio

syndrome patients have normal vital signs at rest including oxygen saturation, end tidal CO_2, and age-dependent heart rate (apart from 2 patients), 2) there are reductions in FEV_{TOT} starting at age 10 [481], 3) the FEV_1/FEV_{TOT} ratios are within normal predicted range, 4) non-invasive assessment of IOS resistance demonstrated that peripheral resistance (R_5) and central airway resistance (R_{20}) were not a function of age (pre- vs. post-puberty), where R_5 was higher than R_{20}, 5) air flow and CO_2 values were normal, 6) IOS resistances were consistent with normal respiratory physiology when correlated with lung volume, and 7) RIP reveals that the skeletal dysplasia, a characteristic finding in Morquio syndrome, provides a limited impact on the worsening respiratory function with age if corrected for stature.

Our results were consistent with higher values of R_5 than R_{20} in younger patients due to frequency dependence [482, 483]. As expected, as lung volume increases, resistances (R_5 and R_{20}) decrease due to the increase in airway caliber [484]. A greater number of Morquio patients should be analyzed to elucidate the age effect on resistance in this patient population. Nonetheless, there is a trend of worsening lung function in young patients to adulthood.

Morquio syndrome patients display restrictive lung characteristics due to their small lungs, stature, and skeletal dysplasia, compared with the age-matched controls; however, there were no clinical symptoms or dysfunction consistent with restrictive/obstructive lung disease (normal oxygen saturation, CO_2 values, synchronous breathing pattern—abdomen and rib cage move simultaneously in concert). Based on predicted values for spirometry, without taking into account that

Morquio patients do not have a normal structure or a normal chest wall, the patients would present a restrictive lung disease pattern, however considering these issues and the results from the non-invasive PFTs our results are not consistent with restrictive and obstructive lung disease, suggesting that the Morquio patients analyzed here have "small lungs" due to their small thoracic size.

The present series of pulmonary function tests demonstrated that the Morquio patients studied herein have normal functional lungs. However, it should be noted that since all of the testing procedures were analyzed at rest, these patients might not have a substantial oxygen reserve in the event the patients are stressed due to exercise or respiratory disorders.

According to our findings of normal lungs in this patient population, some of the associated complications in Morquio syndrome such as—difficulty during anesthesia, cervical spinal cord compression, tracheobronchial abnormalities with tortuous airways, large tongue, and adenoidal, tonsillar and vocal cord hypertrophies—could be associated with tracheal/anatomical abnormalities and obstructive narrow upper airways.

Non-invasive assessment of IOS resistance demonstrated that R_5 and R_{20}, indicative of peripheral and central resistances, respectively, were higher than predicted compared to normal values for patients with average stature. As previously demonstrated by Briscoe and DuBois [484], as lung volume increases resistance decreases. Both R_5 and R_{20} demonstrated a similar relationship with lung volume.

Finally, with respect to non-invasive RIP studies, we have demonstrated that

the resonant frequency, phase angle (Ø) and %RC in Morquio syndrome patients were within normal limits when compared to normal healthy values (average stature, gender, age). Typically, when any of these parameters are out of range, they are associated with mechanical dysfunction of the lungs and chest wall due to compliance or resistance abnormalities [384, 398].

The validation of non-invasive testing with vital signs has provided valuable clinical information about the pulmonary status of Morquio patients. All the non-invasive PFTs were validated with conventional spirometry, and these non-invasive PFTs provide the only clinical evaluation in pulmonary function for patients that were not able to perform spirometry (younger, unable or lack of cooperation).

This study is the first systematic study of non-invasive pulmonary testing in Morquio syndrome, a prototypical skeletal dysplasia, with deformed upper airway, small rib cage, and short stature. Taken together, these data support the concept that Morquio syndrome patients have small but normal functioning lungs, as previously demonstrated in patients with achondroplasia in which the lung function was normal for small adults of similar age [384, 485-487].

The small lung is most likely a result of the skeletal dysplasia providing an impact on the developing lung and chest wall. Thus, we conclude that the lungs are not significantly affected by Morquio syndrome, and consequently, lung function is not likely to respond to therapeutic approaches (ERT and HSCT), unless the treatment results in an improvement in lung/chest growth or prevention of lung disease associated with aging. The overall small population of Morquio patients in the United

States limits this study and it may not be representative of the entire MPS IV population; however, we were successful in testing 22 patients ranging in age from 3 to 40 years of age.

A greater number of patients with broad clinical phenotypes (from mild to severe) and with various stages of the disease need to be studied to further strengthen our results. It is also critical to expand the population to conclude how frequent patients with Morquio syndrome may have obstructive and/or restrictive disease. However, our results indicate that lung function will not have a major impact on risks of anesthesia during surgery.

3.3.6 Conclusion

In conclusion, patients with the Morquio syndrome have small lungs, but do not always exhibit signs and dysfunction of restrictive and obstructive lung disease as presented in prior case reports [415, 466-469, 488]. The proposed non-invasive pulmonary function-testing program presented in this study provides physicians and the Morquio community critical information to clarify key clinical conditions and to monitor therapeutic effects, which can be applied to other types of MPS and skeletal dysplasia.

3.4 Overall Conclusions for Non-invasive Assessments on MPS

The development and establishment of non-invasive tests are essential for disease progression and treatment evaluation. Although bone mineral density

evaluations as well as pulmonary function tests have been established and validated, their utility in MPS IV was not clear.

We were able to demonstrate that BMD is a valuable tool to evaluate the natural history of MPS IV patients. We have also described the technical challenges in lumbar spine and whole body and we have shown the validity of lateral distal femur as an alternative body site for patients with MPS IV. It is well known that patients with MPS IV have severe bone impairment that was confirmed by low BMD at all body sites measured. Interestingly, the low BMD scores were not associated with fractures and ambulation was associated as a major role in preserving BMD.

We have also demonstrated that non-invasive pulmonary function tests different than conventional spirometry can accommodate a broad range of MPS IV patients from younger patients to handicapped patients. We also suggested that MPS IV patients have small lungs, but not always have obstructive and restrictive lung disease.

In conclusion, the use of non-invasive assessment tests such as bone mineral density and pulmonary function tests are viable and valuable tools when evaluating natural history in MPS IV. More studies with larger cohorts and other MPS subtypes should be conducted to further elucidate the natural history of MPSs and as well as to draw the best treatment approaches for patients.

Chapter 4

GLYCOSAMINOGLYCAN ANALYSES BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

Glycosaminoglycans (GAGs) are widely distributed molecules with physiological and pathological roles depending upon specific GAG subclasses. The establishment of accurate, rapid, sensitive and specific methodologies is crucial for diagnosis, disease monitoring and therapeutic efficacy measurements in MPS. Thus the development of a robust methodology for GAG quantification by liquid chromatography tandem mass spectrometry (LC/MS/MS) would expand the diagnosis for MPS patients, which will allow early treatment for a better outcome. Furthermore, it is critical to have a well-established robust diagnostic methodology that allows discrimination of MPS patients from controls samples.

I proposed to test whether a comprehensive panel of GAGs will allow discrimination of MPS patients (prenatal, newborn and infant/adults) from control samples.

4.1 GAGs in Prenatal Diagnosis

4.1.1 Elevation of Glycosaminoglycans in Amniotic Fluid of Fetus with MPS VII[7]

Mucopolysaccharidosis VII (MPS VII; Sly syndrome) (OMIM#253220) is an autosomal recessive lysosomal storage disorder (LSD) caused by deficiency of β-glucuronidase (GUSB). This enzyme deficiency leads to accumulation of chondroitin sulfate (CS), dermatan sulfate (DS), and heparan sulfate (HS) [56]. The first MPS VII case was described in 1973 by Dr. William S. Sly [489].

Patients with MPS VII have a wide range of clinical signs and symptoms including; coarse facies, skeletal dysplasia, short stature, hernias, hepatosplenomegaly, neurological impairment, and corneal clouding. The clinical spectrum ranges from a severe form with lethal hydrops fetalis to attenuated forms with survival into adulthood despite somatic and cognitive impairment [490-499].

GUSB is localized to chromosome 7q21.11 and the 21kb gene contains 12 exons [500-503]. Several groups have independently reported many mutations within

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[7] Published in Prenatal diagnosis

[527] F. Kubaski[†], A.C. Brusius-Facchin[†], R.W. Mason, P. Patel, M.G. Burin, K. Michelin-Tirelli, R.G. Kessler, F. Bender, S. Leistner-Segal, C.A. Moreno, D.P. Cavalcanti, R. Giugliani, S. Tomatsu, Elevation of glycosaminoglycans in the amniotic fluid of a fetus with mucopolysaccharidosis VII, Prenat. Diagn. 1 (2017) 1-15.

[†]The first two authors should be regarded as joint first authors.

GUSB that result in different MPS VII phenotypes [272, 497, 504-517]. Sixty-three mutations have been described according to the Human Gene Mutation database [448] as of November, 2016.

The incidence of MPS VII is not well documented, and many cases are not diagnosed due to spontaneous abortion [419, 446, 447, 518-523]. Clinical manifestations of MPS usually do not appear at birth; however, accumulation of GAGs has been reported histopathologically in the human fetus (MPS I, II, III, IVA) and placenta (MPS II, VI) [524-526] indicating that the disease process starts and can be detected prior to appearance of clinical signs and symptoms.

Prenatal studies for MPS VII have been performed primarily to measure GUSB deficiency and for histopathologic analysis. Until now, no study has reported on the quantification of GAGs in fetal specimens with MPS VII. The aim of this study was to quantify GAGs in amniotic fluid (AF) from an MPS VII fetus and to compare with age-matched AF obtained from normal pregnancies [527]. GAGs were measured as described in section 2.2.

4.1.2 Molecular Analysis

Molecular analyses were conducted by next generation sequencing using Ion Torrent Personal Genome Machine (Thermo Scientific™) with a customized panel (Ion AmpliSeq™ Thermo Scientific™) including the *GUSB* gene at Hospital de Clínicas de Porto Alegre- Brazil. Data was analyzed on Ion Torrent suite and Ion reporter (Thermo Scientific™) version 5.0.

4.1.3 Results

The proband was a female fetus from non-consanguineous parents that presented with hydrops fetalis at 19 weeks of pregnancy and spontaneously died at 25 weeks of pregnancy. The mother, 31 years old, had three previous pregnancies (one stillbirth and two children that died in the first year of life). No investigation was performed on these previous siblings.

Since the most common causes of non-immune hydrops fetalis (as congenital infection, malformations, and chromosomal abnormalities) were ruled out, a lysosomal storage disorder was suspected. Thus, the levels of GAGs in AF at 21 weeks of pregnancy were measured using tandem mass spectrometry.

We observed that the concentration of GAGs found within the patient's AF was greatly elevated (≥10 SD) when compared to age-matched controls (Table 4.1).

Table 4.1: AF GAG Levels from Patient and Age-matched Controls (Mean ± SD)

GAG	GAG levels (ng/mg protein)		
	Age-matched controls (Mean ± SD)	**Patient**	**z-score**
DS	61 ± 44	712	15
C4S	142 ± 56	543	7
C6S	57 ± 19	621	29
HS-0S	59 ± 25	891	33
HS-NS	12 ± 5	142	25
HS-diS_1	4 ± 3	2	-1
mono-KS	131 ± 91	361	2
di-KS	6 ± 2	25	8

AF: amniotic fluid; GAG: glycosaminoglycan; SD: standard deviation; DS: dermatan sulfate; C4S: chondroitin-4-sulfate; C6S: chondroitin-6-sulfate; HS-0S: heparan-0-sulfate; HS-NS: heparan-N-sulfate; HS-diS_1: heparan sulfate di-sulfated 1); mono-KS: mono-sulfated keratan sulfate; di-KS: di-sulfated keratan sulfate.

Levels of DS, HS, and C6S were at least 10 fold higher in MPS VII than those in the age-matched controls, and C4S and KS were over 3 fold higher. C4S, C6S, HS-0S, and HS-NS were elevated as primary storage materials. Levels of mono-sulfated and di-sulfated KS were also elevated, secondarily. HS-diS1 level was not elevated (Table 4.1).

Due to the elevated levels of DS, HS, and C6S, a diagnosis of MPS VII was suspected. Biochemical analysis showed the very low enzymatic activity of GUSB in cultured amniocytes and AF, confirming the initial suspicion. Moderately low activity of β-galactosidase and a slight reduction in the activity of neuraminidase were also observed, but these alterations were not considered clinically relevant (Table 4.2).

Table 4.2: Biochemical Diagnosis of MPS VII by Enzyme Activity Assay in Cultured Amniocytes or Amniotic Fluid Supernatant

	Enzyme Activity	
Enzyme	**Normal Range**	**Patient**
β-glucuronidase[1]	40-254	0.34
α-iduronidase[1]	92-264	156
α-mannosidase[2]	1.25-21	20
Neuraminidase[1]	30-68	25
β-galactosidase[1]	521-1783	281
β-glucosidase[1]	207-596	218
N-acetylgalactosamine 6 sulfate sulfatase[1]	55-212	71
Hexosaminidase[2]	378-2901	979

[1]Enzyme activity in cultured amniocytes (nmol/h/mg protein); [2]Enzyme activity in amniotic fluid supernatant (nmol/h/mL).

α-mannosidase and total hexosaminidase were normal ruling out mucolipidosis II/III [528]. Molecular analyses were conducted by next generation sequencing with a customized panel including the *GUSB* gene. 36,000 reads were obtained with 1,500 reads per amplicon. The p.N379D (c.1135A>G) alteration was found in a homozygous state for the affected fetus and in a heterozygous state in both parents.

This is the first report of p.N379D substitution in *GUSB*.

Asn379 is conserved in GUSB in 50 species including humans, mice, and *E. coli,* and is a buried residue [529]. Consequently, alteration of the neutral Asn to acidic Asp is likely to disrupt the structure of the protein. The PredicSNP[2] program indicates that this change is likely deleterious to the protein structure [530]. More molecular studies are needed to confirm the function of Asn379 in the structure or function of GUSB.

4.1.4 Discussion

We have demonstrated that AF surrounding a fetus with MPS VII at 21 weeks of gestational age has a marked elevation of GAGs, including secondary storage of KS, indicating that AF is valuable for measuring GAG level to detect MPS before birth. In 1993, Chabás et al. described the use of chorionic villus (CVS) as well as amniotic fluid (AF) for GUSB measurement allowing prenatal diagnosis [531].

Also in 1993, Nelson et al. found foamy cytoplasmic changes in the villous Hofbauer cells of the placenta of stillbirths. Informed consent was denied, so post mortem and biochemical analyses could not be performed. Biochemical analyses were performed on tissues from the parents (consanguineous), and both had heterozygote levels of GUSB with normal levels of other lysosomal enzymes suggesting that the fetuses had MPS VII [532].

In 1996, Van Dorpe et al. identified 3 fetuses with MPS VII in which the first two had altered morphology detected by ultrasound (hydrops). The first fetus had pathological examination conducted with further GUSB assay in cultured skin fibroblasts. For the second and third pregnancies, GUSB assay was performed in cultured chorionic villus cells. All three fetuses had similar pathological findings with the presence of vacuolated macrophages in all tissues analyzed [533].

In 1997, Molyneyx et al. also reported a case of MPS VII with reduced GUSB activity after a histopathological examination showed the presence of foamy cells in the fetus and placenta [278]. In 1998, Van Eyndhoven et al. have demonstrated GUSB deficiency in chorionic villus (CVS) samples as early as 11 weeks allowing prenatal

diagnosis in the first-trimester [534]. In 1999, Groener et al. have used two-dimensional electrophoresis to demonstrate elevated DS and CS in fetal blood in a confirmed MPS VII fetus [276].

However, no previous studies have reported on quantification of the levels of GAGs in CVS or AF. Our study shows that all GAGs and their subclasses except HS-diS1 were noticeably elevated in AF of the affected fetus compared with GAGs in AF from age-matched controls and that the biochemical finding of elevated GAGs corroborates prior pathological findings of GAG accumulation in the affected fetuses [524-526].

Others and we have shown that KS is secondarily elevated in plasma (serum) and urine in several types of MPS and LSDs (in addition to MPS IV in which the deficient enzyme is directly involved in the catabolism of KS) [254, 535, 536].

We previously proposed that secondary elevation of KS could be caused by several factors including the release of KS from damaged bone and cartilage [170, 254, 535]. However, newborn DBS from MPS I, II, or III subjects showed no elevation of KS at birth [371]. The elevated KS in AF surrounding the MPS VII fetus in this study could suggest a more severe phenotype, including skeletal damage and/or developmental impairment in utero.

4.1.5 Conclusion

In this first report of quantification of GAGs in AF surrounding an MPS VII fetus using LC/MS/MS, we show that GAG elevation is present at 21 weeks of

gestation. Thus, we suggest that GAG measurements in AF may become a valuable additional tool for the diagnosis of MPS VII and potentially other MPS types.

4.2 GAGs in Newborns

4.2.1 Newborn Screening for MPS: Pilot Study [8]

MPS are inherited progressive and heterogeneous disorders caused by a deficiency of specific lysosomal enzymes responsible for the degradation of GAGs: chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS), and hyaluronan. The MPS are classified according to the deficient enzyme as well as the undegraded GAGs and comprise 11 distinct subtypes, with an estimated combined incidence of 1 in 25,000 live births [56].

Accumulation of undegraded GAGs leads to progressive tissue damage in multiple organs causing disease-specific manifestations (e.g. coarse facial features, skeletal dysplasia, hepatosplenomegaly, corneal clouding, joint rigidity or laxity, cardiac and respiratory complications, and neurological impairment).

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[8] Published in Journal of Inherited Metabolic Disease

[371] F. Kubaski, R.W. Mason, A. Nakatomi, H. Shintaku, L. Xie, N.N. van Vlies, H. Church, R. Giugliani, H. Kobayashi, S. Yamaguchi, Y. Suzuki, T. Orii, T. Fukao, A.M. Montaño, S. Tomatsu, Newborn screening for mucopolysaccharidoses: a pilot study of measurement of glycosaminoglycans by tandem mass spectrometry, J. Inherit. Metab. Dis. 40 (2017) 151-8.

Although clinical manifestations usually do not appear at birth, accumulation of GAGs can be detected in the human fetus (MPS I, II, III, IVA) and placenta (MPS II, VI), [524-526] indicating that the disease process starts and can be detected prior to appearance of clinical signs and symptoms.

Several MPS-subtypes have specific enzyme replacement therapy available, and there are many indications that MPS patients treated at an early age do better than those treated later in life [311, 341, 342, 537-541].

Newborn screening programs for MPSs using dried blood spots (DBS) were proposed in 2001, and high-throughput technologies such as tandem mass spectrometry (MS/MS) are now under investigation [170, 250, 357-361, 363-365, 367, 369, 376, 377, 542]. Methods currently under development include assays for activity of deficient enzymes [367, 369], measurement of accumulated GAGs [201, 244, 249, 543].

In the present study, we analyzed 2,862 de-identified DBS from unselected newborns and 14 DBS from newborns known to have MPS I, II, or III. GAGs were measured as described in section 2.2.

4.2.1.1 Samples

DBS samples of 2,862 newborns were collected during routine newborn screening by Shimane University, Osaka City University, and Nagasaki University after informed consent was obtained. The samples were collected between 3 and 7 days after birth. Fourteen de-identified newborn DBS samples with MPS (MPS I: 7;

MPS II: 2; MPS IIIA: 3, MPS IIIB: 1, MPS IIIC: 1) were provided by the University of Amsterdam (The Netherlands) and St. Mary's Hospital (UK). All samples were shipped to Nemours/AIDHC and stored at -20 °C until the GAG assay was conducted. This study was approved by IRBs at local institutes and Nemours/AIDHC.

4.2.1.2 Statistical Analysis

Sensitivity, specificity, false positive, false negative and median absolute deviation (MAD) were performed using R software [479]. As most of the disaccharides were below the LLOQ (59% for ΔDiHS-0S, 94% for ΔDiHS-NS, 98% for mono-sulfated KS, and 99.8% for di-sulfated KS) in the control samples, raw calculated values were used for statistical analysis. GAG levels in the blood can be elevated for reasons other than MPS, resulting in a non-normal distribution and consequently the standard methodology using mean $\pm$ standard deviation to define cutoff values is not appropriate for this study.

The median absolute deviation (MAD) is a robust method of central tendency, which is not sensitive to outliers [544]. Cutoffs were defined as median + 7x MAD of newborn control samples. False positive samples were defined as controls that were above the cutoff values, and false negatives were defined as MPS newborn samples that were below the cutoffs.

Coefficient of variation (CV) was determined at three different concentrations for the HS-0S standard (std8: 1,000 ng/mL, std5: 125 ng/mL and std3: 31.25 ng/mL). Three separate preparations of each dilution were measured 5 times, and CV

calculated as the standard deviation divided by mean x 100. It is estimated that each 3.3 mm dried blood spot disc corresponds to 3.6 μL of blood [545], and that concentrations of all the GAGs analyzed in this study were expressed as ng/mL of blood.

4.2.1.3 Results

4.2.1.3.1 Coefficient of Variation, Lower Limit of Quantitation, and Lower Limit of Detection

Imprecision was calculated by replicate analysis of three different concentrations (std 8, std 5 and std 3, respectively) of HS-0S, HS-NS and DS standard (Table 4.3). LLOQs and LOD were defined for HS-0S, HS-NS and DS (Table 4.3).

Table 4.3: Lower Limit of Quantitation, Lower Limit of Detection and Coefficient of Variation

GAGs	LLOQ (ng/ml)	LOD (ng/ml)	CV intra 1	CV intra 2	CV intra 3	CV inter 1	CV inter 2	CV inter 3
Di-sulfated-KS	312	156	n/a	n/a	n/a	n/a	n/a	n/a
Mono-sulfated-KS	312	10	n/a	n/a	n/a	n/a	n/a	n/a
HS-NS	31	1	9%	12%	16%	11%	25%	16%
HS-0S	31	1	5%	12%	14%	7%	12%	14%
DS	31	4	2%	5%	11%	3%	3%	13%

LLOQ: lower limit of quantitation; LOD: lower limit of detection; CV: coefficient of variation; intra: intraday; inter: interday; CV1: std8; Cv2: std5; CV3: std 1;
Adapted from: Kubaski et al., 2016 with permission from Springer.

4.2.1.3.2 Median Absolute Deviation

To calculate median and median absolute deviation (MAD) for levels of these disaccharides in control samples, it was necessary to use calculated concentrations of these samples even though the accuracy of measurement will be low. MAD values for the control newborn samples were 10 ng/mL for HS-0S, 2 ng/mL for HS-NS, 10 ng/mL for DS, 47 ng/mL for mono-sulfated KS, 10 ng/mL for di-sulfated KS, and 2 % for ratio di-sulfated KS in total KS. To assure no false negatives, cutoffs were determined as values higher than median + 7x MAD (all patients were above the cutoff for all GAGs). 7 x MAD is equivalent to more than 4 standard deviations for normally distributed data (Table 4.4).

Table 4.4: Values for DS, HS-NS, HS-0S, Mono-sulfated KS, Di-sulfated KS, and Ratio di-KS/total KS% in Newborn MPS (I, II, III) Patients

	DS (ng/mL)	HS-NS (ng/mL)	HS-0S (ng/mL)	Mono-KS (ng/mL)	Di-KS (ng/mL)	ratio Di-KS (%)
I-1	209#	61#	337#	97	24	35
I-2	160#	31#	195#	171	38	29
I-3	276#	68#	374#	213	63	41
I-4	170#	76#	431#	258	65	34
I-5	99#	40#	336#	220	87	42
I-6	150#	50#	259#	242	21	22
I-7	226#	35#	216#	54	27	58
II-1	91#	42#	240#	196	82	40
II-2	453#	34#	266#	65	12	16
III-1[a]	31	57#	397#	115	24	31
III-2[a]	11	45#	212#	62	37	32
III-3[a]	56	30#	145#	71	37	26
III-4[c]	15	35#	187#	116	22	17
III-5[b]	58	72#	464#	66	26	49

Samples above the cutoffs
[a]: MPS IIIA, [b]: MPS IIIB, [c]: MPS IIIC
Adapted from: Kubaski et al., 2016 with permission from Springer.

Cutoffs were >90 ng/mL for HS-0S, and > 23 ng/mL for HS-NS, >88 ng/mL for DS, >445 ng/mL for mono-sulfated KS, > 89 ng/mL for di-sulfated KS and >32% for di-sulfated KS in total KS. Values for MPS newborn samples ranged from 145 to 463 ng/mL for HS-0S, 30 to 76 ng/mL for HS-NS, 54 to 257 ng/mL for mono-sulfated-KS, 12 to 87 ng/mL for di-sulfated KS, and 16 to 58% for ratio di-sulfated KS in total KS. DS values for newborn samples with MPS I and II were between 91 and 453 ng/mL. All 9 MPS I and II newborn patients had a significant elevation of DS while none of 5 MPS III patients showed the elevation of DS, consistent with the catabolic pathway of DS.

4.2.1.3.3 Sensitivity and Specificity

Sensitivity was 100% for HS-0S, HS-NS, and DS (0% false negative rate). MPS I, II and III do not affect KS levels, so the significance of cutoffs for KS cannot be determined. Specificity was 97% for HS-0S, 94% for HS-NS, 98% for DS. Thus, the positive predictive value (PPV) for HS-0S was 16%, 7% for HS-NS and 18% for DS. The negative predictive value (NPV) was 97% for HS-0S, 94% for HS-NS, and 98% for DS (Table 4.5).

Table 4.5: Predictive Positive Value and Negative Predictive Values, Sensitivity and Specificity

Table 4.5A: HS-0S

HS-0S	Condition positive	Condition negative	
Test outcome positive	TP=14	FP=72	PPV=16%
Test outcome negative	FN=0	TN=2790	NPV= 97%
	Sensitivity= 100%	Specificity= 97%	

TP: true positive; FP: false positive; FN: false negative; TN: true negative; PPV: positive predictive value; NPV: negative predictive value.
Adapted from: Kubaski et al., 2016 with permission from Springer.

Table 4.5B: HS-NS

HS-NS	Condition positive	Condition negative	
Test outcome positive	TP=14	FP=179	PPV=7%
Test outcome negative	FN=0	TN=2683	NPV= 94%
	Sensitivity= 100%	Specificity= 94%	

TP: true positive; FP: false positive; FN: false negative; TN: true negative; PPV: positive predictive value; NPV: negative predictive value.
Adapted from: Kubaski et al., 2016 with permission from Springer.

Table 4.5C: DS

DS	Condition positive	Condition negative	
Test outcome positive	TP=9	FP=42	PPV=18%
Test outcome negative	FN=0	TN=2820	NPV= 98%
	Sensitivity= 100%	Specificity= 98%	

PPV: predictive positive value; NPV: negative predictive value; HS: heparan sulfate; DS: dermatan sulfate;
Adapted from: Kubaski et al., 2016 with permission from Springer.

HS-0S ($p = 2.25576E-07$), HS-NS ($p = 4.15E-07$), DS ($p = 0.002$), and ratio di-KS in total KS ($p = 0.0001$) were significantly higher in DBS from this cohort of MPS patients, compared to control DBS.

2.5% of the controls samples were above the cutoffs (median + 7xMAD) for HS-0S (Fig. 4.1), 6.2% for HS-NS (Fig. 4.2), 1.5% for DS (Fig. 4.3), 0.3%

mono-sulfated KS, 1.6% for di-sulfated KS, and 2.3% for ratio di-sulfated KS in total KS.

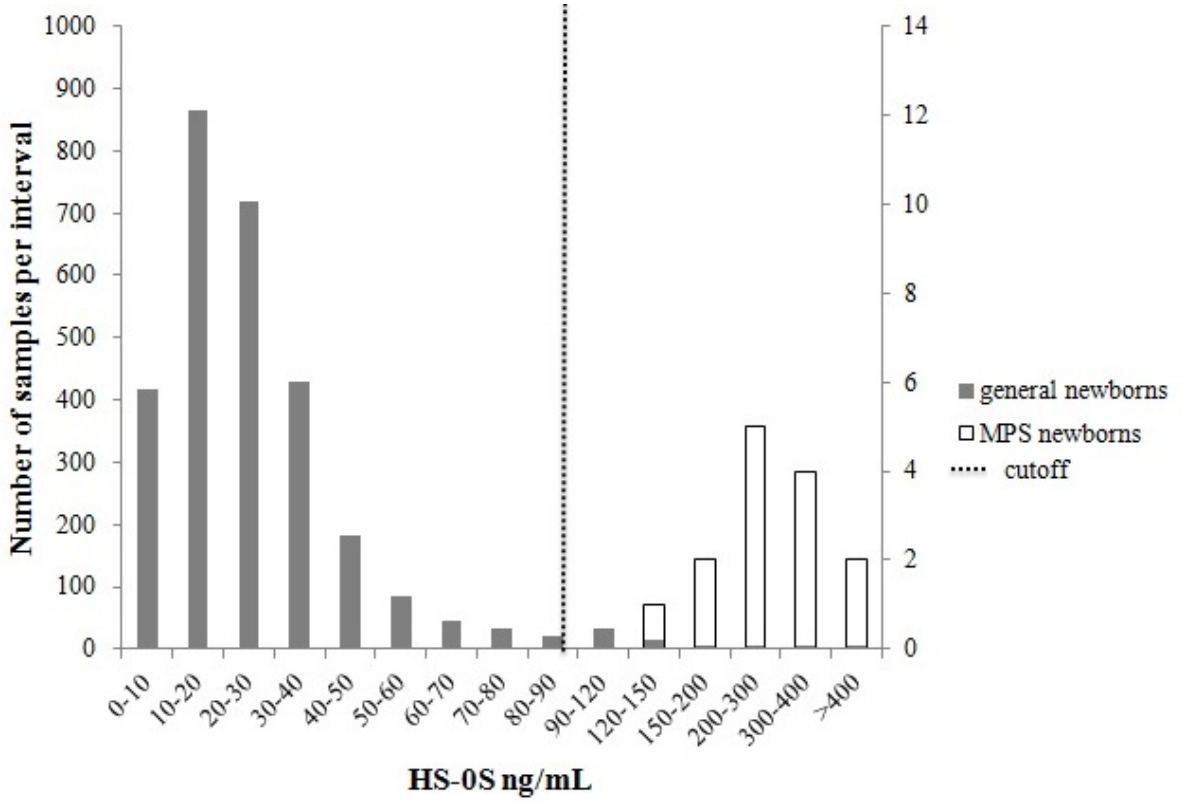

Figure 4.1: HS-0S in General Newborns and MPS Newborns
Glycosaminoglycan analyses in 2,862 general newborns were compared to 14 MPS newborns. 97.5% of general newborns had HS-0S levels below the cutoffs, while all MPS newborns had levels of HS-0S above the cutoffs. Samples were divided according to different intervals. Primary left y-axis represents solid bars for the number of general newborns (n=2,862) among the different intervals; the secondary right y-axis represents open bars for the number of MPS newborns (n=14) among the different intervals; dashed line represents the cutoff (median + 7x MAD for control newborns).
Adapted from: Kubaski et al., 2016 with permission from Springer.

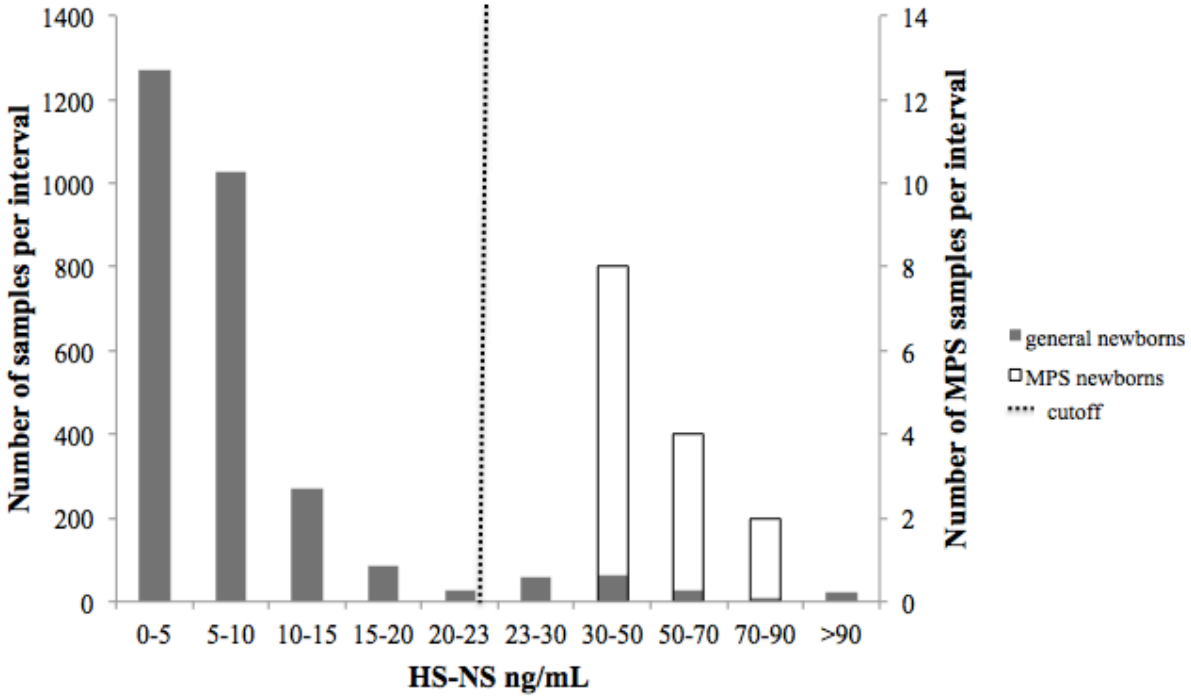

Figure 4.2: HS-NS in General Newborns and MPS Newborns
Glycosaminoglycan analyses in 2,862 general newborns were compared to 14 MPS newborns. 93.8% of general newborns had HS-NS levels below the cutoffs, while all MPS newborns had levels of HS-NS above the cutoffs. Samples were divided according to different intervals. Primary left y-axis represents solid bars for the number of general newborns (n=2,862) among the different intervals; the secondary right y-axis represents open bars for the number of MPS newborns (n=14) among the different intervals; dashed line represents the cutoff (median + 7x MAD for control newborns).
Adapted from: Kubaski et al., 2016 with permission from Springer.

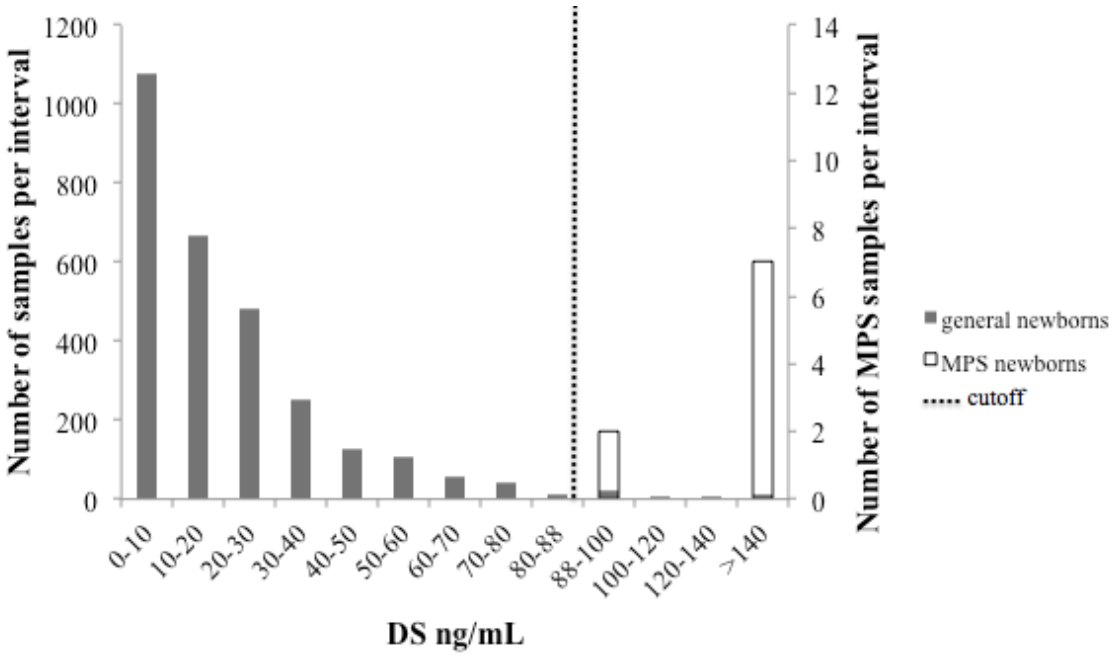

Figure 4.3: DS in General Newborns and MPS Newborns
Glycosaminoglycan analyses in 2,862 general newborns were compared to 9 MPS newborns. 98.5% of general newborns had HS-0S levels below the cutoffs, while all MPS I and II newborns had levels of DS above the cutoffs. Samples were divided according to different intervals. Primary left y-axis represents solid bars for the number of general newborns (n=2,862) among the different intervals; the secondary right y-axis represents open bars for the number of MPS newborns (n=9) among the different intervals; dashed line represents the cutoff (median + 7x MAD for control newborns).
Adapted from: Kubaski et al., 2016 with permission from Springer.

However, after a combination of elevated levels for HS-0S, HS-NS, and DS, the false positive rates decreased to 0.03% for MPS I and II and 0.9% with a combination of HS-0S and HS-NS for MPS III (Table 4.6). There were no false negative samples in the patient population.

Table 4.6: Number of Samples above the Cutoffs

	Control samples (n=2,862)	MPS patients (n=14)
> Cutoff HS-0S and HS-NS	26	14
> Cutoff HS-0S and DS	6	9*
> Cutoff HS-NS and DS	8	9*
> Cutoff HS-0S, HS-NS, and DS	1	9*

Cutoffs were defined as (median + 7x MAD) of general newborns; MPS patients (I-7; II-2; III-5); * MPS III patients not included. Adapted from: Kubaski et al., 2016 with permission from Springer.

4.2.1.3 Discussion

A major advantage of developing a measurement of GAGs as a newborn screen for MPS is that levels of one or more GAGs should be elevated in any MPS, making a single initial screen more sensitive and cost-effective for these rare disorders. A potential limitation is that GAGs can be elevated due to other conditions unrelated to MPS giving false positives [e.g.: Mucolipidosis [535]; diabetes [546]; arthritis [547]; cancer [548]], as seen in the general NBS samples in this study.

However, MPS I and II cause an elevation in three GAGs and MPS III causes an elevation in two GAGs, reducing the number of false positives. For this study we were able to obtain newborn DBS from 14 MPS I, II, or III patients to compare GAG levels in these samples with the ones observed in 2,862 DBS from a random control sample using a cutoff of median + 7x MAD of the control sample. We were able to distinguish all of the patient samples from 99.1% of the controls (26 out of 2,862 samples) by measuring levels of HS-0S in combination with HS-NS and could distinguish the MPS I and II patients from 99.97% (1 out of 2,862 samples) when levels of DS, HS-NS, and HS-0S were combined.

Considering that the combined incidence of MPS is approximately 1:20,000, it is unlikely that positive samples in the control sample set are due to MPS. The control samples were all de-identified, so that we were not able to determine the cause of elevated GAGs in these samples. None of the newborn MPS samples had levels of mono-sulfated KS or di-sulfated KS that were distinguishable from controls, but eight patient samples (57%) showed elevated levels of ratio di-sulfated KS in total KS. The ratio of di-sulfated KS in total KS was significantly higher in MPS newborn patients compared to general newborns ($p = 0.0001$) (Table 4.4).

We have previously shown a secondary elevation of total KS and increase in the ratio of di-sulfated KS in blood from older patients with other MPS types, where 40% for MPS II patients and 54% of MPS IVA had significantly higher levels of ratio di-sulfated KS in total KS [170, 254, 535]. We did not see a secondary elevation of KS in the MPS II newborn samples, possibly due to low accumulation during early stages of bone development. KS levels are age-dependent with expected accumulation during growth and development of the skeleton. MPS IV samples would be expected to show elevated KS due to impaired GALNS metabolism, but MPS IVA newborn samples were not available to test this hypothesis.

The standard deviation for the control samples is abnormally high due to a pronounced skew of the data towards higher levels, most likely due to elevated GAG levels in some newborns due to other unidentified conditions. For a population with a normal distribution, the number of false positives at mean + 2SD

would be 5% and mean and median values would be the same. Using the MAD, we were able to limit the effects of the high outliers.

To ensure that the screen includes the majority of the patients, we based cutoffs on values from known patient samples. Availability of newborn patient samples is limited; preventing detailed statistical analysis of patient data. For MPS I and II, by measuring three GAGs (HS-0S, HS-NS, and DS) we were able to distinguish all 9 patients from all but one of the 2,862 controls. We did not determine whether this "control" had MPS I or II and for the purposes of this study, we considered this to be a false positive. This false positive rate of 0.03% is much lower for single newborn screens for LSDs of Pompe, Fabry, Gaucher, and MPS I that give false positive rates of 0.17, 0.4, 0.23 and 0.8 percent respectively [369].

When MPS I, II, and III were included the false positive rate increased to 0.9%. Chace et al (2010) and Turgeon et al. (2010) suggest that a first-tier newborn screen could be performed with less selective methodologies if they give few false negatives and limited false positives that could then be followed by a more selective second-tier screen (Fig. 1.7) [352, 378]. A better understanding of the cause of high levels of GAGs in the "control" population might help eliminate some false positive, but a first screen that eliminates 99% of the unaffected samples would be particularly valuable in reducing the costs of a more expensive enzyme or genetic tests to define the specific metabolic defect.

We have previously shown that blood from patients with other forms of MPS (IV, VI, and VII) contain elevated levels of other GAGs, but we do not have

access to newborn DBS from such patients at this time and cannot directly determine GAG levels expected in newborn DBS from all MPS types [170, 254, 535, 542, 549]. Nevertheless, based on data from older patients, this newborn screen is likely to capture patients with a severe form of any MPS.

Prenatal lysosomal GAG storage has been demonstrated in MPS patients and animal models. Initial clinical signs and symptoms in newborn patients with MPSs include sacral dimple, gibbus, and abnormal shape of vertebrae in X-ray images [550]. In human, fetuses aged 18–30 weeks gestation (MPS I, II, III, and IVA) have storage vacuoles in major organs [525, 526]. Newborn mice with MPS I, II, IVA, or VII, have storage vacuoles as well [551, 552]. Skeletal abnormalities represent the earliest clinical observations in MPS VII mice. Histological analysis of the growth plate, articular cartilage and cortical bone showed early pathology and progressive bone lesion [145]. It is noteworthy that all 14 MPS newborns have an elevation of GAGs consistent with the previous findings, [250, 363, 542, 553] demonstrating that accumulation of GAGs has already started before birth and that therapy should start at a newborn stage to prevent irreversible damage especially in bone and brain.

A limitation of this study is the low number of DBS from newborn MPS patients. Very few samples are available because MPS is not usually identified at birth and consequently DBS are not usually available. Thus while we were able to establish stringent cut-off values to distinguish all patient samples in this study from controls, we are not able to predict false negative rates for larger screening purposes.

Another limitation is that the run time of the current LC-MS/MS (4 to 5 minutes per sample) is still a challenge for application to mass screening, compared to high-throughput MS/MS (10 to 12 seconds per sample) [253]. Although the main drawback of HT-MS/MS is that disaccharides with identical molecular weights cannot be distinguished, this may not be crucial for a method that is proposed as a first-tier screen to identify a high-risk group that may have an MPS. Further feasibility studies are required to compare LC-MS/MS and HT-MS/MS.

4.2.1.4 Conclusion

In conclusion, the combination of elevated HS-0S, HS-NS and DS seems to be a good biomarker for newborn screening of MPS I and II and HS-0S and HS-NS for MPS III. Elevated levels of GAGs will be valuable as a first-tier screen to identify a high-risk group that may have an MPS, and can be confirmed in a second-tier screen using a specific enzyme or genetic assays.

4.2.2 Newborn Screening for MPS: 16,727 Samples

Based on the results obtained in our pilot study, I have analyzed 16,727 de-identified DBS from unselected newborns and 14 DBS from newborns known to have MPS I, II, or III. Samples were collected as described in section 4.2.2, GAGs were measured as described in section 2.1. Samples were analyzed based on cutoffs established in our pilot study [371] (see section 4.2.3).

4.2.2.1 Results

2.8% of the DBS samples were above the cutoffs (median + 7xMAD) for HS-0S (Fig. 4.4), 3.5% for HS-NS (Fig. 4.5), 1.2% for DS (Fig. 4.6).

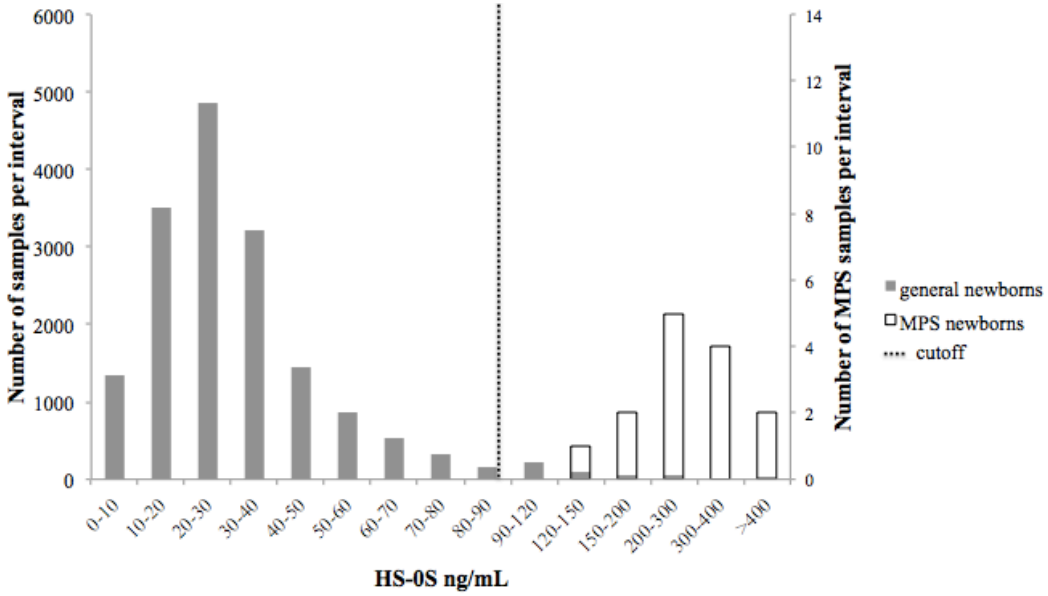

Figure 4.4: HS-0S in General Newborns and MPS Newborns
Glycosaminoglycan analyses in 16,727 general newborns were compared to 14 MPS newborns. 97.2% of general newborns had HS-0S levels below the cutoffs, while all MPS newborns had levels of HS-0S above the cutoffs. Samples were divided according to different intervals. Primary left y-axis represents solid bars for the number of general newborns (n=16,727) among the different intervals; the secondary right y-axis represents open bars for the number of MPS newborns (n=14) among the different intervals; dashed line represents the cutoff (median + 7x MAD for control newborns).

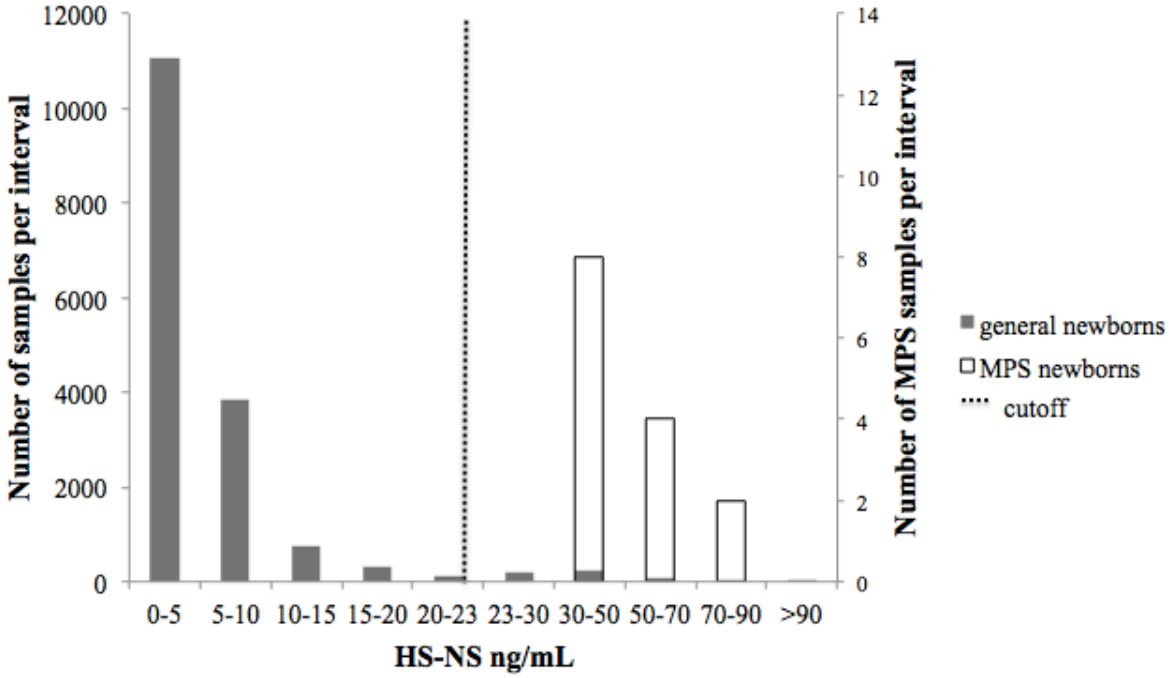

Figure 4.5: HS-NS in General Newborns and MPS Newborns
Glycosaminoglycan analyses in 16,727 general newborns were compared to 14 MPS newborns. 96.5% of general newborns had HS-NS levels below the cutoffs, while all MPS newborns had levels of HS-0S above the cutoffs. Samples were divided according to different intervals. Primary left y-axis represents solid bars for the number of general newborns (n=16,727) among the different intervals; the secondary right y-axis represents open bars for the number of MPS newborns (n=14) among the different intervals; dashed line represents the cutoff (median + 7x MAD for control newborns).

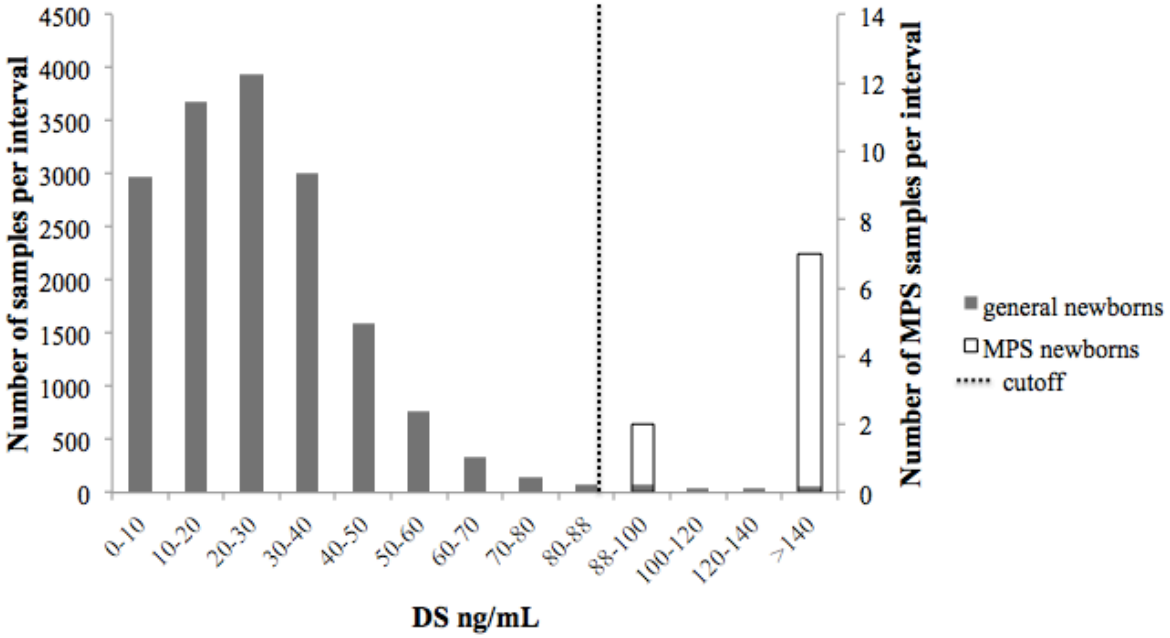

Figure 4.6: DS in General Newborns and MPS Newborns
Glycosaminoglycan analyses in 16,727 general newborns were compared to 9 MPS newborns. 98.8% of general newborns had DS levels below the cutoffs, while all MPS newborns had levels of HS-0S above the cutoffs. Samples were divided according to different intervals. Primary left y-axis represents solid bars for the number of general newborns (n=16,727) among the different intervals; the secondary right y-axis represents open bars for the number of MPS newborns (n=9) among the different intervals; dashed line represents the cutoff (median + 7x MAD for control newborns).

However, after a combination of elevated levels for HS-0S, HS-NS, and DS, the positive rates decreased to 0.04% for MPS I and II and 1.2% with combination of HS-0S and HS-NS when MPS III is added (Table 4.7). There were no false negative samples in the know patient population.

Table 4.7: Number of Samples above the Cutoffs

	DBS samples (n=16,727)	MPS patients (n=14)
> Cutoff HS-0S and HS-NS	203	14
> Cutoff HS-0S and DS	11	9*
> Cutoff HS-NS and DS	25	9*
> Cutoff HS-0S, HS-NS, and DS	7	9*

Cutoffs were defined as (median + 7x MAD) of general newborns; MPS patients (I-7; II-2; III-5); * MPS III patients not included.
Adapted from: Kubaski et al., 2016 with permission from Springer.

4.2.2.2 Discussion

As previously discussed, MPS are progressive, and GAG accumulation is constant. We have demonstrated in our pilot study that our GAG assay can distinguish MPS samples from control samples; however, we are limited by false positive samples that can have elevated GAG levels due to other conditions.

The combination of more than one elevated GAG reduced the number of positive samples in this screening to 0.04% for MPS I and II and 1.2% for MPS III (Table 4.7). The combination of elevated GAGs by this methodology has lead us to distinguish all of the patient samples from 98.8% of general newborns (203 out of 16,727 samples) by combination of HS-NS and HS-0S as well as 99.96% of MPS I and II patients from general newborns (7 out of 16,727) by combination of DS, HS-NS and HS-0S. All 203 positive samples will be followed-up by enzyme assay to distinguish potential MPS patients from false positives.

4.2.2.3 Conclusion

In conclusion, the combination of elevated DS and HS are a good biomarker for newborn screening of MPS I, II & III. Elevated levels of GAGs are

valuable as a first-tier screen to identify a high-risk group that may have MPS that will then be followed-up for enzyme assay (second-tier screen) (Fig. 1.7).

4.3 GAGs in Children and Adults[9]

The MPSs and ML are progressive LSDs that share many clinical features such as: coarse facies, neurological impairment (MPS I, II, III, VII and ML II), skeletal dysplasia (all, but may be mild in MPS III), hepatosplenomegaly, joint rigidity, and heart valvular disease [554]. MPSs are usually asymptomatic at birth, and the initial signs and symptoms appear with the progression of the disease during the two years of age. Mucolipidoses II (ML II; I-cell disease) is fatal during childhood or the first decade of life, and can even produce intra-uterine fractures, while ML III has a milder somatic phenotype with slower progression throughout childhood but leads to severe neurodegeneration with a fatal outcome during adulthood [555-557].

ML II and III are caused by impaired trafficking of several lysosomal enzymes [555, 558]. The prevalence of ML is variable among different populations: 0.3 cases per 100,000 live births in Australia, 0.16 per 100,000 live births in Portugal, and 0.08 per 100,000 live births in the Netherlands [559, 560].

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[557] F. Kubaski, Y. Suzuki, K. Orii, R. Giugliani, H.J. Church, R.W. Mason, V.C. Dũng, C.T.B. Ngoc, S. Yamaguchi, H. Kobayashi, K.M. Girisha, T. Fukao, T. Orii, S. Tomatsu, Glycosaminoglycan levels in dried blood spots of patients with mucopolysaccharidoses and mucolipidoses, Molecular Genetics and Metabolism 1 (2016) 1-8.

The incidence in Quebec, Canada is very high, 1:6184, due to a founder effect [561]. The combined incidence of MPSs is 1:25,000 live births, and therefore more common than ML [363].

The development of ELISA methods in early 90's made it possible to measure HS and KS in blood and urine of MPS and ML patients [535, 558, 562]. An ELISA method was used to show that KS levels in blood are elevated not only in MPS IV, but also in other types of MPS and ML [535]. However, ELISA assays are expensive and cannot distinguish subclasses of HS and KS.

Since 2001, protocols have been developed for GAG analysis using tandem mass spectrometry (MS/MS). Two main branches of GAG detection methods by MS/MS have been developed: detection of digested disaccharides (direct or labeled with aniline) [238, 242-244, 249, 563] and chemically depolymerized GAGs by methanolysis and/or butanolysis [255, 256, 536, 564, 565].

Such MS/MS methods have been used to measure specific GAGs in blood and urine of MPS and ML patients [249, 254, 255, 291, 536, 553, 566-568]. In this study, we have simultaneously determined levels of dermatan sulfate (DS), heparan sulfate (HS-0S, HS-NS), and keratan sulfate (mono, di-sulfated, and ratio di-sulfated in total KS) in DBS of control subjects and patients with MPS I, II, III, IV, VI, VII; and ML II and III by liquid chromatography tandem mass spectrometry (LC/MS/MS).

4.3.1 Samples

Whole blood was collected with EDTA by venipuncture and 150 μL of blood was spotted onto filter paper to create DBS. DBS from 106 untreated MPS

and ML patients (MPS I = 7; MPS II = 21; MPS IIIA = 12, MPS IIIB = 17, MPS IIIC = 6, MPS III (undefined) = 2; MPS IVA = 28, MPS IVB = 2; MPS VI = 7; MLII = 3; ML III = 1), and 115 control subjects. Diagnosis of MPS and ML was made with enzyme assay.

DBS from MPS patients were provided by Shimane University (Japan), Gifu University (Japan), St. Mary's Hospital (UK), and Kasturba Medical College Manipal University (India). Control samples were obtained from 15 volunteer subjects from Hospital de Clínicas de Porto Alegre (Brazil) and from subjects who had blood draws for clinical testing for non-metabolic disease from Shimane University (Japan). Informed consent was obtained at each Institute for all patient and control samples according to IRB approval at each institute.

All de-identified samples were shipped to Nemours/AIDHC and stored at − 20 °C until the GAG assay was conducted. This study was approved by the Nemours IRB (protocol # 281498). GAGs were measured as described in section 2.2.

4.3.2 Statistical Analysis

For KS (mono and di-sulfated KS, ng/mL; ratio di-sulfated KS in total KS, %) analyses, patients were grouped by age as follows: 0–5 (years), 5–10 (years), 10–15 (years), 15–30 (years), and > 30 years. Age-matched data was summarized using mean and standard deviation (SD). Other GAGs did not change with age in either controls or untreated patients, so these could be compared without dividing into age groups. Untreated patients were compared to controls using Student's *t*-test at the level of significance of 0.05 performed using Graphpad Prism 7.0a.

4.3.3 Results

Ages of the patients with MPS were as follows: untreated MPS I (mean: 3 ± 2 years; range: 3 months to 5 years; n = 7), untreated MPS II (mean: 9 ± 7 years; range: 1.1 to 29 years; n = 21), untreated MPS III (mean: 13 ± 8 years; range: 1 to 34.2 years; n = 37), untreated MPS IVA (mean: 15 ± 14 years; range: 7 months to 56 years; n = 28), untreated MPS IVB (ages 9 months and 48 years; n = 2); untreated MPS VI (mean: 6 ± 7 years; range: 2 months to 22 years; n = 7), untreated ML (mean: 6 ± 6 years; age range: 0.2 to 14 years; n = 4); controls (mean: 12 ± 12 years; age range: 1 month to 57 years; n = 115).

4.3.3.1 Distribution of GAG Levels in Untreated MPS and ML Patients Compared to Controls

No age-dependent differences in DS, HS-0S or HS-NS were detected in control subjects, or MPS and ML patients. Consequently, levels of these GAGs in all untreated patients with each MPS or ML were compared with levels in control subjects.

Mean levels of DS were higher than controls in untreated MPS I ($p<0.01$), MPS II ($p<0.0001$), MPS III ($p<0.01$) (Fig. 4.7). Levels of DS in 6 of 7 MPS I, 20 of 21 MPS II, 2 of 37 MPS III, 3 of 6 MPS VI, and 3 of 4 ML were higher than the mean + 2SD of the control group. DS was not significantly higher in untreated MPS IV patients.

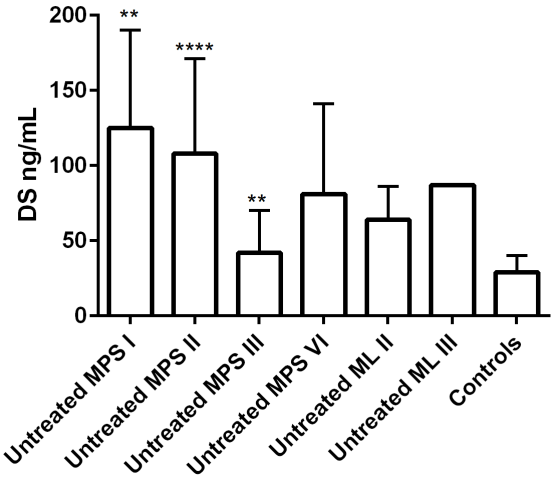

Figure 4.7: DS Levels in Untreated Patients *vs.* Controls
Untreated MPS I, II and III patients had significantly higher levels of DS than controls ($p<0.01$, $p<0.0001$ and $p<0.01$, respectively).
**$p<0.01$; **** $p<0.0001$
Adapted from: Kubaski et al., 2016 with permission from Elsevier.

Levels of HS-0S were higher than controls in untreated MPS I ($p<0.005$), MPS II ($p<0.0001$), MPS III ($p<0.0001$), MPS VI ($p<0.05$) (Fig. 4.8). Levels of HS-0S in 5 of 7 MPS I, 20 of 21 MPS II, 24 of 37 MPS III, 4 of 6 MPS VI, and 3 of 4 ML were higher than the mean + 2SD of the control group.

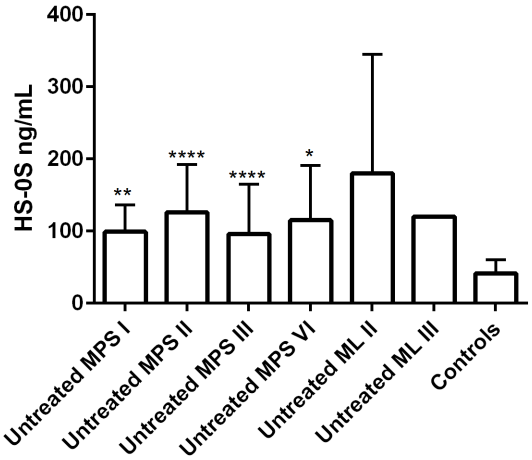

Figure 4.8: HS-0S Levels in Untreated Patients *vs*. Controls

Untreated MPS I, II, III, and VI patients had significantly higher levels of HS-0S than controls ($p<0.05$, $p<0.0001$, $p<0.0001$, and $p<0.05$, respectively).
*$p<0.05$; **$p<0.005$; ****$p<0.0001$
Adapted from: Kubaski et al., 2016 with permission from Elsevier.

Levels of HS-NS were also higher than controls in untreated MPS I ($p<0.005$), MPS II ($p<0.0001$), MPS III ($p<0.0001$), MPS IV ($p<0.05$) (Fig. 4.9). Levels of HS-0S in 6 of 7 MPS I, 19 of 21 MPS II, 30 of 37 MPS III, 10 of 30 MPS IV, 4 of 6 MPS VI, and all 4 ML were higher than the mean + 2SD of the control group.

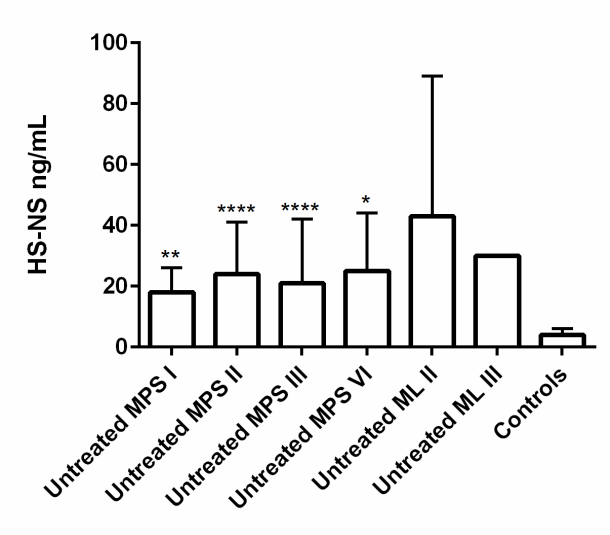

Figure 4.9: HS-NS Levels in Untreated Patients *vs.* Controls
Untreated MPS I, II, III, and VI patients had significantly higher levels of HS-NS than controls ($p<0.005$, $p<0.0001$, $p<0.0001$, and $p<0.05$, respectively).
*$p<0.05$; **$p<0.005$; ***$p<0.0001$
Adapted from: Kubaski et al., 2016 with permission from Elsevier.

Di-sulfated KS levels vary with age in control samples, being high from newborn until 15 years of age and then decreasing with age (Table 4.8). Although

the numbers of patients in each age group are low, di-sulfated KS levels are also lower in older patients. Di-KS levels in MPS patients were higher than in age-matched controls (Table 4.8) although only 12 of 21 MPS II, 10 of 37 MPS III, 12 of 30 MPS IV, and 4 of MPS VI were more than mean + 2SD of the control groups.

Table 4.8: Di-sulfated KS (ng/mL)

Age (years)	Controls	MPS I	MPS II	MPS III	MPS IV	ML
Newborns	27 ± 47	39 ± 20	84	29 ± 7	n/a	n/a
0–5	33 ± 21	55 ± 30	245 ± 474	92 ± 88	107 ± 139	42
5–10	40 ± 20	n/a	84 ± 41	44 ± 23	126 ± 200	25
10–15	41 ± 19	n/a	295 ± 557	106 ± 165	497 ± 869*	46
15–30	21 ± 11	n/a	122	44 ± 20*	38	n/a
> 30	19 ± 15	n/a	n/a	56	56 ± 37	n/a

MPS: mucopolysaccharidoses; KS: keratan sulfate;
Newborn data from Kubaski et al., 2016;
Adapted from: Kubaski et al., 2016 with permission from Elsevier.

Mono-sulfated KS levels also varied with age in the control group, with lower levels after the age of 15 years (Table 4.9). Levels of mono-KS were generally higher in MPS patients than in age-matched controls, although differences were only significant in older MPS III and IV patients (Table 4.9). Only 12 of 21 MPS II, 7 of 37 MPS III, 4 of 30 MPS IV, 2 of 6 MPS VI were more than mean + 2SD of the age-matched control groups.

Table 4.9: Mono-sulfated KS (ng/mL)

Age (years)	Controls	MPS I	MPS II	MPS III	MPS IV	ML
Newborns	128 ± 161	172 ± 82	208	86 ± 27	n/a	n/a
0–5	188 ± 75	216 ± 127	398 ± 415	257 ± 186	332 ± 408	100
5–10	152 ± 63	n/a	313 ± 163	106 ± 49	225 ± 190	115
10–15	140 ± 69	n/a	560 ± 720	315 ± 383	259 ± 177	182
15–30	67 ± 32	n/a	317	121 ± 60*	96	n/a
> 30	49 ± 24	n/a	n/a	143 ± 85	123 ± 61*	n/a

MPS: mucopolysaccharidoses; KS: keratan sulfate;
Newborn data from Kubaski et al., 2016;
Adapted from: Kubaski et al., 2016 with permission from Elsevier.

The ratio of di-sulfated KS in total KS gradually increased with age in the control group (Table 4.10). As seen previously in newborn samples [371], this ratio was higher in DBS from MPS I, II, and III patients aged 0–5 than in age-matched controls. This was also the case for MPS IV children in this age group. Differences were not significant in older patients.

Table 4.10: Ratio of Di-sulfated KS in Total KS (%)

Age (years)	Controls	MPS I	MPS II	MPS III	MPS IV	ML
Newborns	17 ± 6	26 ± 15***	27 ± 12***	28 ± 11***	n/a	n/a
0–5	15 ± 8	23 ± 5*	26 ± 10*	25 ± 5***	23 ± 4**	30 ± 2
5–10	21 ± 7	n/a	21 ± 1	24 ± 4	29 ± 8*	18
10–15	23 ± 5	n/a	26 ± 8	30 ± 9*	32 ± 21	20
15–30	23 ± 7	n/a	28	26 ± 4	29	n/a
> 30	28 ± 7	n/a	n/a	29	24 ± 9	n/a

MPS: mucopolysaccharidoses; KS: keratan sulfate;
Newborn data from Kubaski et al., 2016;
Adapted from: Kubaski et al., 2016 with permission from Elsevier.

4.3.4 Discussion

This study demonstrates the usefulness and significance of assay of disaccharides from DBS by LC/MS/MS as a diagnostic approach for MPS and ML. I measured several GAGs (DS, HS, and KS) simultaneously and found that the majority of untreated MPS and ML patients had higher levels of at least one GAG compared to age-matched controls.

An important consideration when selecting a method to analyze GAG levels is related to the choice of specimens. Urine has been extensively used in clinical practice due to convenience for collection and accessibility [568-571]. DBS may offer a useful alternative to urine for measurement of GAGs, including easy access to specimens, the simplicity of transport, and the potential for use in multiple assays including measurement of enzyme activity. DBS samples are routinely used for newborn screening for several metabolic disorders [572].

Limitations for the use of DBS are related to procedures for DBS preparation. Although each 3.3 mm disc from a DBS corresponds to approximately 3.6 μL of blood [373], variations in sample collection methods among different centers might affect blood volume per spot. Also, protocols are typically standardized for serum or plasma use, and consequently, extra validation is required for the use of DBS.

Another important consideration for clinical use is that measurements of GAGs in DBS will reflect the concentration of analytes in both the plasma and the cellular fraction of whole blood, whereas the cells are removed in serum or plasma. Consequently levels of GAGs in DBS may not directly correspond with levels in

plasma or serum. Another disadvantage of DBS is that the volume of sample is small and may not be sufficient for early stage research that typically requires more samples for protocol validation [573].

As expected, and described previously in serum (or plasma) and urine samples [253, 574-577], the analysis of DBS samples revealed that MPS I, II, III, VI, and ML patients had an elevation of DS compared to controls (Fig. 4.7). These patients also had elevated HS-0S and HS-NS, although elevations were not statistically significant for the ML patients. Data support the use of DBS to measure DS and HS levels to screen for MPS and ML patients. DS was not significantly elevated in DBS from MPS IV patients, but levels of HS-NS were elevated, indicating that these patients may also be detected in a screen for HS in DBS.

Levels of KS are age-dependent, and results of this study confirm that levels of KS are lower in both controls and patients > 15 years old. MPS II and MPS IV patients generally had higher levels of mono- and di-sulfated KS than controls.

However, due to limited numbers of patients in each age group, most differences were not statistically significant. By contrast, the ratio of di-sulfated KS in total KS was significantly higher than controls for all MPS patients (I, II, III, and IV) in the 0–5 year age group. These results are consistent with our previous findings that the ratio of di-KS in total KS is elevated in newborn DBS of MPS I, II, and III patients. This ratio was also higher in the ML patients, consistent with our previous observation of a patient with ML II who had elevated KS levels [535].

It is well-established that each type of MPS results in characteristic accumulated of specific GAG(s) based on the deficient enzyme (Table 1.1) [56]. More recent studies show that secondary elevations of other GAGs also occur. For example, elevation of KS level in blood and urine is diagnostic for MPS IV since the deficient enzyme is directly involved in the catabolism of KS; but KS is also elevated in several other types of MPS [254, 535, 536, 574, 578]. Notably, in the current study the secondary KS elevation in DBS of MPS II patients is as high as the levels seen in patients with MPS IVA. Elevation of the ratio of di-KS in total KS is not a specific biomarker for MPS IV, but is a marker in newborns and young patients for several forms of MPS including I, II, III, and IV.

Although MPS are progressive disorders that often take years to present clinically, there is considerable evidence from both humans [524, 525, 579, 580] and animal models [327, 581] that biochemical storage commences in the fetus. Results of this study showing the elevation of GAGs in young patients support these observations.

Because most MPS patients are not diagnosed at birth, DBSs of newborn MPS patients are rare and consequently it is difficult to define cutoff values of GAGs for newborn screening. In this study, we show that as levels of DS and HS do not vary significantly with age, most older patients still have levels of DS and HS that are distinguishable from controls using a cutoff of mean + 2SD of the controls. With a limited number of newborn MPS samples, we were able to define cutoffs for HS and DS that effectively discriminated MPS I, II, and III patients from controls [371].

Previous studies have reported the validity of GAG measurement in urine and CSF in which a good discrimination between MPS patients and controls were seen [255, 256, 536, 565, 566, 568]. However, some of the older patients in the present study were indistinguishable from the controls. The reason for low levels of GAGs in our patients is not known, but the management of their condition by palliative care or anti-inflammatory treatments could lower GAG levels in the bloodstream. It is also possible that their genotype and phenotype were less severe with lower GAG accumulation.

4.3.5 Conclusion

In conclusion, this study has demonstrated that DBS, when available, can be used as a convenient source of patient samples for rapid and simultaneous measurement of multiple GAGs by MS/MS and that they could be used for diagnosis of severe forms of MPS and ML. Furthermore, it is important to note that MPS patients with attenuated phenotypes are likely to have lower GAG levels and that this could be a potential limitation, with potential false negative results when using DBS for diagnosis and/or treatment monitoring of those patients. Longitudinal studies should be conducted to elucidate the feasibility of GAG monitoring with DBS samples.

4.4 Overall Conclusions for GAG Measurement in MPS

The development and establishment of a panel of comprehensive GAGs allow discrimination of MPS patients from control samples being an extremely useful methodology for diagnosis and disease monitoring.

We were able to demonstrate that GAGs can be quantified in a variety of samples such as: amniotic fluid, blood (dried blood spots or serum/plasma) and urine. We also demonstrated that this methodology is useful for prenatal diagnosis in which quantification of GAGs in amniotic fluid is very sensitive showing that MPS fetus has high GAG levels as early as 21 weeks of gestation.

We have also demonstrated the feasibility of this methodology for newborn screening of MPS patients in which the combination of several high GAG levels seems to decrease false positive rates and high-risk groups can be followed-up by enzyme assay for diagnostic confirmation.

This methodology is not only useful for prenatal and newborn diagnosis, but also for older MPS patients (infants and or adults) follow-up in which GAG quantification in DBS seems to be a convenient source and can aid diagnosis and disease progression.

In conclusion, GAG analysis by tandem mass spectrometry is a viable and feasible tool for diagnosis and disease monitoring in MPS. More studies with larger cohorts and other MPS subtypes (e.g. newborn MPS IV, VI, and VII) should be conducted to define cutoffs for newborn screening. GAG quantification will aid not only diagnosis but also prognosis and disease monitoring of MPS patients.

Chapter 5

GAG MEASUREMENT BY TANDEM MASS SPECTROMETRY CAN MEASURE EFFICACY OF THREE DIFFERENT TREATMENT OPTIONS FOR MPS PATIENTS

There is no current cure for MPSs, but there are available treatments including: enzyme replacement therapy (ERT) [311], hematopoietic stem cell transplantation (HSCT) [312] and anti-inflammatory therapy [182, 582].

Although there is no current consensus on which treatment approach is the best, it is well known that if treatment is effective a decrease in GAG levels is expected. Thus, I proposed to test whether this comprehensive method of GAG detection can measure the efficacy of three different treatment options (ERT, HSCT and anti-inflammatory therapy with pentosan polysulfate-PPS).

5.1 Treatment in MPS I, III, IVA, VI, and VII Patients [10]

Enzyme replacement therapy (ERT) is available commercially an approved for treatment of MPS I, II, VI, and IVA [281, 283, 284, 343]. Hematopoietic stem

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cell transplantation (HSCT) is recommended for MPS I [583, 584]. Several studies indicate that HSCT will also improve outcomes for MPS II [320, 477, 585], MPS IVA [586, 587], MPS VI [588] and MPS VII [498]. In this study, we have evaluated GAG levels in ERT and HSCT treated patients with MPS I, III, IVA, VI, and VII. GAGs were measured as described in section 2.2.

5.1.1 Samples

Whole blood was collected in EDTA by venipuncture and 150 µL of blood was spotted onto filter paper to create DBS. DBS from 106 untreated MPS and ML patients (MPS I = 7; MPS II = 21; MPS IIIA = 12, MPS IIIB = 17, MPS IIIC = 6, MPS III (undefined) = 2; MPS IVA = 28, MPS IVB = 2; MPS VI = 7; MLII = 3; ML III=1), 18 treated MPS (MPS I with ERT = 6, MPS I with ERT+HSCT = 2, MPS I with HSCT = 1; MPS IIIA with HSCT = 1, MPS IIIB with HSCT = 2; MPS IVA with HSCT = 2; MPS VI with ERT = 2, MPS VI with HSCT = 1; MPS VII with HSCT = 1), and 115 control subjects. Diagnosis of MPS and ML had been made using enzyme assays.

DBS from MPS patients were provided by Shimane University (Japan), Gifu University (Japan), St. Mary`s Hospital (UK), and Kasturba Medical College Manipal University (India).

Control samples were obtained from 15 volunteer subjects from Hospital de Clínicas de Porto Alegre (Brazil) and from 100 subjects who had blood draws for clinical testing for non-metabolic disease from Shimane University (Japan). Informed consent was obtained at each Institute for all patient and control samples according to IRB approval at each institute.

All de-identified samples were shipped to Nemours/AIDHC and stored at -20 °C until the GAG assay was conducted. This study was approved by the Nemours IRB (protocol # 281498).

5.1.2 Statistical Analysis

Untreated patients were compared to controls and treated patients (ERT, HSCT, and/or ERT+HSCT) using student *t test* at the level of significance of 0.05 performed using Graphpad Prism 7.0a.

5.1.3 Results

5.1.3.1 GAG Levels in Untreated vs. Treated Patients with MPS and ML

Levels of HS-0S and HS-NS were lower in all treated patients compared to untreated patients although due to the limited sample size, only the ERT treated MPS I patients showed a significant reduction in these GAGs (Fig. 5.1 & 5.2).

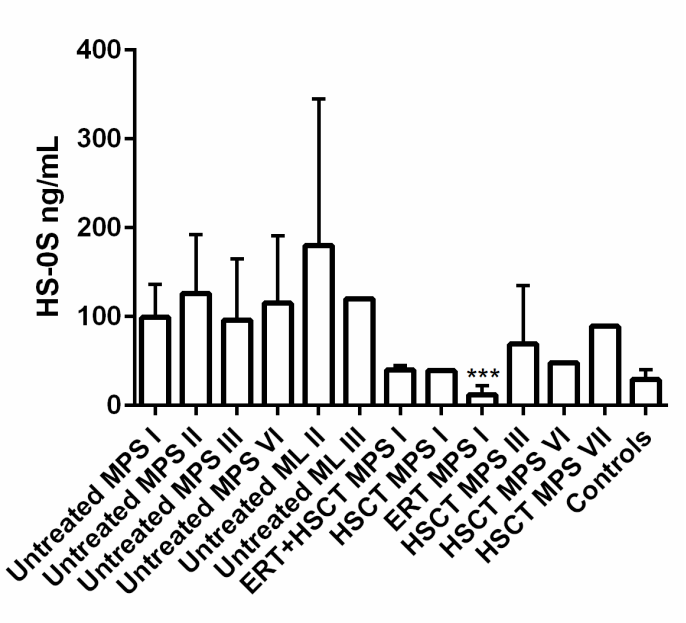


Figure 5.1: HS-0S Levels in Untreated *vs.* Treated Patients
Treated patients had lower levels of HS-0S compared to untreated patients. ERT MPS I was significantly lower ($p<0.001$) in comparison to untreated MPS I. Due to limited sample size statistical analysis could not be performed in the other treated groups.
***$p < 0.001$
Adapted from: Kubaski et al., 2016 with permission from Elsevier.

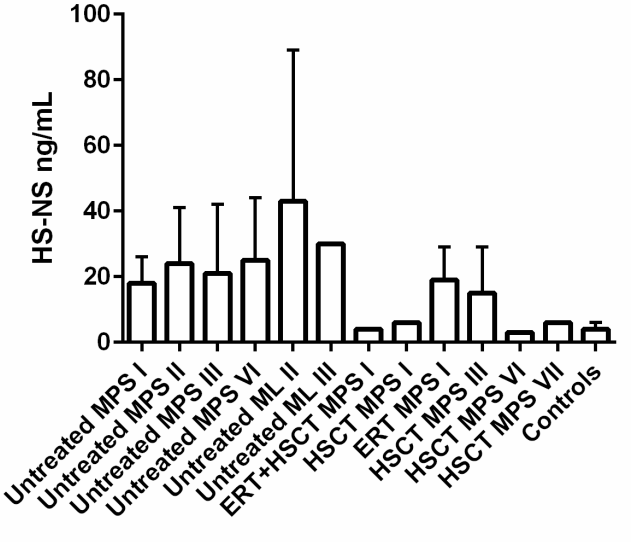


Figure 5.2: HS-NS Levels in Untreated *vs.* Treated Patients
Treated patients had lower levels of HS-NS compared to untreated patients. Due to limited sample size statistical analysis could not be performed.
Adapted from: Kubaski et al., 2016 with permission from Elsevier.

Levels of DS were less affected by treatment although lower levels were seen in HSCT treated MPS I and VI patients (Fig. 5.3).

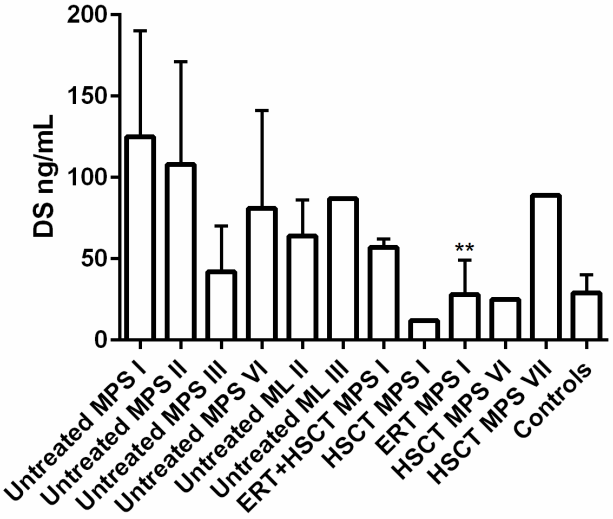


Figure 5.3: DS Levels in Untreated *vs.* Treated Patients
Treated patients had lower levels of DS compared to untreated patients. ERT MPS I was significantly lower ($p<0.01$) in comparison to untreated MPS I. Due to limited sample size statistical analysis could not be performed in the other treated groups.
** $p<0.01$
Adapted from: Kubaski et al., 2016 with permission from Elsevier.

Two MPS IVA patients were treated by HSCT. Patient 1 was 25 years old (transplanted at 4 years of age) and patient 2 was 26 years old (transplanted at 15 years of age). In patients 1 and 2 respectively, levels of di-sulfated KS were 21 ng/mL, 4 ng/mL; levels of mono-sulfated KS 56 ng/mL, 79 ng/mL; and the ratio of di-sulfated KS 27% and 5%. Levels of mono-sulfated, di-sulfated KS, and ratio of di-sulfated KS were more similar to age-matched controls than untreated MPS IV patients (Table 4.8, 4.9 & 4.10).

5.1.4 Discussion

We have shown a reduction of GAGs in patients treated with ERT and/or HSCT in MPS I, VI, and VII when compared to other untreated patients, suggesting the potential use of this method for treatment monitoring. Samples from individual patients pre- and post-therapy were not available for this study.

It is well known that total urinary GAG is reduced by ERT [281, 283, 284, 343, 382]. However, studies examining the effects of either ERT or HSCT treatment on specific GAG levels are limited. We previously showed that HS levels are more effectively reduced by HSCT than by ERT in MPS II patients [253].

In the present study, we evaluated the effect of ERT and/or HSCT on specific GAGs in MPS I, III, VI, and VII. For all treated patients, levels of HS-0S and HS-NS were similar to control levels, indicating efficient reduction of GAGs in blood for both types of treatment and each form of MPS. The results are less clear for DS, with values remaining high for the two ERT treated MPS VI patients and the HSCT-treated MPS VII patient.

More extensive studies are required with many patients treated with ERT and/or HSCT to determine whether levels of HS, and possibly other GAGs, are consistently reduced and whether this reduction translates into better outcomes for patients.

5.1.5 Conclusion

In conclusion, this study has demonstrated that DBS can be used as a convenient source for therapeutic monitoring and that our method can be used to assess treatment efficacy in MPS patients.

5.2 ERT *vs.* HSCT in MPS II Patients

Mucopolysaccharidosis II (MPS II; Hunter syndrome) is an X-linked recessive lysosomal storage disorder (LSD) caused by a deficiency of iduronate-2-sulfatase (I2S) responsible for the catabolism of glycosaminoglycans (GAGs): dermatan sulfate (DS) and heparan sulfate (HS) [56, 56, 589]. In the absence of I2S, those GAGs accumulate in multiple organs including the central nervous system (CNS) and skeletal system, leading to impairment of cognitive and motor functions and to several physical handicaps [320, 447, 590].

The incidence is variable among different populations, ranging from 1:100,000 to 1:320,000 male births [90, 447, 591]. In Japan, the incidence of MPS II between 1982 and 2009 was 1:119,000 births (unpublished data). The clinical spectrum ranges from severe (with cognitive impairment) to attenuated phenotype (no cognitive impairment, although some patients may present severe somatic manifestations) [56, 592].

The common therapeutic approach for MPS II is enzyme replacement therapy (ERT) [593], which improves the visceral organ function and physical activity. ERT uses recombinant human I2S that is internalized by cells via mannose-6-phosphate (M6P) receptors and clears accumulated GAGs from visceral organs [594, 595]. ERT is superior to hematopoietic stem cell transplantation (HSCT) in

ease of treatment and safety, but ERT does not cross the blood-brain barrier (BBB) and leads to antibody formation that neutralizes the infused enzyme and thereby reducing therapeutic efficacy. ERT is very expensive, costing $400,000 (considering a patient weighing 25 kg) annually in Japan while HSCT costs $70,000 to 120,000 for a one-time procedure [590, 593-596].

HSCT is commonly used for patients with a severe form of MPS I (Hurler syndrome) and has been widely recognized as a therapeutic option at an early stage [597, 598]. In early studies of HSCT for MPS, the major concern was the high mortality rate (approximately 20-25%) due to the pre-transplant conditioning and graft-versus-host disease (GVHD) [599, 600]. Advancements in treatment regimens and earlier introduction of HSCT for MPS reduced the mortality risk to 11.5% in 13 years (1990-2003), [590, 596] and a more recent report described a 5% mortality rate for MPS patients [601].

Current HSCT guidelines and early studies suggested that HSCT is not suitable for MPS II due to little improvement of CNS impairment [592, 597, 602-607]. However, the number of reports remain limited without a systemic analysis. More recent studies have shown positive data in HSCT for MPS II. HSCT donor cells were found in the brain of a MPS II patient with HSCT [320], and brain magnetic resonance imaging (MRI) showed improvements in hydrocephalic changes and perivascular enlargement of HSCT patients [590]. We showed that ERT and HSCT have a similar impact on growth [608], and that activity of daily living (ADL) for HSCT patients is better than that in ERT-treated patients [609]. A report on HSCT for adult MPS II mice proved therapeutic efficacy in somatic

tissues [610]. Furthermore, a recent study suggested improvements in speech and in neurological impairments for MPS II patients treated with HSCT [585].

Thus, HSCT for MPS II became a therapeutic option for patients in Japan although survival rates depend on the clinical condition of the patient, the donor type, and the skills of trained staffs [590, 601, 608, 609].

In this study, we evaluated the efficacy of HSCT for MPS II by investigating clinical, biochemical, and radiological findings in 27 new cases from Japan and 119 reported cases, compared with ERT and untreated groups.

5.2.1 ADL Questionnaire

Activity of daily living (ADL) was evaluated in severe phenotype patients: 22 HSCT patients, 35 ERT patients, and compared to 142 age-matched controls by use of an ADL questionnaire that was validated in an earlier study [609]. Questionnaires were received from Gifu, Osaka City, and Tokai Universities. The questionnaire had three main domains ("Movement," "Movement with cognition," and "Cognition") where the maximum score for each domain is 20, and consequently, the maximum total score is 60 [609]. ADL scores for age-matched controls were compared to patients with a severe phenotype.

5.2.2 DBS Samples

We collected DBS samples from 22 cases who received HSCT (2, attenuated; 20, severe), 51 ERT (20, attenuated; 31, severe), and from 15 untreated cases. DBS were from Gifu University (Japan), Shimane University (Japan), St. Mary's Hospital (UK), Kasturba Medical College (India), and Vietnam

National Children's Hospital (Vietnam). 115 control DBS samples were received from the Blood Bank at the Hospital de Clínicas de Porto Alegre (Brazil), Shimane University, and Vietnam National Children's Hospital.

Most HSCT cases received ERT before the transplant, and all ERT cases had been treated for at least over one year. This study was approved by IRBs at local institutes and Nemours/AIDHC (protocol: 281498-4) and all subjects provided written informed consent. GAGs were measured as described in section 2.2.

5.2.3 Retrospective Study

Survival rates, transplant-associated complications, and clinical benefits of HSCT were analyzed from 19 published studies including 119 cases between 1984 and 2016 [320, 585, 590, 592, 605-608, 611-621].

5.2.4 Brain MRI

Brain MRI was assessed in the new cohort of patients and patients that had MRI at least twice over 2 years were included in this study (n=13). We have analyzed the findings of brain MRI according to four distinct types (categories I–VI) (Table 5.1) and graded it according to the scoring system [622].

Table 5.1: Brain MRI Evaluation of Lesions

Category	MRI finding	Grading
I	Cystic or cribriform lesions	Graded based on T1-weighted MRI as follows: 0 = none; 1 = mild (≤10 cystic lesions of <3 mm); 2 = moderate (>10 small cystic lesions of <3 mm); and 3 = severe (many cystic lesions including those >3 mm).
II	White matter signal changes	These changes observed on T2-weighted MRI were graded as follows: 0 = none; 1 = mild (a few limited to the periventricular area); and 2 = severe (in most parts of the periventricular area and other white matter areas).
III	Ventricular enlargement	Graded as follows: 0 = none; 1 = mild (<3 mm widening of the third ventricle without temporal horn dilatation); 2 = moderate, (>5–10 mm widening of the third ventricle); and 3 = severe (>10 mm dilatation of the third ventricle with bulbous configuration).
IV	Brain atrophy	Graded as follows: 0 = none, 1 = mild (mild widening of Sylvian and interhemispheric fissures by <3 mm, but not all of the sulci are involved); 2 = moderate (widening of all tissues and sulci by 3–5 mm); and 3 = severe (widening of all tissues and sulci by >5 mm with definite loss of cortex and white matter).

Sulci: depression or groove in the cerebral cortex.

5.2.5 Statistical Analysis

GAG levels and ADL scores were evaluated by student t-test at the levels of significance of 0.05 performed using Graphpad Prism v.7.0a. GAG levels in untreated patients were compared to treated patients (ERT, ERT+HSCT). ADL scores for severe phenotype patients were normalized by age-matched control

scores (individual score x 100/ control score). Average score for each domain was compared between ERT and ERT+HSCT treated groups by student t-test.

5.2.6 Results

5.2.6.1 Demographics and Clinical Status

We examined 146 HSCT cases of MPS II, comprised of 27 previously unreported cases and 119 previously reported cases. Distribution of new cases was: 74% with severe (n=20) and 26% with attenuated phenotype (n=7). Distribution of published cases was similar: 74% with a severe phenotype (n=88), 11% with an attenuated phenotype (n=13), 1% with an intermediate phenotype (n=1), and 14% with undefined phenotype (n=17). The mean ages at HSCT were 5.5 years (2 to 21.4 years) for the newly reported cases and 5.5 years of age (10 months to 19.8 years) for the published cases. Source of donor cells were 74% from bone marrow (BM), 15% from cord blood (CB), and 11% unknown for newly reported cases, while for previously reported cases cases were 65% from BM, 7% from CB, 11% from peripheral blood stem cells (PBSC), and 17% from unknown sources. For the newly reported cases, 52%, 21%, and 28% of the donors were related, unrelated, and unknown, respectively while 48%, 24%, and 28% of the donors were matched, mismatched, and unknown, respectively.

Average follow-up time after HSCT was 7 years (range: 1 to 22.9 years). Average follow-up time after ERT was 4.8 years (range: 1.7 to 12 years). For ERT+HSCT group average time of ERT before HSCT was 3.6 years (range: 1.3 to 5.3 years) and average time of ERT was 5.1 years (range: 1.7 to 12 years).

5.2.6.2 Survival Rates

None of 27 new cases died of transplant-associated complications, although three of these patients (11%) had acute GVHD. A similar rate of GVHD was published previously (8 out of 85 patients, 9%) [320, 590, 606, 618]. Nine of the previously published 119 cases died of transplant-associated complications [320, 606, 620] resulting in 8% mortality rate among published cases from 1984 to 2016. Of the deceased patients, 7 had received transplants from unrelated BM donors; [606] and two had received unrelated umbilical cord transplantation (UCBT) [320, 620].

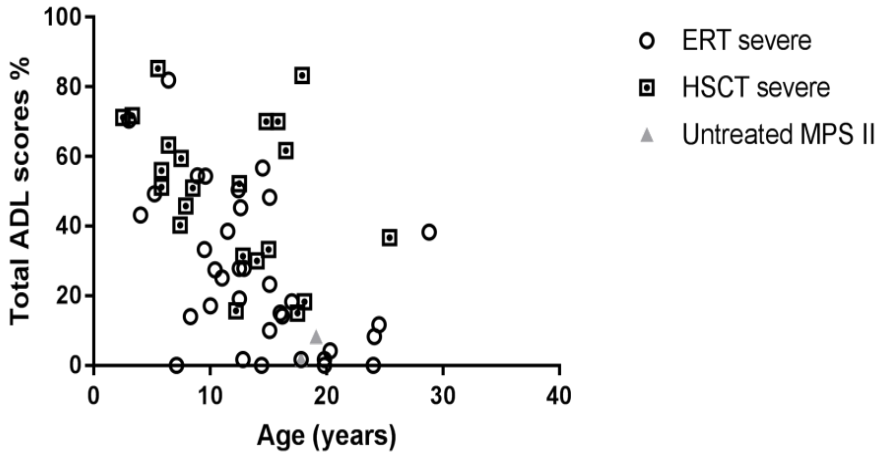
5.2.6.3 ADL

ADL scores were combined with those from a previous study to extend and enhance interpretation of relative efficacy of HSCT and ERT [609]. Average total ADL scores for patients with an attenuated phenotype were 55 ± 7 in ERT patients (n=20) and 58 in ERT+HSCT patients (n=2). Thus as ADL is not significantly impacted in patients with an attenuated phenotype treatment has no significant effect.

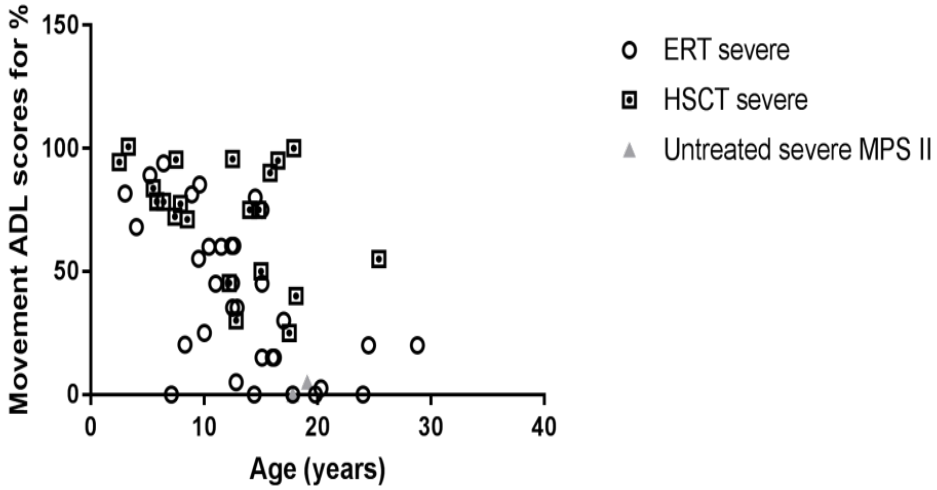
Scores for patients with a severe phenotype were much lower, 3 ± 3 for untreated patients (n=2), 15 ± 12 for ERT patients (n=35) and 28 ± 11 in ERT+HSCT patients (n=21). Although both treatments gave higher ADL scores than the two untreated patients, a statistical comparison between treatments and untreated is not possible. To compare the two different treatments for severe patients, ADL scores were normalized to age-matched controls. Total normalized

scores were significantly higher in ERT+HSCT ($p<0.0005$) compared to ERT only (51± 21% and 27 ± 22%, respectively). ERT+HSCT was significantly higher than ERT alone in all three domains (movement: $p<0.00001$; movement with cognition: $p<0.001$; cognition: $p<0.05$; respectively). Scores were generally higher for HSCT than ERT regardless of patient age (Figure 5.4).

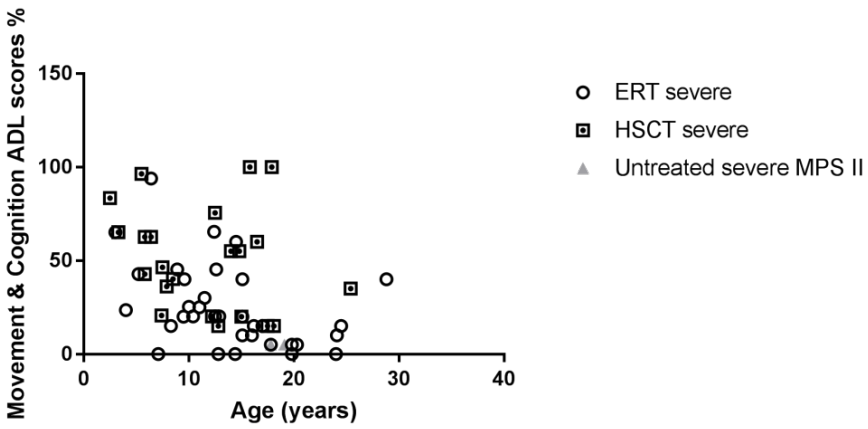
A.



B.



C.



D.

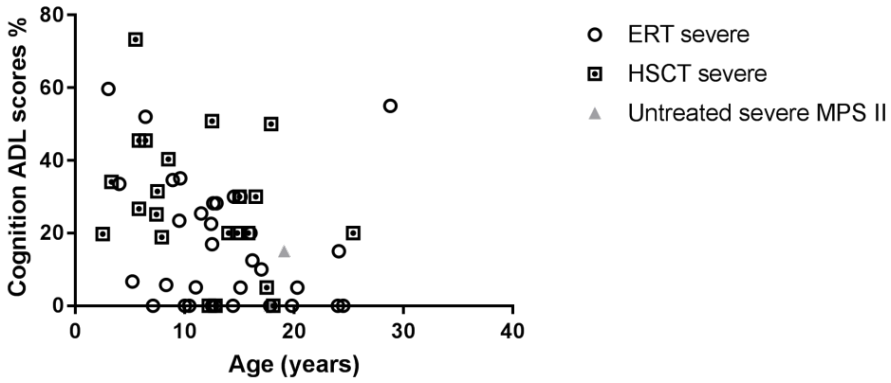


Figure 5.4: ADL Scores Normalized by Age-matched Controls
Figure 5.4 A: Total ADL Scores
Total ADL scores were obtained from ERT and ERT+HSCT MPS II treated patients. All scores were normalized by age-matched controls. ERT+HSCT had significantly higher ADL scores in comparison to ERT only ($p<0.0005$).
Figure 5.4 B: Movement Scores
The movement domain evaluates basic motor skills required for daily function (walking, movement on stairs, grasping/finger movement, endurance in a 6-minute walk). Movement ADL scores were normalized by age-matched controls. ERT+HSCT had significantly higher scores in comparison to ERT only ($p<0.00001$).
Figure 5.4 C: Movement with Cognition Scores
The movement & cognition domain evaluates basic motor skills in which different levels of cognition are required (toileting, getting dressed, bathing and eating).

Movement & cognition ADL scores were normalized by age-matched controls. ERT+HSCT had significantly higher scores in comparison to ERT only ($p<0.001$).
Figure 5.4 D: Cognition Scores
The cognition domain evaluates impairment in cognitive function (understanding of everyday conversation, conversation skills, social participation and problem solving skills). Cognition ADL scores were normalized by age-matched controls. ERT+HSCT had significantly higher scores in comparison to ERT only ($p<0.05$).

5.2.6.4 GAG Levels

DS, HS, and KS levels in 88 DBS samples with MPS II (22 HSCT, 51 ERT, and 15 untreated) were compared with 115 control DBS samples. For DS, the mean value in untreated patients was 97 ± 57 ng/mL ($p<0.0001$ compared to controls, 11 ± 19 ng/mL). Levels for treated patients were significantly lower than untreated patients: 22 ± 6 ng/mL in ERT-attenuated patients ($p<0.0005$), 27 ± 9 ng/mL in ERT-severe patients ($p<0.0005$), 21 ng/mL in ERT+HSCT-attenuated patients, 17 ± 7 ng/mL ($p< 0.0001$) in ERT+HSCT-severe patients. In severe phenotype patients ERT+HSCT had lower GAG levels ($p<0.0001$) than ERT only. However, levels of DS in treated patients (ERT, ERT+HSCT) remained higher than levels of controls ($p<0.0001$) (Fig. 5.5).

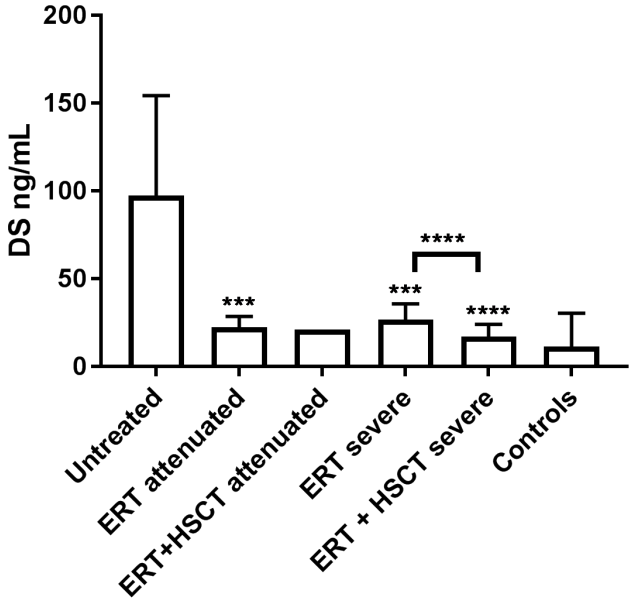


Figure 5.5: DS levels in Treated *vs.* Untreated MPS II Patients
DS levels in treated patients (ERT and ERT+HSCT) were compared to untreated patients. ERT and ERT+ HSCT had decreased DS levels compared to untreated patients. In patients with severe phenotype, both treatments reduced DS levels, but the combination of ERT+HSCT lead to higher reduction ($p<0.0005$).
***$p < 0.0005$; **** $p < 0.0001$;
DS: dermatan sulfate; ERT: enzyme replacement therapy; HSCT: hematopoietic stem cell transplantation. ERT and HSCT had decreased GAG levels compared with untreated patients.

The mean value of HS-NS in untreated patients was 33 ± 16 ng/mL ($p < 0.0001$, compared to controls, 2 ± 1 ng/mL). Levels for treated patients were all significantly lower than untreated patients: 10 ± 7 ng/mL in ERT-attenuated patients ($p< 0.0001$), 10 ± 8 ng/mL in ERT-severe patients ($p<0.0001$), 16 ng/mL in ERT+HSCT-attenuated patients, and 8 ± 6 ng/mL in HSCT-severe patients ($p<0.0001$). However, levels of HS-NS in treated patients (ERT, ERT+HSCT) remained significantly higher than levels of controls ($p<0.0005$) (Fig. 5.6).

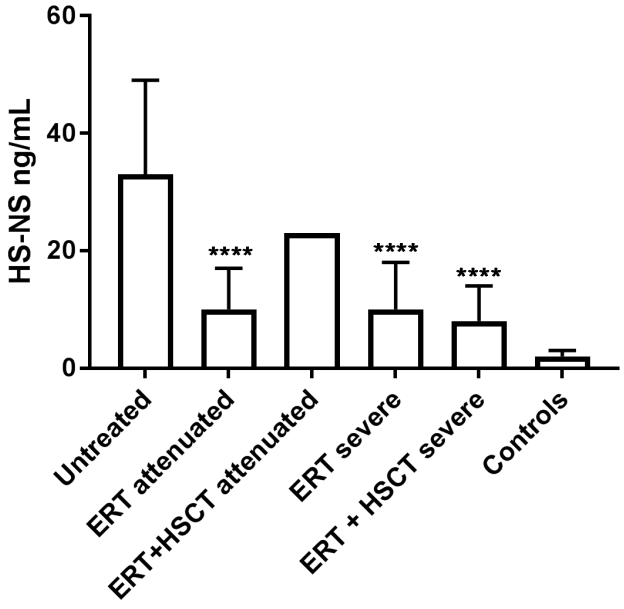


Figure 5.6: HS-NS Levels in Treated *vs.* Untreated MPS II Patients
MPS II patients HS-NS levels in treated patients (ERT and ERT+HSCT) were compared to untreated patients. ERT and ERT+ HSCT had decreased HS-NS levels compared to untreated patients.
**** $p < 0.0001$;
ERT: enzyme replacement therapy; HSCT: hematopoietic stem cell transplantation; HS: heparan sulfate;

For HS-0S, untreated patients had an average of 99 ± 50 ng/mL ($p<0.0001$ compared to controls, 13 ± 6 ng/mL). Levels for treated patients were all significantly lower than untreated patients: 18 ± 9 ng/mL in ERT attenuated ($p<0.0001$), 20 ± 7 ng/mL in ERT severe ($p<0.0001$), 21 ng/mL in ERT+HSCT-attenuated, 14 ± 5 ng/mL in ERT+HSCT severe ($p<0.0001$). In severe phenotype patients ERT+HSCT had lower GAG levels ($p<0.0005$) than ERT only. Levels of HS-0S in HSCT treated patients were indistinguishable from controls, but remained significantly higher for ERT treated patients ($p<0.0001$) (Fig. 5.7).

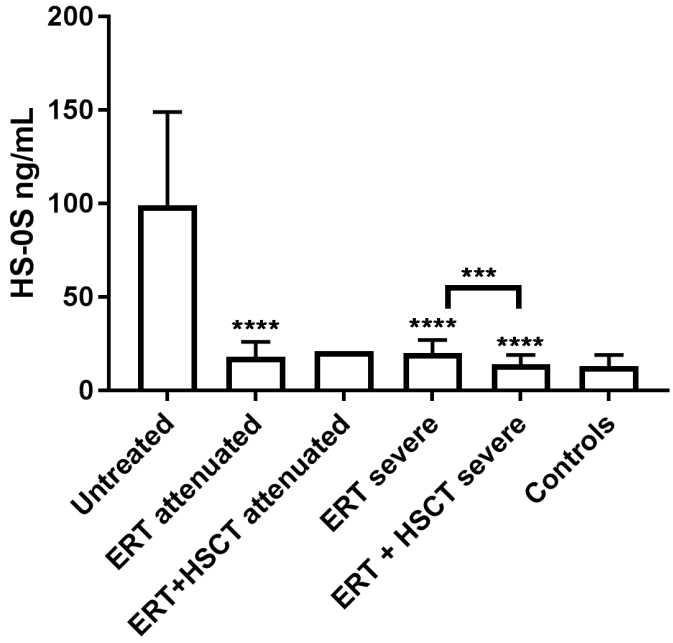


Figure 5.7: HS-0S Levels in Treated *vs.* Untreated MPS II Patients
HS-0S levels in treated patients (ERT and ERT+HSCT) were compared to untreated patients. ERT and ERT+ HSCT had decreased HS-0S levels compared to untreated patients. In patients with severe phenotype, both treatments reduced HS-0S levels, but the combination of ERT+HSCT lead to higher reduction ($p<0.0005$).
**** $p < 0.0001$;*** $p<0.0005$;
ERT: enzyme replacement therapy; HSCT: hematopoietic stem cell transplantation; HS: heparan sulfate.

For mono-KS, the mean value in untreated patients was 265 ± 149 ng/mL ($p<0.0001$ compared to controls, 62 ± 32 ng/mL). Levels for treated patients were significantly lower in majority of treated groups compared to untreated patients: 156 ± 78 ng/mL in ERT-attenuated patients ($p<0.05$), 208 ± 83 in ERT severe (NS), 230 ng/mL in ERT+HSCT attenuated, 149 ± 52 ng/mL in ERT+HSCT severe ($p<0.01$). In severe phenotype patients, ERT+HSCT had lower GAG levels ($p<0.005$) than ERT only. Levels of mono-KS in treated patients (ERT, ERT+HSCT) remained higher than levels of controls ($p<0.0001$) (Fig. 5.8).

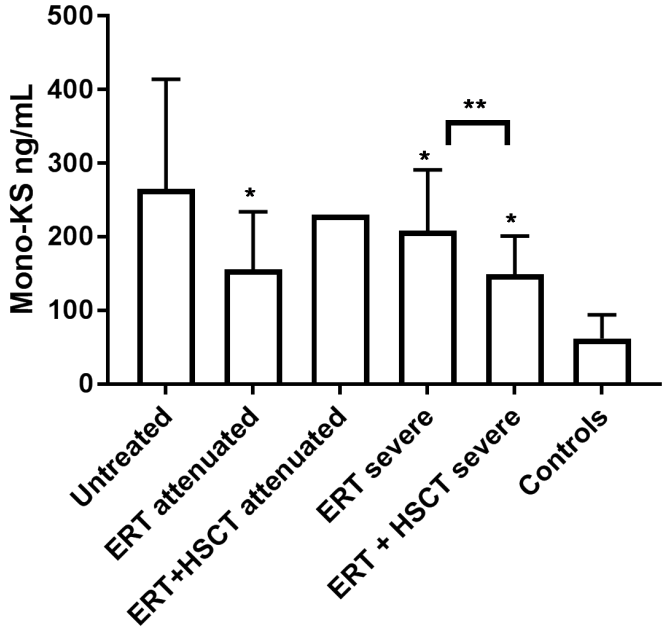


Figure 5.8: Mono-KS Levels in Treated *vs.* Untreated MPS II Patients
Mono-KS levels in treated patients (ERT and ERT+HSCT) were compared to untreated patients. ERT and ERT+ HSCT had decreased mono-KS levels compared to untreated patients. In patients with severe phenotype, both treatments reduced mono-KS levels, but the combination of ERT+HSCT lead to higher reduction ($p<0.005$).
*$p<0.05$; **$p<0.005$;
ERT: enzyme replacement therapy; HSCT: hematopoietic stem cell transplantation; KS: keratan sulfate;

For di-KS, the mean value in untreated patients was 110 ± 64 ng/mL ($p<0.0001$ compared to controls, 19 ± 12 ng/mL). Levels for treated patients were significantly lower in treated groups compared to untreated patients: 60 ± 47 ng/mL in ERT-attenuated patients ($p<0.05$), 54 ± 27 ng/mL in ERT severe ($p<0.005$), 88 ng/mL in ERT+HSCT attenuated, 46 ± 22 ng/mL in ERT+HSCT severe ($p<0.005$), but again levels of di-KS in treated patients remained higher than seen in controls ($p<0.0001$) (Fig. 5.9).

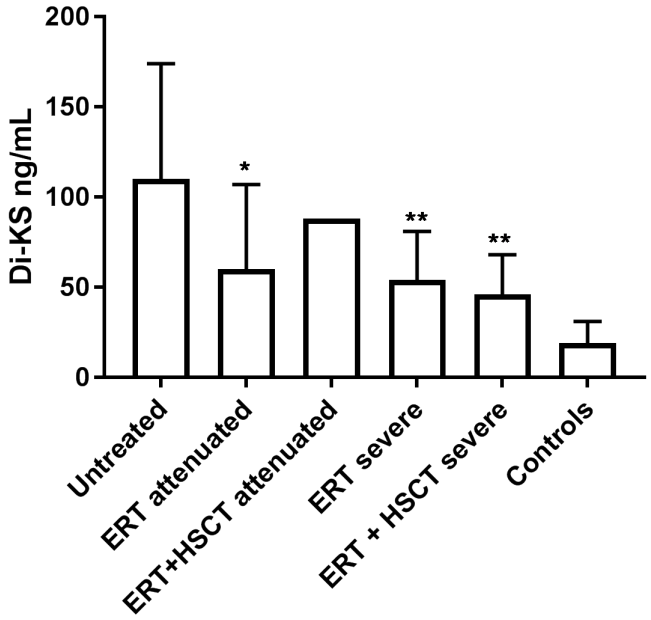


Figure 5.9: Di-KS Levels in Treated *vs.* Untreated MPS II Patients
Di-KS levels in treated patients (ERT and ERT+HSCT) were compared to untreated patients. ERT and ERT+ HSCT had decreased di-KS levels compared to untreated patients.
*p<0.05; **p<0.005;
ERT: enzyme replacement therapy; HSCT: hematopoietic stem cell transplantation; KS: keratan sulfate;

5.2.6.5 MRI Findings

Brain MRIs at least 2 years apart were available for 13 of the newly reported cases (6 ERT treated only and 7 HSCT treated) (Figs. 5.10-22; Table 5.2).

Table 5.2: Summary of Brain MRI

		Age (years)			Category of lesions								ADL	
		MRI			I			II		III	IV			
Case	Phenotype	earliest	latest	Treatment	Basal ganglia	White matter	Corpus callosum	Patchy	Diffuse		Interhemispheric	Sylvian fissure	before	after
1	mild	12	24	ERT	3 _ 3	2 _ 3	3 _ 3	2 _ 2	1 _ 1	3 _ 3	2 _3	3 _ 3	n/a	56
2	mild	26	34	ERT	1 _2	2 _ 2	2 _ 3	2 _ 2	1 _ 1	2 _ 2	1 _1	3 _ 3	n/a	59
3	mild	31	39	ERT	2 _ 3	1 _ 2	1 _ 1	1 _ 1	0 _ 0	2 _ 2	0 _ 0	0 _0	n/a	52
4	severe	2	6.3	HSCT	2 _ 1	3 _ 3	0 _ 0	2 _ 0	0 _ 0	2 _ 2	2 _ 0	3 _2	26	42
5	mild	5	16.8	HSCT	1 _ 0	2 _ 1	0 _ 0	1 _ 1	0 _ 0	0 _ 0	0 _ 0	1 _0	n/a	60
6	mild	21	24.3	HSCT	3 _ 2	3 _ 3	3 _ 1	0 _ 1	3 _ 0	3 _ 3	3 _ 3	3 _3	56.5	60
7	mild	8	33	HSCT	1 _ 1	0 _ 0	0 _ 0	2 _ 3	0 _ 0	1 _ 1	0 _ 0	0 _ 0	n/a	60
8	mild	5	24	HSCT	1 _ 0	3 _ 1	0 _ 0	0 _ 0	0 _ 0	0 _ 0	0 _ 0	0 _ 0	n/a	47
9	severe	11	17	ERT	1 _ 2	1 _ 2	2 _ 2	1 _1	1 _1	2 _ 3	2 _ 3	2 _ 3	14	11
10	severe	5	15	HSCT	0 _ 0	0 _ 0	0 _ 0	0 _ 0	0 _ 0	0 _ 0	0 _ 0	0 _ 0	n/a	20
11	severe	5	14	HSCT	0 _ 0	0 _ 0	1 _ 0	1 _ 0	0 _ 0	1 _ 1	0 _ 2	1 _ 2	n/a	18
12	severe	3	10	ERT	0 _ 0	3 _ n/a	2 _ n/a	1 _ 1	1 _ 1	1 _ 3	1 _ 3	1 _ 3	n/a	10
13	severe	3	11	ERT	0 _ 0	2 _ 2	0 _ 0	1 _ 0	0 _ 0	0 _ 3	0 _ 3	0 _ 3	n/a	15

ERT: enzyme replacement therapy; HSCT: hematopoietic stem cell transplantation; ADL: activity of daily living; n/a: not available.

5.2.6.5.1 ERT Cases

Three ERT-treated patients with an attenuated form (cases 1-3) were 24, 34, and 38 years old, respectively, when the last MRI image was taken (Fig. 5.10-5.12). They had been treated with ERT for 8, 9, and 7 years, respectively. A comparison of MRIs before and after treatment showed increased cystic (or cribriform) lesions, white matter signal changes, ventricular enlargement, and/or brain atrophy with age. MRI changes were particularly severe for case 1, with progressive ventricular dilatation and severe brain atrophy correlated with the neurological signs of recurrent seizures (Fig. 5.10).

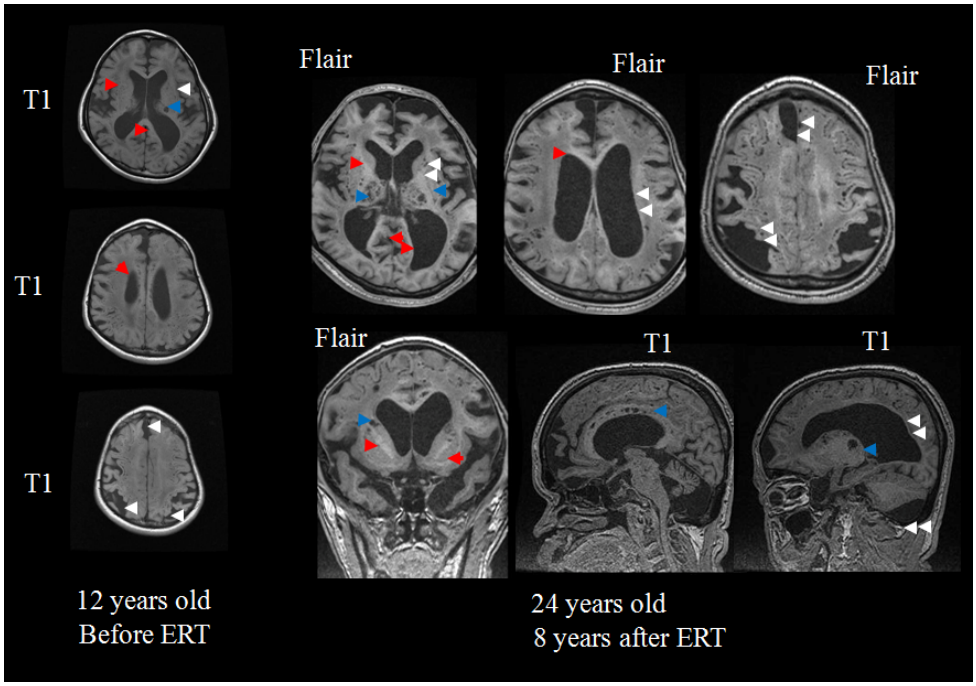


Figure 5.10: MRI Findings of Case 1
Case 1: A 24-year-old patient with over 8 years of ERT (attenuated): His intellect is normal, but he has recurrent episodes of seizures, shivering, and dizziness with regurgitation of the heart valves. Brain atrophy, ventricular dilatation, white matter signal changes, cribriform changes, and cystic lesions are present at 12 years. At 24 years, multiple and larger cystic lesions are seen in basal ganglia, thalamus, the corpus callosum as well. Large cisterna magna and ventricular enlargement and brain atrophy are more prominent. Red arrows represent white matter signal changes; blue arrows represent cystic or cribriform regions; white arrows represent ventricular enlargement and brain atrophy.

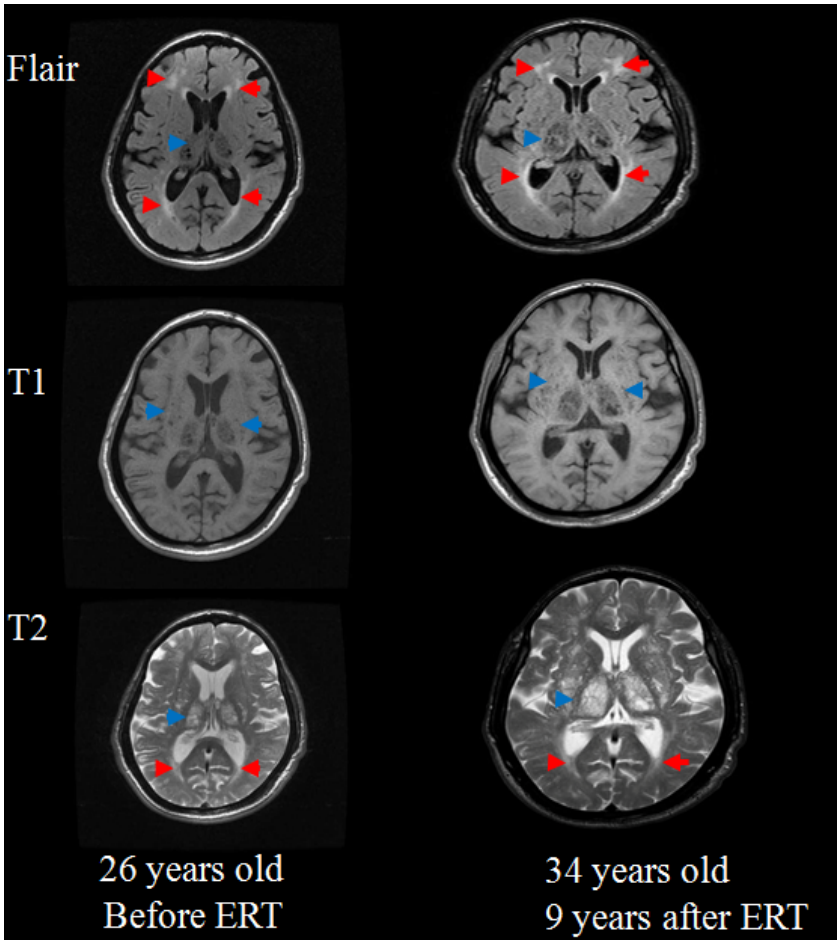


Figure 5.11: MRI Findings of Case 2
Case 2: A 34-year-old patient with over 9 years of ERT (attenuated): His intellect is normal, but he has regurgitation of the heart valves needed for the surgical intervention. Symmetrical high signal intensity lesions are seen on the FLAIR and T2 images in the peri trigonal white matter (single arrows). Cribriform changes are present in the basal ganglion and thalamus. Red arrows represent white matter signal changes; blue arrows represent cystic or cribriform regions.

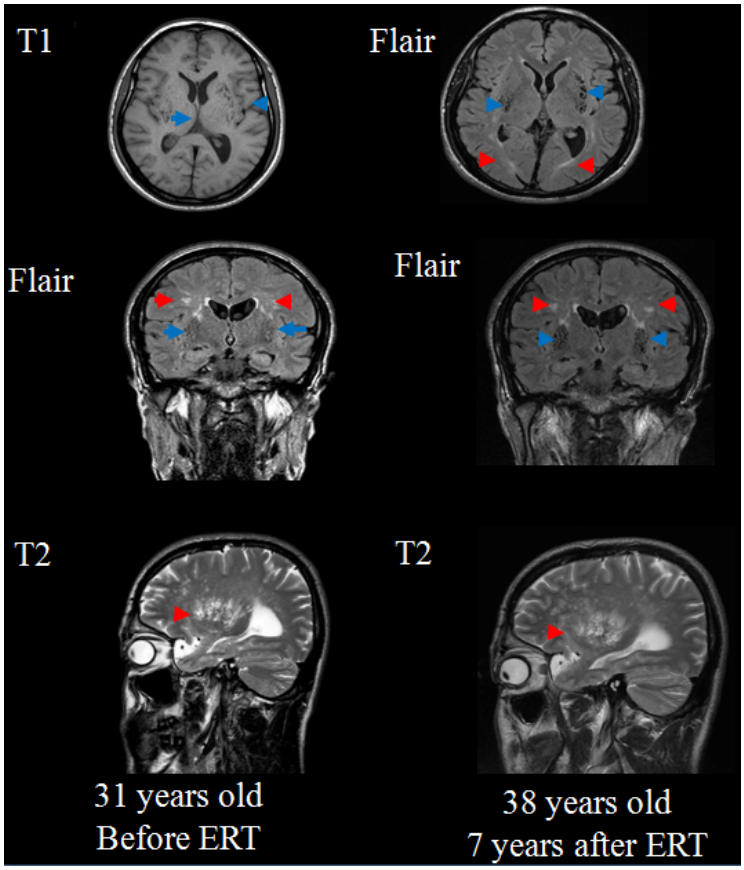


Figure 5.12: MRI Findings of Case 3
Case 3: A 38-year-old patient with over 7 years of ERT (attenuated): His intellect is normal, but he has progressive retinal degeneration leading to near blindness. Symmetrical high signal intensity lesions are seen on the FLAIR images in the peri trigonal white matter and on the T2 image in the thalamus (red arrows). Cribriform changes are present in the basal ganglion and thalamus. Red arrows represent white matter signal changes; blue arrows represent cystic or cribriform regions; white arrows represent ventricular enlargement and brain atrophy.

Three patients with a severe form (cases 4-6) had received ERT for over 5 years and were 17, 10, and 11 years old at the last MRI. MRIs showed progressive ventricular dilatation and severe brain atrophy with age (Fig. 5.13-15). Case 4 had normal development until 1 year and 2 months. At 2.5 years speech delay was noticed, and at 3 years joint rigidity appeared with a confirmed diagnosis of MPS II. He could comprehend words but over time became speechless. His physical

activity decreased due to reduced ability to walk, slowed ability to climb stairs, and progressive joint rigidity. He started ERT at 11 years old; but became wheelchair bound with slow walking and loss of body weight (Fig. 5.13). At 19 years old, he had an epileptic seizure with disease progression (ADL score; 11). His condition continued to worsen with age.

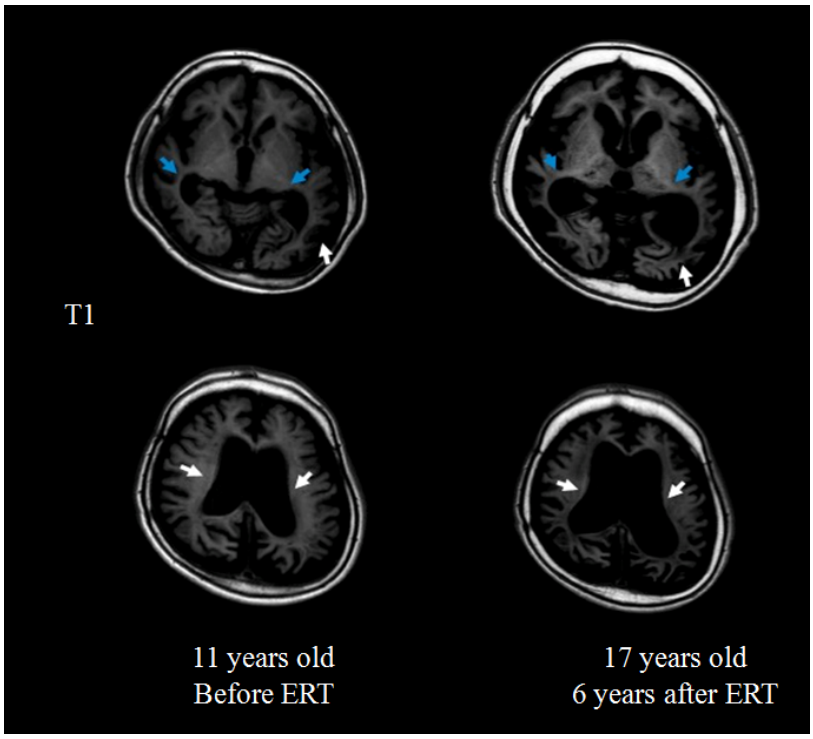


Figure 5.13: MRI Findings of Case 4
Case 4: A 17-year-old patient with over 6 years of ERT (severe): MRI before ERT shows small cystic lesions in basal ganglia and white matter with white matter signal changes, moderately enlarged ventricle, and moderate brain atrophy. After over 6 years of ERT, these lesions are increased and worsening when he is 17 years old. Blue arrows represent cystic or cribriform regions; white arrows represent ventricular enlargement and brain atrophy.

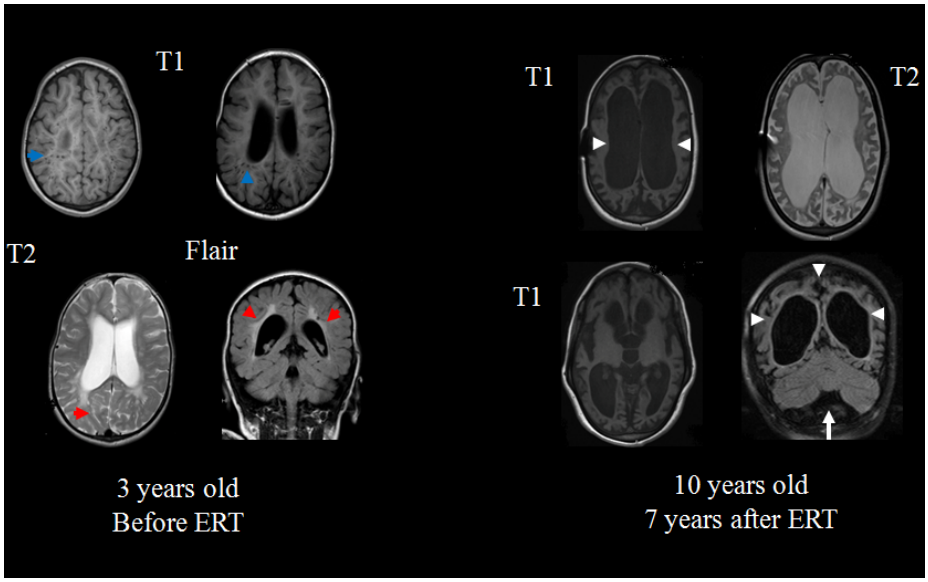


Figure 5.14: MRI Findings of Case 5
Case 5: A 10-year-old patient with over 7 years ERT (severe); MRI before ERT shows small cystic lesions and white matter signal changes, moderately enlarged ventricle. At 10 years old, severe atrophic brain and enlarged ventricle are observed. Red arrows represent white matter signal changes; blue arrows represent cystic or cribriform regions; white arrows represent ventricular enlargement and brain atrophy.

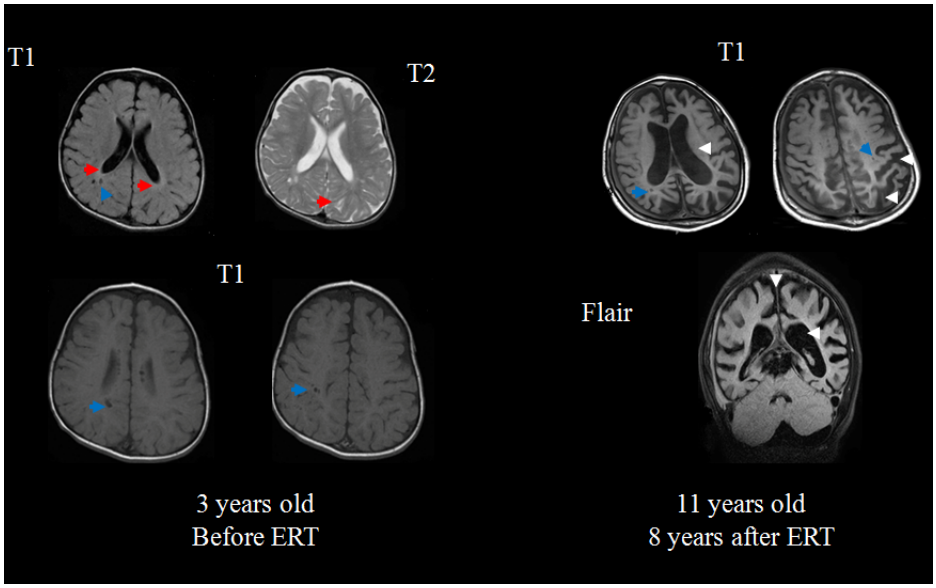


Figure 5.15: MRI Findings of Case 6
Case 6: A 11-year-old patient over 8 years ERT (severe); MRI before ERT shows small cystic lesions and white matter signal changes. At 11 years old, moderate

atrophic brain and enlarged ventricle are observed. Red arrows represent white matter signal changes; blue arrows represent cystic or cribriform regions; white arrows represent ventricular enlargement and brain atrophy.

5.2.6.5.2 HSCT Cases

MRIs were obtained from four patients with an attenuated form who had HSCT (cases 7-10; Fig. 5.16-19). Case 7 was transplanted at 5.4 years. At 16.8 years, MRI showed preserved fine structure in white matter and improvement of cribriform changes. The patient could speak and succeeded intellectually at school (Fig. 5.16).

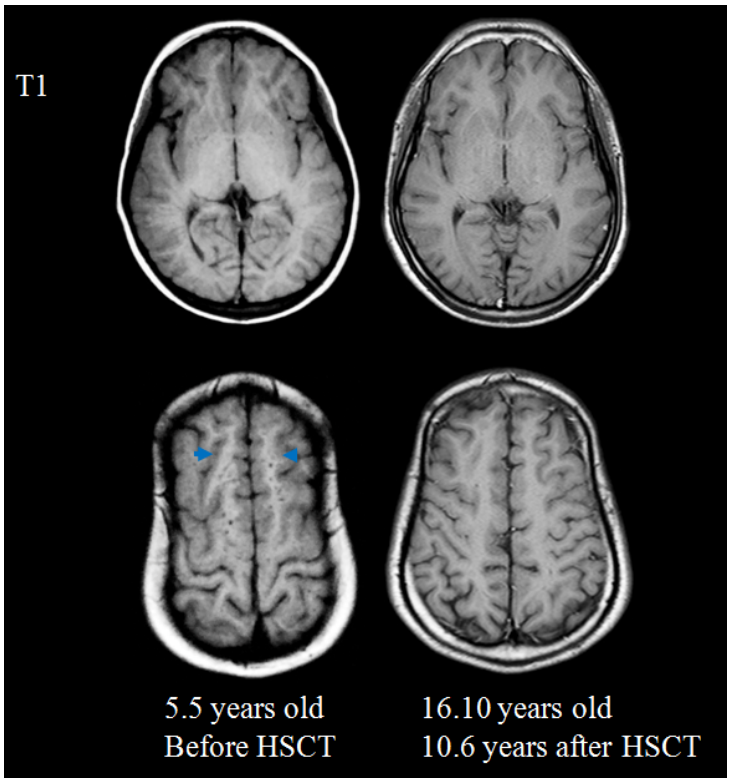


Figure 5.16: MRI Findings of Case 7
Case 7: A 16-year- and 8-month-old patient with HSCT at 5 years and 5 months (attenuated): Fine structure of white matter is preserved or improved, and cystic (or cribriform) change is improved. Blue arrows represent cystic or cribriform regions.

Case 8 was diagnosed as MPS II at 6 years old. He received ERT since the age of 15 years old and showed a slight improvement in rigidity of joints in hands. Although there was no clinical indication for HSCT based on phenotype and age, the patient and his family requested HSCT to improve clinical outcomes. The patient underwent HLA-C one allele-mismatched unrelated BMT at 21.4 years and had full engraftment followed by mixed chimerism that was corrected by donor-lymphocyte infusion. Although both acute and chronic GVHD developed, they were successfully treated with immunosuppressive therapy. Two years post-HSCT, MRI showed no change in ventricular size and fine structure in white matter, especially thalamus, was preserved. Signal of the high-density area was decreased. Three years post-HSCT, the patient's difficulty in conversation improved (due to reduced size of tongue), along with improvements in range of motion, hair and skin coarseness, finger motor skills, MRI, and ADL. The patient is completely independent and mobile and has an ADL score of 60 (Fig. 5.17).

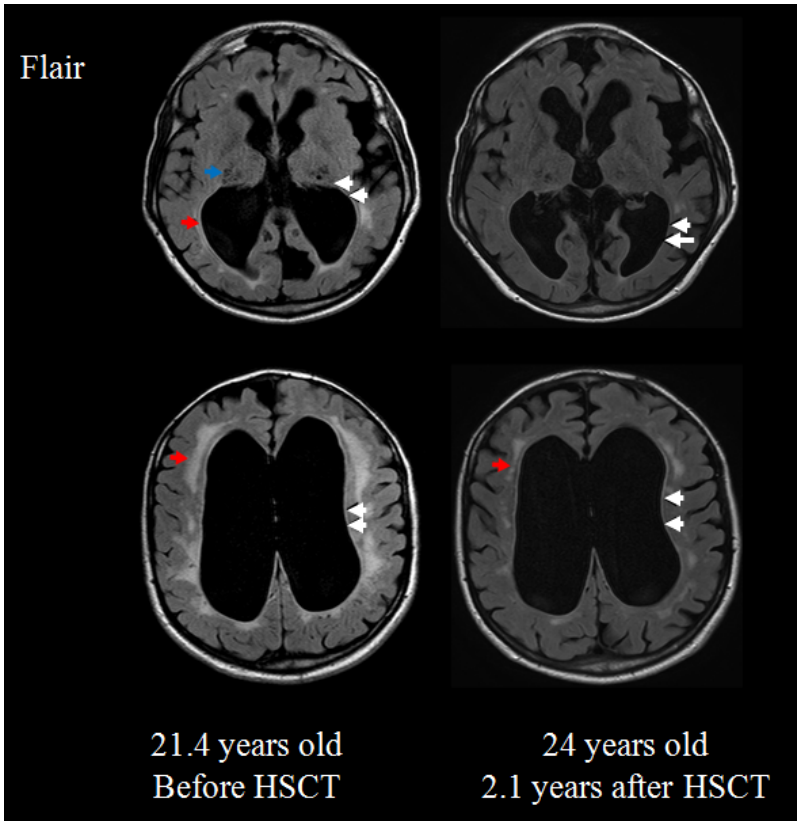


Figure 5.17: MRI Findings of Case 8
Case 8: A 24-year-old patient with HSCT at 21 years (attenuated): Ventricular size is not changed and fine structure in white matter, especially thalamus, is improved. The decrease of the signal of high-density area is seen. Red arrows represent white matter signal changes; blue arrow represents cystic or cribriform regions; white arrows represent ventricular enlargement and brain atrophy.

Cases 9 and 10 were followed up for > 20 years after HSCT. Both patients had no intellectual disabilities before receiving HSCT. Case 9 received HSCT at 9 years old from his carrier sister and MRIs showed cribriform changes in the corpus callosum, mild ventricular enlargement, and patchy intensity changes of the periventricular white matter prior to HSCT. 24 years post-HSCT, MRI findings show little change and his intellectual ability remained normal despite psychological symptoms (Fig. 5.18).

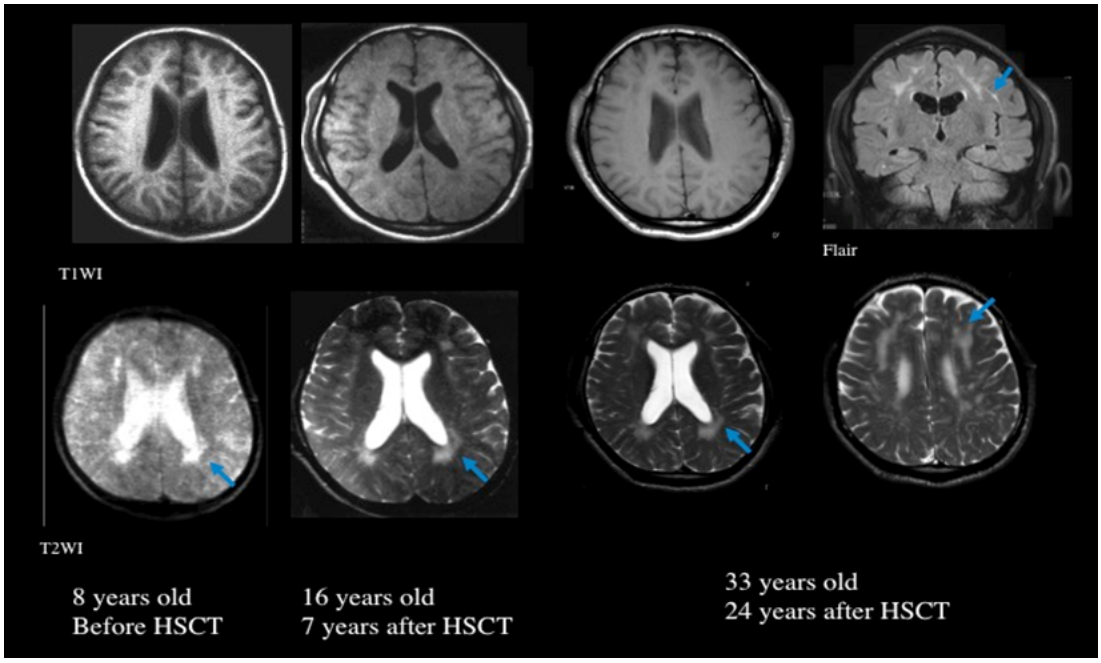


Figure 5.18: MRI Findings of Case 9
Case 9: A 33-year-old patient with HSCT at 9 years (attenuated): No significant intensity change of the periventricular area is observed, and fine structure of white matter is preserved. Ventricle dilatation is not changed. Blue arrows represent cystic or cribriform regions.

Case 10 received HSCT from his healthy brother at 5.5 years old. Brain MRI before HSCT showed multiple cystic lesions in basal ganglia and white matter (Fig. 5.19; Table 5.2). These lesions were diminished when he was 8 years (2.5 years post-HSCT), and his brain MRI at 16 years old was normal (Fig. 5.19).

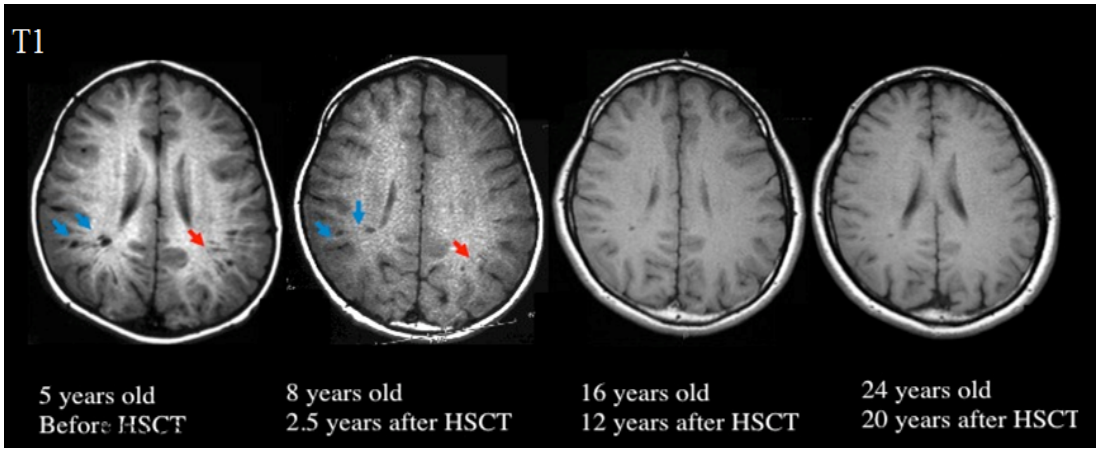


Figure 5.19: MRI Findings of Case 10
Case 10: A 24-year-old patient with HSCT at 6 years (attenuated): MRI before HSCT shows multiple cystic lesions in basal ganglia and white matter. These lesions are diminished when he is 8 years old and 2.5, 12, and 20 years after the HSCT in pentagons and circles. Red arrows represent white matter signal changes; blue arrows represent cystic or cribriform regions.

Three patients with a severe form of MPS II underwent HSCT (cases 11-13; Fig. 5.20-22). These patients had received BM at 1 year and 11 months from a HLA-identical sibling, at 3 years old from a carrier mother, and at 6 years old from one HLA -mismatched brother, respectively. 2.5 years post-HSCT, case 11 had no change in ventricular size, and fine structure of white matter was preserved. At present, the patient can talk and has an IQ of 64 at 5 years and 10 months (Fig. 5.20).

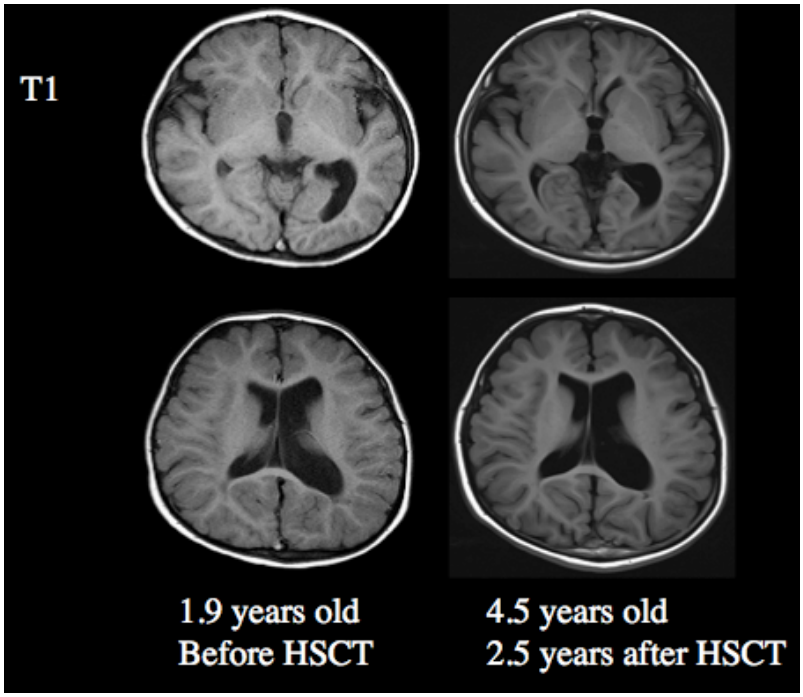


Figure 5.20: MRI Findings of Case 11
Case 11: A 4.5-year-old patient with HSCT at 1 year and 11 months (severe). No significant intensity change is observed before and after HSCT. No progressive brain atrophy, ventricular dilatation, white matter signal changes are present. The cribriform or cystic lesion is not found in basal ganglia and thalamus. Fine structure of white matter is preserved or improved.

Cases 12 and 13 showed little change in MRI images for over 12 and 8 years, respectively (Fig. 5.21 & 5.22).

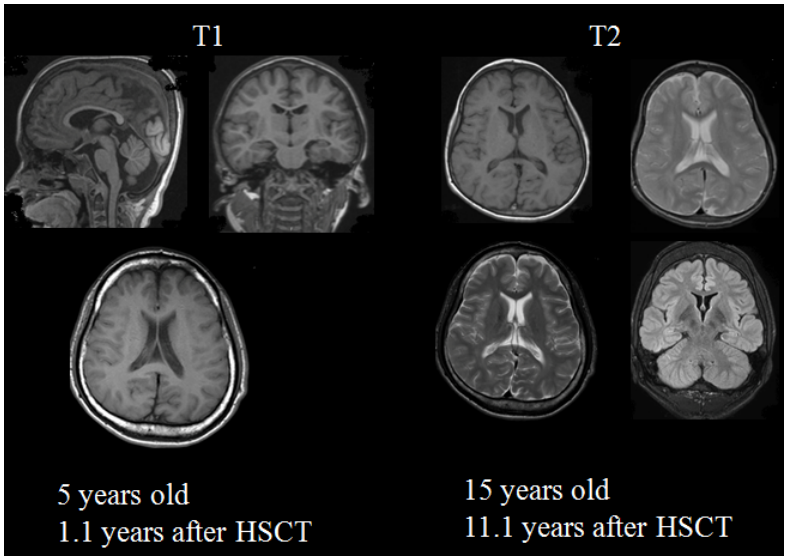


Figure 5.21: MRI Findings of Case 12
Case 12: A 15-year-old patient with HSCT at 4 years old (severe): MRI before and after HSCT did not show any abnormal finding without change.

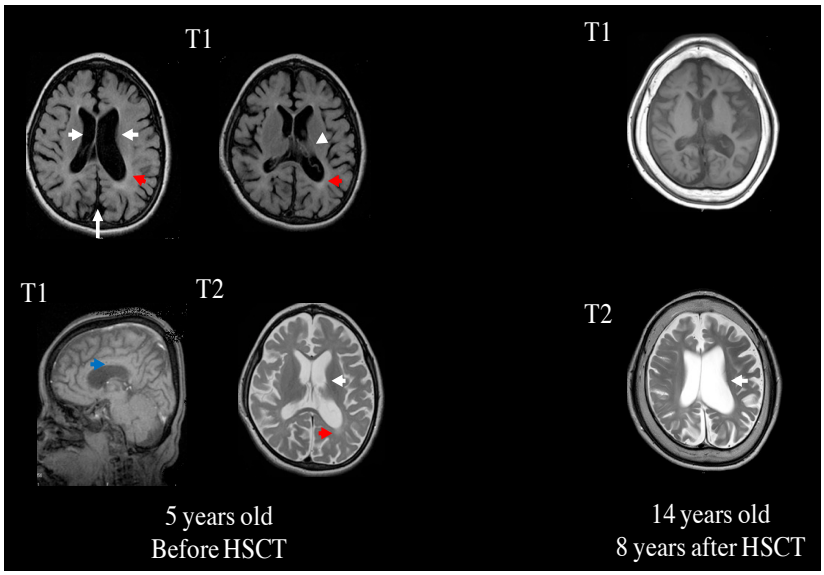


Figure 5.22: MRI Findings of Case 13
No significant intensity change is observed before and after HSCT. Brain atrophy and ventricular dilatation are slightly changed before and after HSCT. White matter signal changes are improved. The cribriform or cystic lesion is also improved. Red arrows

represent white matter signal changes; blue arrows represent cystic or cribriform regions; white arrows represent ventricular enlargement and brain atrophy.

Overall, while MRI showed that brain structures worsen with age in patients treated with ERT, brain deterioration does not progress and may improve in some cases after HSCT.

5.2.6.6 Modulation of High Immune Response against ERT by HSCT

A patient diagnosed as MPS II with a severe form at 2 years old started ERT at 2.3 years. Before ERT, total urinary GAG was 263 mg/g Cre, reduced to 70.9 mg/g Cre 8 months post-ERT. However, a high titer of positive IgG antibody response to IDS (1: 1,310,720) was detected. 24 months post-ERT, urinary GAG increased to 232 mg/h Cre, and the patient had hepatosplenomegaly, indicating that the treatment was no longer effective.

The patient underwent HSCT from an unrelated donor at 4.5 years old (HLA-C, HLA-DRB1 mismatched) with pretreatment using Rituximab. Full donor engraftment was achieved without acute GVHD. Urinary GAG was reduced to 71.5 mg/g Cre 14 months post-HSCT. Hepatosplenomegaly was markedly reduced 6 months post-HSCT (5 years old). At 6.1 years old, urinary GAG, hepatosplenomegaly, and clinical condition (ADL score; 26) all improved. The patient is stable and can walk normally.

5.2.7 Discussion

We have demonstrated that MPS II patients treated with HSCT have better outcomes than patients treated with ERT according to GAG levels, ADL scores, and MRI findings. Although three of 27 new cases of MPS II patients treated with HSCT showed GVHD, no patients died due to the treatment. To date, more than 900 patients with various forms of MPS have been treated with HSCT, showing an increase in acceptance of this therapy [104, 498, 586, 587, 590, 596, 601, 603, 623-626].

HSCT guidelines highly indicate HSCT for patients with MPS I when the procedure is made at an early stage [319, 597, 620, 625, 627]. Positive effects have also been shown for MPS IVA and MPS VI including increase of endurance, joint mobility, growth, lung function, ADL, and survival period [584, 586, 587, 626].

Publications before 2000 demonstrated high mortality rates of HSCT for MPS (27%), but the majority of deaths were caused by progressive stages of the disease, poor donor selection, infection and/or organ failure caused by tissue rejection or GVHD, and/or complications from the conditioning regimens [585, 606]. During early development of HSCT (1974-1998), low survival rates were recognized for most conditions, including leukemia, severe aplastic anemia, and inborn errors of metabolism [628].

The survival of all of the MPS II patients newly reported in this study is partly due to early transplant ages (76% of the new cases were treated when under 6 years old). Survival rates for HSCT of MPS I (85%) [629] and MPS VI (67% to 78%) [102, 626] will likely improve with careful selection of donors and more rigorous patient

care. Wang et al. recently reported 100% survival rates for 12 MPS II patients given HSCT in China [585].

Prasad and Kurtzberg defined successful transplantation as requiring not only longevity but also an improvement in morbidity and quality of life [600]. In our study, most patients with a severe form of MPS II showed improvements in organ function, ADL, and brain MRI. This contrasts with earlier studies of Shapiro et al. who reported that the IQ of 15 out of 16 MPS II cases who received HSCT between 2 and 3 years years remained lower than 50, leading them to conclude that HSCT has no impact on CNS involvement [607]. Furthermore, Guffon et al. showed worsening of DQ/IQ in a group of MPS II patients treated with BMT when older than 3 years old (mean: 5.7 years; 3-16.4 years) [592]. Two patients who had undergone HSCT when older and had pre-existing CNS involvement, developed progressive deterioration in locomotor and performance skills [606].

The rate of transfer of donor cells (microglia) to the brain is disease-specific, so limited effect of HSCT on MPS II could be due to insufficient enzyme uptake and expression in the neurons [320, 630]. However, long-term follow-up of a patient treated with HSCT at 10 months old using a matched sibling donor showed remarkably fewer clinical problems than untreated patients and the patient maintained an IQ score of 78 at 7 years and 10 months. In a more recent study, 4 (33%) of 12 MPS II patients treated with HSCT showed improvement in motor skills, and 2 cases (17%) showed improvement in speech skills[585]. These findings are consistent with

results for patients with MPS I; those with an IQ < 70 respond to HSCT less favorably than those with an IQ > 70 at the time of transplantation (under 2 years old) [631]. Data presented in this study support a positive effect of HSCT on neurological outcomes for MPS II patients, showing reduced degeneration by MRI and more favorable outcomes in cognitive functions. Changes in MRI categories I and II are direct consequences of the accumulation of GAGs in the CNS. Changes in category I were identified previously in patients with MPS types I, II, and VI [622].

They were observed in patients with both normal and abnormal intellectual abilities, suggesting that they may not directly affect the neurons but occupy the perivascular spaces through the accumulation of substances. Category II changes (white matter signal changes) are speculated to be due to demyelination, gliosis, or accumulation of foamy cells. Category III and IV (ventricle enlargement and brain atrophy) may correlate with the cognitive function. ERT patients with MPS II showed progressive changes in brain MRI with age. By contrast, HSCT patients showed either no progression or improvement of these lesions.

HSCT also improved brain MRI findings and GAG levels in patients with an attenuated phenotype. Patients with an attenuated form do not normally develop neurological symptoms and retinal degeneration until later in life, so we did not see impairment of ADL for the attenuated patients in our cohort. However, the MRI improvements indicate that HSCT may prevent or delay the onset of neurological symptoms in these patients and consequently HSCT may be a valuable treatment for patients with an attenuated phenotype as well.

Thus HSCT may be as effective for MPS II as it is for MPS I if patients are treated before the onset of neurological symptoms [320, 592]. More data in patients under 2 years old with MPS II should clarify therapeutic efficacy in cognitive function compared with patients with MPS I.

Another advantage of HSCT over ERT relates to the production of a high-titer antibody against infused enzyme. A high titer of antibody production correlates with the poor efficacy of ERT. We have demonstrated that one patient with a high-titer antibody had worsening of clinical condition with hepatosplenomegaly and increased in GAG levels, but these issues were overcome by subsequent treatment with HSCT. Similar observations have been seen in the treatment of patients with MPS I [632]. Once the immune system is replaced in transplanted patients with MPS I, the donor`s cells do not recognize the recombinant enzyme from ERT as exogenous and consequently do not form antibodies, leading to improvement of therapeutic efficacy [632].

Several factors can improve the impact of HSCT as follows: timing of transplantation (age, clinical severity, and stage), primary pathology, graft source (HLA matching, donor source), preparative regimen, and well-trained institutes [596]. The international HSCT guidelines recommend that to achieve high overall survival and event-free survival with low toxicity, a non-carrier matched sibling donor, matched unrelated UCB, and matched unrelated donor are preferred donors. Complying with these guidelines in HSCT for MPS II patients should result in improved safety and efficacy, allowing extension of HSCT to more attenuated MPS

types [601]. Since a younger age at HSCT correlates with reduction of HSCT-related toxicity and increase of therapeutic efficacy, newborn screening for MPS II will improve safety and treatment outcomes.

5.2.8 Conclusion

In conclusion, the compiled data of 146 patients indicates that HSCT may be a therapeutic option for MPS II, potentially for a broad range of clinical phenotypes, and is probably even more effective than conventional ERT. Selection of the most suitable MPS II patients would probably lead to even better HSCT outcomes than reported here.

5.3 Pentosan Polysulfate in MPS

Another alternative treatment for MPS is based on the use of anti-inflammatory agents. Inflammation has been implicated as a major cascade that contributes to disease progression, with increased apoptosis and up-regulation of cytokines [182, 633] (Fig. 1.1).

Schuchman et al. (2013) demonstrated positive effects of the anti-inflammatory compound, pentosan polysulfate (PPS) in the treatment of MPS VI rats. PPS treatment reduced levels of GAGs and also showed chondrogenic properties [143]. These findings led to pre-clinical trials in a MPS I dog model.

5.3.1 PPS in MPS I Dogs[11]

A pre-clinical trial has been performed by treating MPS I dogs with PPS, both orally and by subcutaneous injection, by our collaborators at Mount Sinai. The study was designed to evaluate the safety of chronic use and effectiveness of PPS in a larger animal model. The dog model had a homozygous null mutation in intron 1 of *IDUA* [634, 635]

5.3.1.1 Animals and Treatment

Three weeks old MPS I dogs were divided into two treatment groups: oral (daily treated for 17 months) and subcutaneous (SubQ) (bi-weekly for 12 months). Powdered PPS was dissolved in sterile saline (10mg/mL), and animals were treated at a dose of 1.6mg/kg (human equivalent dose). Treated animals were compared with untreated littermates, and all groups had 5 animals each [146].

At the end of treatment blood, urine, cerebrospinal fluid (CSF) and tissues were collected to assess treatment efficacy. GAG analysis in urine and CSF were performed as described in section 2.1 with a run time of 4.5 minutes per sample [146].

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[11] Published in PLoS One

[146] C.M. Simonaro, S. Tomatsu, T. Sikora, F. Kubaski, M. Frohbergh, J.M. Guevara, R.Y. Wang, M. Vera, J.L. Kang, L.J. Smith, E.H. Schuchman, M.E. Haskins, Pentosan Polysulfate: Oral Versus Subcutaneous Injection in Mucopolysaccharidosis Type I Dogs, PLoS ONE 11 (2016) e0153136.

5.3.1.2 Statistical Analysis

Student`s t-test was used to compared values between treated groups and untreated groups.

5.3.2 Results

5.3.2.1 Urinary GAGs

Mass spectrometry analyses of urine samples were performed to confirm GAG reduction in PPS-treated dogs (Fig. 5.23).

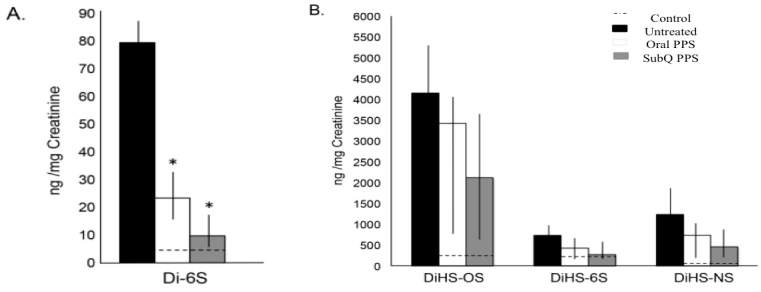

Figure 5.23: Urinary GAGs in Age-matched PPS-treated MPS I Dogs
Figure 5.23 A: DS Levels
Di-6S was significantly reduced with both modes of PPS administration in comparison to untreated MPS I dogs ($p<0.05$). SubQ administration had a higher reduction in comparison to the untreated group (*$p=0.0141$).
*$p<0.05$; Adapted from: Simonaro et al., 2016 with permission from Creative Commons Attribution License.

Figure 5.23 B: HS Levels
HS disaccharides (DiHS-0S, DiHS-6S, DiHS-NS) also were reduced with PPS treatment when compared to untreated animals, although the reductions were not significant. Adapted from: Simonaro et al., 2016 with permission from Creative Commons Attribution License.

Heparan sulfate disaccharides (DiHS-0S, DiHS-6S and DiHS-NS) and the dermatan sulfate disaccharide (Di-6S) were each reduced in the PPS-treated animals. The greatest response was seen with Di-6S, a primary GAG fragment that also accumulates in MPS VI [146].

5.3.2.2 CSF GAGs

I have also assessed GAGs in CSF from: control (n=1), untreated (n=8), oral (n=4) and subQ (n=4) treated MPS I dogs to determine whether PPS can reduce GAG levels in CNS (Fig. 5.24, 5.25 A & B). Mass spectrometry analyses of GAGs in CSF were performed as described in section 2.1.

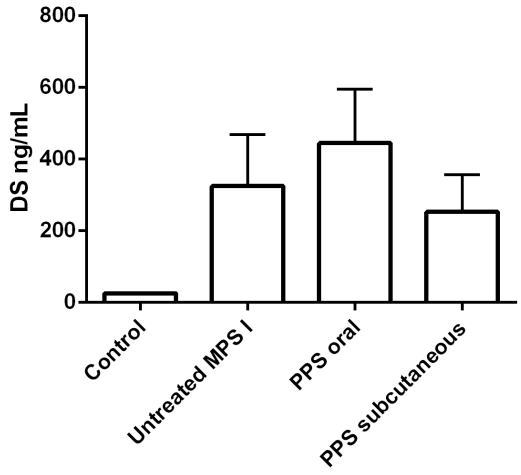

Figure 5.24: CSF DS Levels in Treated *vs.* Untreated MPS I Dogs
DS levels (Di-4S) were analyzed in CSF of MPS I PPS-treated (oral and subcutaneous) and untreated MPS I dogs. Overall, PPS administrations (oral or SubQ) did not significantly reduce levels of DS in MPS I treated dogs in comparison with untreated.

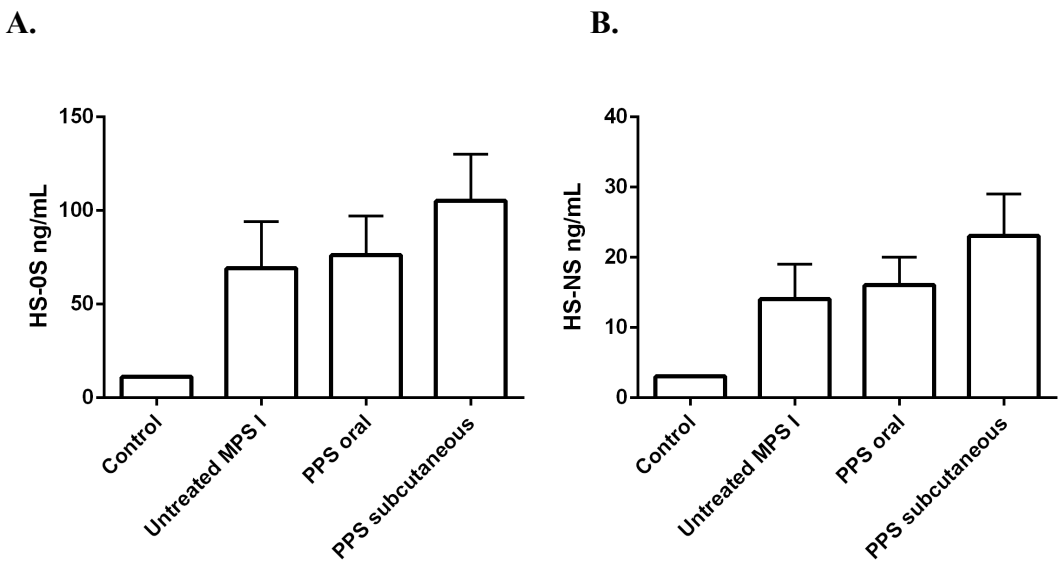

Figure 5.25: CSF HS Levels in CSF of Treated *vs.* Untreated MPS I Dogs
Figure 5.25 A: HS-0S Levels
HS-0S levels were analyzed in CSF of MPS I treated and untreated dogs. PPS administrations (oral or SubQ) did not reduce levels of DS in MPS I treated dogs, and PPS subcutaneous group had even higher levels in comparison with untreated dogs.
Figure 5.25 B: HS-NS Levels
HS-NS levels were analyzed in CSF of MPS I treated and untreated dogs. PPS administrations (oral or SubQ) did not reduce levels of DS in MPS I treated dogs, and PPS subcutaneous group had even higher levels in comparison with untreated dogs.

Overall, PPS administrations (oral or subcutaneous) did not reduce levels of DS and HS. Dogs treated with subcutaneous injections had higher levels of HS in comparison with untreated dogs (Fig. 5.25 A & B). A larger sample set is needed to determine whether PPS significantly affects GAG levels in CSF.

5.3.3 Discussion

As previously reported with MPS VI treated rats, MPS I dogs also have reduced GAG storage after PPS treatment [146, 636]. Tandem mass spectrometry analysis of GAG disaccharides confirmed lower levels in PPS-treated dogs compared to untreated. DS and HS were reduced by PPS treatment. However DS had a greater reduction ($p=0.0141$) in urinary GAGs [146].

We were not able to show any significant effect of PPS on GAG levels in CSF of treated (oral and subQ) compared to untreated dogs. Although small sample groups limit this study, our results indicate that PPS does not improve neurological accumulation of GAGs, probably because the highly charged polymer does not cross the blood-brain barrier.

5.3.4 Conclusions

We conclude that PPS should be investigated as a potential treatment for MPSs. Studies conducted in two animal models (MPS VI rats and MPS I dogs) have shown that PPS is a safe treatment for MPS with an effective decrease in GAG levels likely due to modulation of inflammatory cytokines [146, 636]. Other anti-inflammatory agents that cross the BBB may be needed to be more effective in treating aspects of MPS.

5. 4 Overall Conclusions for Treatment Assessments

Treatment approaches are crucial for patients with MPS, and the timing of treatment can highly affect outcomes. Although GAG decrease and somatic improvements are expected with treatment management, constant monitoring is required.

Due to the constant GAG accumulation seen in MPSs, a methodology that enables GAG assessments is a very useful tool for evaluation of treatment efficacy. We were able to demonstrate that ERT, HSCT, ERT+HCT and PPS are successful therapies in decreasing GAG levels. We used our GAG detection protocol to compare therapy efficacy. Although pre and post-treatment samples were not available from individual patients at the time of this study, we were able to conclude that for the majority of MPS types analyzed HSCT or HSCT+ERT more effectively lowered GAG levels than ERT alone.

In conclusion, GAG analyses by tandem mass spectrometry can be used to determine treatment efficacy in MPSs. More studies with pre and post-treatment samples are required to clarify exact treatment efficacy.

Chapter 6

DISCUSSION AND CONCLUSIONS

6.1 Non-invasive Assessments for MPS

Natural history studies are required to elucidate disease progression from onset to resolution without intervention. Although it is important to understand how diseases progress, it is even more essential to have treatment approaches that will be able to not only improve but also change the evolution of a disease. In this context, tests that can assess disease evolution from infancy through adulthood are valuable.

Mucopolysaccharidoses were first described in the early 20th century by Dr. Charles Hunter and Dr. Gertrude Hurler [55, 637]. Despite the progress that has been made over the past century, no cure is currently available for MPSs [522]. Furthermore, the development of new therapies strongly relies on assessment methodologies that can measure disease progression with or without treatment, highlighting the need for new disease measurements and biomarkers.

In the current study, I have analyzed the use of bone mineral density (BMD) measurements and non-invasive pulmonary function tests (PFT) in order to establish if they are useful in disease evaluation. Both methodologies are non-invasive, pose minimal risk to the patients and can be tracked long-term throughout life.

In order to assess disease progression and evolution, clinicians must determine which tests can be performed in specific disease populations of patients, and which techniques are most appropriate based on risk factors and complications associated with such pathologies. MPS I, II, IV, VI and VII are all associated with bone impairment to some extent. Thus they are associated with risk for bone and growth complications, indicating that BMD evaluations may provide clinically relevant data on disease progression.

From BMD evaluations by DXA of MPS IV (A & B) patients, we concluded that BMD is a valuable tool for non-invasive assessment of bone when measured at the lateral distal femur (LDF). The standard body sites that are typically recommended for BMD evaluation are technically limited due to several aspects: 1) lumbar spine is limited by abnormal vertebral shape, 2) the whole body is a challenging body site due to presence of metallic hardware and it is not well tolerated due to positioning difficulties (patients are not able to breathe when laying down on a flat surface), and 3) hip DMB examination is only recommended for adults and due to disease progression most patients already had hip replacement by adulthood. This study was the first to evaluate the use of LDF in MPS IV patients, and we showed that the LDF is a feasible and reliable body site for this patient population.

We were also able to identify that ambulation plays a key role in preserving BMD in MPS IV. This fact is extremely important for patient management as most of the patients choose to use a wheelchair for convenience rather than pain. It is important for patients to know that if they can ambulate, then they can preserve their

BMD, which can have a strong positive impact on their future. Furthermore, in the present study, we also showed that MPS IV patients have low BMD at all body sites analyzed, as expected, but no fractures were reported despite the low BMD. Longitudinal studies need to be conducted to determine any association of Z-scores with fractures in MPS. Other types of MPS have similar orthopedic issues and consequently, examination of BMD in the LDF may be of value in monitoring disease progression for MPS in general.

MPS patients are also susceptible to the development of respiratory complications due to a variety of factors, including constant GAG accumulation in the respiratory system, inflammation, tracheal abnormalities, and deformities of the chest wall. Pulmonary function evaluations are essential to avoid life-threating complications as well as to provide an accurate assessment of progression of lung dysfunction aiding effective treatment management (e.g. pharmacological intervention, mechanical ventilatory support and or surgery).

Airway management is crucial in most MPS subtypes, as obstructive, restrictive and or tracheobronchial manifestations can occur, leading to death [480]. Treatment management of MPS patients includes several surgical interventions requiring anesthesia, which can be associated with high mortality risk [638, 639].

Spirometry is most commonly used to assess lung disease in cooperative patients, but it cannot be performed in young children, wheelchair-bound patients, non-cooperative patients or in patients after surgical interventions [640]. Another limitation of spirometry is that spirometry data must be compared against normative

data (reference values or predicted values) that are obtained from population surveys that do not include abnormal height or skeletal morphology seen in MPS patients. Also, due to the low incidence of MPS no normative data are available for proper interpretation. As no reference values have been established for MPS patients, it is useful to have non-invasive methods that can be used in patients throughout their lifespan to evaluate their own progression.

We have performed non-invasive PFTs and conventional spirometry (when possible) in MPS IV patients to determine which are valuable for measuring lung function. Using PFT, we are the first to show that, although MPS IV patients are short and have chest wall abnormalities making their lungs small, their lung function is normal when compared to similar sized lungs of unaffected people. It appears that the skeletal dysplasia seen in MPS IVA has limited impact on the deterioration of their lung capacities. It is also important to note that the non-invasive tests are not only helpful in determining the pulmonary status of MPS patients, but also allow for clinical evaluation of patients who are not able to perform spirometry, the current standard test for lung function. Unlike spirometry, these non-invasive PFTs can be used to follow patients throughout their whole life.

In conclusion, MPS IV patients have small, but functioning lungs; the small lungs are likely the result of skeletal dysplasia that limits the size of the chest cavity. Our study shows that restrictive and obstructive lung disease is less common in MPS IVA patients than previously reported.

6.2 Glycosaminoglycan Analyses by Mass Spectrometry

6.2.1 Untreated Patients

GAG analysis is particularly valuable for studying clinical progression in mucopolysaccharidoses (MPS). GAG degradation pathways are disrupted due to an enzyme deficiency that causes undegraded GAGs to accumulate in multiple tissues leading to organ dysfunction represented by a variety of clinical signs and symptoms such as skeletal dysplasia, short stature, mental retardation, heart valve disease, hearing loss, corneal clouding, hepatosplenomegaly, umbilical and inguinal hernias. Untreated patients with severe forms of MPS die of respiratory failure, heart disease, and brain damage within the first two decades of life [56, 641]. The establishment of GAG measurements facilitates diagnosis, prognosis, therapeutic monitoring (biomarkers), and disease screening [205, 642].

Therefore, the establishment of accurate, rapid, sensitive, and specific measurements of specific GAGs is needed urgently. MS/MS based GAG assays are applied to not only diagnosis and therapeutic efficacy for MPS, but also other disorders such as: mucolipidoses [535, 558], cancer [643, 644], osteoarthritis [645], rheumatoid arthritis [646], diabetes [647, 648], infectious diseases [649], and spinal cord injury [650] where GAG(s) are down or up-regulated [205].

In this study, I have shown MPS patients can be discriminated from control subjects by measurement of disaccharides derived from CS, DS, HS and KS in different sample types (AF and blood-DBS) and at all age ranges (prenatal to adults). I

also showed that this GAG assay method can measure therapeutic efficacy in patients treated by ERT, HSCT, ERT+HSCT and PPS.

I made the first report to show the elevation of GAGs in the amniotic fluid of a 21-week fetus with MPS VII using LC/MS/MS. Prenatal diagnosis is crucial for early treatment interventions. Though the fetus analyzed here spontaneously died at 25 weeks of pregnancy due to a very severe disease phenotype, it is expected that prenatal diagnosis by GAG assay could aid early intervention (pre-symptomatic), allowing better outcomes for patients.

One of the largest pitfalls of symptomatic diagnosis of MPS patients is that several patients are not diagnosed before two years of age; by which time several irreversible disease manifestations have already arisen. Newborn screening programs would allow diagnosis at birth, before irreversible disease manifestations are established, enabling early treatment for maximum therapeutic benefit.

The main aspects of NBS programs are analytical specificity (the ability of a test to correctly classify as sick or healthy) and sensitivity (probability of testing the positive in presence of the disease). The majority of primary screening (first-tier) methods aim to identify as many abnormal newborns as possible, which favors sensitivity over specificity, and elevates the number of false positives (non-affected individuals who tested positive). Second tier tests are designed to eliminate false positives [378].

In my dissertation, I focused on a first-tier assay to measure GAG levels in DBS; a second-tier test measuring enzyme activity would be needed to improve

selectivity (Fig. 1.7). I hypothesized that GAG analysis would be valuable for both diagnosis and therapeutic efficacy evaluation. In the pilot study conducted with 2,862 and 14 newborn MPS samples, cutoffs were set to achieve 100% sensitivity and resulted in up to 94% specificity for DS and HS. The positive rates for the general newborn screening samples were 2.5% for HS-0S, 6.2% for HS-NS and 1.5% for DS. A combination strategy was developed based on the knowledge that affected MPS patients usually have an elevation of several GAGs. The combination of cutoffs for DS and HS (0S & NS) identified all MPS I and II patients and reduced positive rates in the screened samples to 0.03% (MPS I and II). Cutoffs for HS (0S & NS) identified all MPS patients in this study (I, II and III) and 0.9% of the screened samples [371].

Using cutoffs established in the pilot study, I have analyzed over 16,000 screen samples. The positive rates are 2.8% for HS-0S, 3.5% for HS-NS and 1.2% for DS. The combination of all three GAGs gives a positive rate of 0.04% to identify MPS I and II and 1.2% to identify all three forms of MPS. Elevated samples will be analyzed by the enzyme assay (second-tier) to exclude or confirm an MPS diagnosis.

Second-tiers NBS programs are usually designed to increase the specificity of positive predicted values (PPV), which represents the number of true positive results and consequently decrease the number of false positives. The inclusion of second-tier to the screen avoids unnecessary sample re-collection, thereby avoiding adding stress to a parent who might be concerned that there is a problem before a true diagnosis is confirmed [651, 652].

A review of national data on NBS by the Council of Regional Networks for Genetic Services from 1990 to 1994 revealed that more than 50 false-positive results are obtained for each true-positive in the United States emphasizing the challenge of FP rates [653].

The FP rate for MPS I and II (0.04%) and MPS I, II and III (1.2%) using GAG assays in our study compares favorably with FP rates for other single NBS programs for LSD such as: MPS I (0.05%) [376]; Pompe (0.009%), Gaucher (0.020%), Fabry (0.025%), MPS I (0.037%) [369]; Krabbe disease (0.02%) [654]; and congenital adrenal hyperplasia (0.065%) [655].

Another crucial rate is the number of false negatives (true positive samples that tested negative). We designed our pilot study to have no false negatives, although we cannot rule out the possibility that some MPS patients will be missed. However, as the test is a measure of disease severity, any missed MPS patients are likely to be of an attenuated phenotype that may not need early intervention.

Overall, this first-tier newborn screen has acceptable false positive rates with no false negatives to date. These GAG assays will also be valuable for measuring GAGs throughout a patient`s life, allowing long-term follow-up of disease progression and treatment efficacy.

6.2.2 Treatment of Patients

Research on natural history and disease pathogenesis has lead to advancements in supportive care and the development of disease-specific treatments for MPSs.

Currently, treatment management techniques can be divided into two main categories: supportive treatments (symptom-based mostly composed of surgical interventions and palliative care) and disease-specific treatments (ERT, HSCT, SRT, PCT and gene therapy). Clinically, ERT and HSCT are the standard of care, while other treatment options are under investigation [656].

Both current treatment options require a multidisciplinary team and regular follow-up to identify positive and or negative outcomes. Biomarkers help clinicians monitor disease progression, prognosis and treatment outcomes [211]. Due to continuous storage, GAGs are considered primary biomarkers for MPS.

We demonstrated the efficacy of ERT and HSCT by showing GAG reduction in treated MPS I, II, IV, VI and VII patients in comparison with untreated patients. Our GAG detection method by tandem mass spectrometry has potential use for treatment monitoring in MPSs in different sample types (CSF, DBS, and urine). A limitation of this study was the lack of pre and post-treatment samples from the same patient ant the small sample sizes, but a comparison of treated patients with age-matched controls and age-matched untreated MPS patients clearly showed efficacy [557].

HSCT is a standard care therapy for MPS I in which guidelines suggest its use in patients younger than two years of age with an IQ > 70. Patients with a significantly impaired IQ (<70) are much less likely to benefit from HSCT. There is much controversy over the use of HSCT for MPS II patients due to reported poor improvements in neurocognitive impairment and high mortality rates in studies

conducted before 2000. However, a few studies published in the last few years have indicated that HSCT may be beneficial for MPS II based on: detection of donor-derived cells in brain parenchyma [320], improvement in brain or heart involvement if HSCT is performed before brain atrophy or valvular heart valvular regurgitation [477], higher ADL scores in HSCT treated patients compared to ERT treatment only [609] and improvement of motor skills and speech skills [585]. My study has shown that HSCT in MPS II reduces GAG levels and treated patients showed clinical improvements in ADL and brain MRI. Furthermore, GAG levels were lower ($p<0.005$) and ADL scores were higher ($p<0.0001$) in HSCT treated patients than ERT treated patients.

We conclude that if HSCT is performed at an early stage, MPS II patients will have better outcomes than if only treated with ERT. HSCT is often used for MPS II in Japan, and our examination of current clinical outcomes indicate that this should now be considered as a viable treatment option for all MPS II patients and treatment decisions should be similar to those developed for HSCT for MPS I patients.

Inflammation exacerbates deleterious aspects of MPS, and consequently anti-inflammatory therapy is proposed to improve outcomes for patients. My contribution to testing of this type of therapy showed that PPS reduced levels of GAGs in the urine of treated MPS I dogs. However, levels of GAGs in CSF were not reduced. The mechanisms by which PPS decrease GAGs are not completely understood at present time but it has been shown to reduce pain and improve bone and cartilage impairment. It seems that PPS cannot cross the blood-brain barrier. Thus it will not be effective in

decreasing high degrees of inflammation in the CNS due to GAG storage. Administrations of PPS orally or subcutaneously were not effective in decreasing GAG levels in the cerebrospinal fluid of MPS I dogs, suggesting that PPS might not be able to correct or decrease inflammation in CNS. Other membrane permeant anti-inflammatory agents may have better efficacy for neurological effects of MPS.

The GAG detection protocol developed in this study can also be used to help monitor efficacy of new therapies that are still needed due to limitations of the current therapies (high cost; impermeable to BBB; immune-responses as antibody formation or GVHD; no reversal of disease pathology if treatment is performed at later disease stages).

We specifically demonstrated the potential of this GAG quantification protocol to identify biomarkers for MPS such as chondroitin-6- sulfate (C6S) and di-sulfated KS (di-KS) [254, 542]. C6S was demonstrated to be a novel biomarker for MPS IVA and VII in which blood levels were higher than in age-matched controls, while di-sulfated KS was elevated in MPS II, IVA and IVB [254, 542].

Our current GAG detection method includes a panel of five screening GAGs (DS, HS-NS, HS-0S, di-KS, mono-KS) that appear useful for diagnosis, prognosis and treatment follow-up. Early diagnosis obtained by prenatal or newborn screenings will provide profound impact in disease outcomes as well as drastically change natural history.

6.3 Overall Conclusions and Impact of the Work

MPSs are devastating disorders with progressive, multisystemic impairment in which a spectrum of disease manifestations and clinical courses classically categorize patients into two main disease forms (attenuated and severe). As previously described, the attenuated form has a milder progression with later disease onset, while the severe form has rapid progression (first few years of life) and can even lead to death in the first or second decade of life. The severe form is also accompanied by life-threatening, irreparable disease manifestations such as neurological impairment. Furthermore, due to the rapid evolution and the irreversible character, early detection and treatment is required to improve survival and quality of life for patients.

Classically, in the absence of family history of MPS, patients with MPS would receive a clinical diagnosis after disease manifestations had arisen that would then be confirmed by loss of enzyme activity. By this time irreversible disease manifestations are usually prominent and treatment cannot effectively reverse the damage.

My work has led to the development and validation of a GAG assay that can be used for diagnosis of both symptomatic and asymptomatic patients. This methodology is not only successful in identifying patients at any age, including newborns and pre-term, but it is also a valuable tool for monitoring disease progression (natural history) and treatment efficacy.

Newborn screening for rare disorders such as MPSs is considered to be a laborious search for a “needle in a haystack”. However, recent studies have

demonstrated that MPSs are more prevalent than previously expected (1:25,000 live births), likely due to misdiagnosis. As mentioned in section 1.4, approximately 160 new MPS patients are expected to be born annually in the United States. The methodology developed here to diagnose MPSs before irreversible disease manifestations appear has the potential to drastically change the quality of life, prognosis and life expectancy of those patients if they receive early treatment. In addition to improving outcomes for patients, early diagnosis and treatment will also improve the life of caregiver`s and reduce public health costs.

If we can validate this methodology for all forms of MPS, it has the potential to be included in National newborn screening laboratories as per recommendations of the American College of Medical Genetics to include MPSs in NBS. This GAG assay could also be used to monitor other conditions that elevate GAGs.

Chapter 7

FUTURE DIRECTIONS

Based on literature and previous findings, I developed a hypothesis that diseases progression of MPSs could be measured using non-invasive clinical tests and analysis of GAGs using liquid chromatography tandem mass spectrometry (LC/MS/MS). In addition, I proposed that these techniques could be used to understand the natural of MPSs, measure the efficacy of different treatment options and, for LC/MS/MS, identify MPS patients in newborn screening. Notably, my studies showed that unlike current physiological tests used to monitor disease progression and treatment efficacy, BMD of the lateral distal femur (LDF) and non-invasive PFTs could be performed on nearly all MPS IV patients regardless of physical ability.

In future, these techniques can be used in longitudinal studies to monitor disease progression as patients age and to determine longer term effect of treatment (e.g. ERT, HSCT, ERT+HSCT and/or PPS). Although our focus has been on MPS IV, other forms of MPS may also benefit from disease evaluation using these non-invasive techniques, particularly, MPS VI in which patients have similar bone and cartilage involvement as that seen in MPS IV. The non-invasive techniques may also be

valuable in the study of other patient populations, including achondroplasia and Duchenne muscular dystrophy.

My studies have demonstrated that a GAG detection method by mass spectrometry should be a valuable approach to screen for MPS patients in dried blood spots obtained at birth. I have clearly demonstrated its value for MPS I, II and III patients and it is likely that it can also be applied to identify most MPS types. Future work is needed with newborn DBS from MPS IV, VI and VII patients to establish cutoff values for diagnosis of these patients.

To distinguish true positive from false positives, enzyme assays (second-tier) need to be performed. Several groups have established the measurement of enzyme activities by the use of deuterated chemically identical internal standards. Enzyme measurements by tandem mass spectrometry enable high sensitivity and specificity, and the final quantification of enzyme level will enable definitive MPS diagnosis. We have already received substrates and internal standards for enzyme assay for some MPS, and we plan to standardize the method in our current routine. Some centers realize molecular analysis post-enzyme assay measurement to elucidate the molecular basis of the disease, help with prognosis, and aid with family counseling. After GAG and enzyme assays are performed, we also would like to perform genotype analysis in our population of patients.

In addition, further research is required to determine whether GAGs can be elevated in some false positives due to other medical complications at birth. After confirmation by enzyme assay that newborns with elevated GAG levels have a normal

activity for lysosomal enzymes, clinical follow-up will aid to establish what other pathological conditions are responsible for the elevation of GAGs in the newborn period.

A limitation of the current procedure is the long running time per sample (5 minutes). To overcome this limitation, we have previously tested the feasibility of a high-throughput system (Rapid-fire) [253], in which the chromatographic step is eliminated, thereby reducing the run time to 10 seconds per sample. We estimate that with our current method with LC/MS/MS approximately 59,136 samples can be analyzed in a year, while the high-throughput (HT) system could analyze around 525,600 samples. We have recently acquired a high-throughput system at our facility at AIDHC and plan to run samples with this new system. In our previous study, only HS was analyzed and now we would like to establish our entire panel of GAGs (DS, HS, and KS) at the high-throughput system; samples above the cutoffs at the HT/MS/MS will be further tested by LC/MS/MS and confirmed by enzyme assay (second-tier screen).

Another limitation of our current newborn screen is the lack of cutoff values for KS. To determine those cutoffs, MPS IV newborn samples are required. However, at the present moment such samples are not available because by the time most of MPS IV patients are diagnosed they are already 2 to 3 years of age. We expect that running a larger number of samples with the HT/MS/MS system will allow us to identify more MPS patients and consequently detect MPS IV newborns.

We have also measured GAGs in post-newborn untreated and treated samples to monitor therapeutic efficacy. However, pre and post-treatment samples from the same patients were not available at the time of this study, limiting further conclusions about treatment effect. Longitudinal studies with more samples would be ideal to monitor treatment. The small number of treated patients also limited us; larger cohorts should be analyzed to elucidate effects of different therapies and to help the development of new therapies.

Lastly, current treatment approaches are limited by several factors. ERT is only available for MPS I, II, IVA and VI and majority of severe complications (bone, CNS, heart and respiratory impairment) are not improved by ERT. This treatment approach is also very expensive (€150,000 to 450,000 per year per patient) [657] impacting national health costs and also requires constant infusions affecting patients and caregivers routines.

HSCT is the standard care for severe MPS I types, and studies have suggested its potential use for MPS II, VI and VII. Although, it is still considered a higher risk procedure, it requires the availability of donors (BM, UCB, or PBSC) and its effects are highly depend on age and disease status at the time of the procedure.

Research on emerging treatments such as gene therapy holds a significant promise for a cure or better outcomes for MPS patients if such therapy is proven to be safe and effective as it was shown in animal models. Our GAG assay protocol has great potential to analyze the effect of such therapies in a variety of samples and

tissues allowing direct correlation of disease biomarkers that can be associated with clinical findings.

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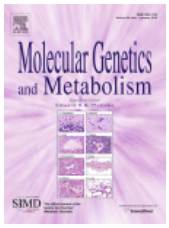

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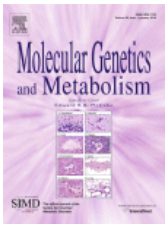

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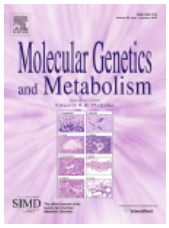

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Nemours Office of Human Subjects Protection
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MEMORANDUM

DATE:	September 24, 2013
TO:	Robert Mason, PhD
FROM:	Nemours IRB 1
STUDY TITLE:	[281498-4] Newborn screening and biomarkers for mucopolysaccharidoses - Aim 2: Control vs mucopolysaccharidosis patients
IRB #:	281498
SUBMISSION TYPE:	Continuing Review/Progress Report
ACTION:	APPROVED
APPROVAL DATE:	September 20, 2013
EXPIRATION DATE:	December 20, 2013

Thank you for your submission of Continuing Review/Progress Report materials for this research study. Your submission received Full Committee Review based on the applicable federal regulation and meets all DHHS [and FDA] criteria for approval. The above-referenced research study is approved.

The IRB has determined that:

- The team is working on remediating the areas of noncompliance related to processes, samples and clinical data and should be allowed to pursue their goal. They are developing an amendment to redesign selected study elements.
- The study can continue for 3 months with a follow-up continuing review as to the study progress.
- The PI must provide clarification of the status of all of the samples obtained during the study, prior to the suspension. (How many samples have been tested, how many banked and an account of the status of de-identification).
- In addition to the minutes of the weekly meetings, the PI must provide a progress report which specifically addresses implementation of core components of the remedial plan.
- The investigator must provide a copy of all Institutional BioSafety Committee approval/s for Dr. Mason's/Tomatsu's studies related to this grant.
- Nemour's Compliance will be asked to provide a report/s regarding their 3 and 6 months' reviews of the implementation of the remedial plan, detailing areas that remain incomplete or noncompliant.

Reviewed/approved documents in this submission:

• Continuing Review/Progress Report - Continuing review form dated 29 August 2013 (due 06 SEP 2013) (UPDATED: 08/29/2013)

• Cover Sheet - 281498 Submission cover memo dated 29 August 2013 (UPDATED: 08/29/2013)

• Letter - Documentation from IBC that Dr. Tomatsu's previous IBC approval is unaffected by changing human subjects PI to Dr. Mason (email dated 02 July 2013) (UPDATED: 08/29/2013)

• Letter - IBC initial approval memo (approval period 07 March 2012 through 07 March 2015)

(UPDATED: 08/29/2013)

• Other - MPS study group meeting minutes 02 July 2013 through 09 August 2013 (UPDATED: 08/29/2013)

• Other - MTA with Shimane University, executed on 12 January 2012 (UPDATED: 08/29/2013)

Investigator Agreement: As the PI, you have agreed to assure that this research is conducted incompliance with Nemours policy and all applicable federal regulations and ICH standards, including, but not inclusive of:

• All research must be conducted in accordance with this approved submission. Any revision to approved materials must be approved by the IRB prior to initiation.

• Remember that informed consent/parental permission is a process beginning with a description of the study and insurance of participant understanding followed by a signed consent form. Informed consent must continue throughout the study via a dialogue between the researcher and research participant. Federal regulations require each participant receive a copy of the signed consent document.

• All serious and unexpected adverse events and unanticipated problems affecting participants must be reported promptly to the IRB according to NOHSP policy.

• All non-compliance issues or complaints regarding this study must be reported to the Director, NOHSP.

• All research records must be retained for a minimum of three years.

• A Closure Report must be submitted to the IRB when this protocol is completed.

If you have any questions, please contact Camille Varacchi at Nemours AI duPont Hospital for Children 1600 Rockland Road, ARB-Room 160A, Wilmington, Delaware 19803, 302-651-6807 or

cvaracch@nemours.org.

Please include your study title and reference number in all correspondence with this office.

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MEMORANDUM

DATE:	December 24, 2013
TO:	Robert Mason, PhD
FROM:	Nemours IRB 1
STUDY TITLE:	[281498-7] Newborn screening and biomarkers for mucopolysaccharidoses - Aim 2: Control vs mucopolysaccharidosis patients
IRB #:	281498
SUBMISSION TYPE:	Continuing Review/Progress Report
ACTION:	APPROVED
APPROVAL DATE:	December 20, 2013
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Reviewed/approved documents in this submission:

- Continuing Review/Progress Report - Aim 2 Continuing Review form due 06DEC2013 for 20DEC2013 meeting (UPDATED: 12/3/2013)

The study team continues to meet weekly to discuss this study (Aim 2). As indicated in the recent amendment to this Aim's protocol, specimens from international bio-banks may be received and analyzed per fully executed MTAs. This activity has resumed. Additionally, collaborators at Gifu have resumed shipping dried blood spots (DBS) and questionnaire data from individuals with MPS. To date, specimens from 2 MPS patients have been received and questionnaire data from 111 patients have been received. A shipment of approximately 100 DBS from MPS patients seen at Gifu is expected during the week of 12/2/13. A portion of this specimen set will be forwarded to Saint Louis University according to the recently revised protocol.

The study can continue for 6 months with a follow-up continuing review as to the study progress.

Investigator Agreement: As the PI, you have agreed to assure that this research is conducted in compliance with Nemours policy and all applicable federal regulations and ICH standards, including, but not inclusive of:

- All research must be conducted in accordance with this approved submission. Any revision to approved materials must be approved by the IRB prior to initiation.

- Remember that informed consent/parental permission is a process beginning with a description of the study and insurance of participant understanding followed by a signed consent form. Informed consent must continue throughout the study via a dialogue between the researcher and research participant. Federal regulations require each participant receive a copy of the signed consent document.
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- All non-compliance issues or complaints regarding this study must be reported to the Director, NOHSP.
- All research records must be retained for a minimum of three years.
- A Closure Report must be submitted to the IRB when this protocol is completed.

If you have any questions, please contact Camille Varacchi at Nemours AI duPont Hospital for Children 1600 Rockland Road, ARB-Room 160A, Wilmington, Delaware 19803, 302-651-6807 or cvaracch@nemours.org.

Please include your study title and reference number in all correspondence with this office.

Nemours Office of Human Subjects Protection
10140 Centurion Parkway North
Jacksonville, FL 32256
Phone: 904-697-4023 Fax: 904-697-4024

MEMORANDUM

DATE:	May 9, 2014
TO:	Robert Mason, PhD
FROM:	Nemours IRB 1
STUDY TITLE:	[281498-8] Newborn screening and biomarkers for mucopolysaccharidoses - Aim 2: Control vs mucopolysaccharidosis patients
IRB #:	281498
SUBMISSION TYPE:	Continuing Review/Progress Report
ACTION:	APPROVED
APPROVAL DATE:	May 7, 2014
EXPIRATION DATE:	May 6, 2015

Thank you for your submission of Continuing Review/Progress Report materials for this research study. Your submission received Expedited Review based on the applicable federal regulation and meets all DHHS [and FDA] criteria for approval. The above-referenced research study is approved.

The IRB has determined that:

- This is "Research not involving greater than minimal risk per 45CFR46.404 and 21CFR50.51".
- This study is part of a larger, NIH funded project that seeks to develop a test to identify individuals with mucopolysaccharidoses (MPS). This specific portion of the study will evaluate specimens collected from healthy controls from Shimane University and MPS patients seen at Gifu University for levels of certain enzymes that are known to be deficient in this patient population. Participants with MPS will also be asked to complete Quality of Life questionnaires.The study team continues to meet monthly to discuss this study (Aim 2). As stated previously, specimens from international biobanks may be received and analyzed per fully executed MTAs. This activity has resumed. Additionally, collaborators at Gifu continue to ship dried blood spots (DBS) and questionnaire data from individuals with MPS. To date, specimens from 114 MPS patients have been received and questionnaire data from 111 of those patients have been received. A portion of this specimen set will be forwarded to Saint Louis University according to the active protocol.
- The research study team at Nemours does not obtain consent. The samples that are received from the collaborting sites are de-identified.
- To continue, the research requires IRB review and approval on an annual basis. Otherwise, May 6, 2015 is the last day that research may be conducted. The Principal Investigator is responsible for the timely submission of the continuing review application. Please post this date on your research calendar.

Reviewed/approved documents in this submission:

- Continuing Review/Progress Report - 281498 CR due 05.02.2014 for 06.20.2014 meeting (UPDATED: 05/2/2014)

Investigator Agreement: As the PI, you have agreed to assure that this research is conducted in compliance with Nemours policy and all applicable federal regulations and ICH standards, including, but not inclusive of:

- All research must be conducted in accordance with this approved submission. Any revision to approved materials must be approved by the IRB prior to initiation.
- Remember that informed consent/parental permission is a process beginning with a description of the study and insurance of participant understanding followed by a signed consent form. Informed consent must continue throughout the study via a dialogue between the researcher and research participant. Federal regulations require each participant receive a copy of the signed consent document.
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- All non-compliance issues or complaints regarding this study must be reported to the Director, NOHSP.
- All research records must be retained for a minimum of three years.
- A Closure Report must be submitted to the IRB when this protocol is completed.

If you have any questions, please contact Camille Varacchi at Nemours 807 ChilAI duPont Hospital for Children 1600 Rockland Road, ARB-Room 160A, Wilmington, Delaware 19803, 302-651-6807 or cvaracch@nemours.org.

Please include your study title and reference number in all correspondence with this office.

Nemours Office of Human Subjects Protection
10140 Centurion Parkway North
Jacksonville, FL 32256
Phone: 904-697-4023 Fax: 904-697-4024

MEMORANDUM

DATE:	April 29, 2015
TO:	Robert Mason, PhD
FROM:	Nemours IRB 1
STUDY TITLE:	[281498-14] Newborn screening and biomarkers for mucopolysaccharidoses - Aim 2: Control vs mucopolysaccharidosis patients
IRB #:	281498
SUBMISSION TYPE:	Continuing Review/Progress Report
ACTION:	APPROVED
APPROVAL DATE:	April 28, 2015
EXPIRATION DATE:	April 27, 2016

Thank you for your submission of Continuing Review/Progress Report materials for this research study. Your submission received Expedited Review based on the applicable federal regulation and meets all DHHS [and FDA] criteria for approval. The above-referenced research study is approved.

The IRB has determined that:

- This is "Research not involving greater than minimal risk per 45CFR46.404 and 21CFR50.51".
- This study is part of a larger, NIH funded project that seeks to develop a test to identify individuals with mucopolysaccharidoses (MPS). This specific portion of the study will evaluate specimens collected from healthy controls from Shimane University and MPS patients seen at Gifu University for levels of certain enzymes that are known to be deficient in this patient population. Participants with MPS will also be asked to complete Quality of Life questionnaires.The study team continues to meet monthly to discuss this study (Aim 2). As stated previously, specimens from international biobanks may be received and analyzed per fully executed MTAs. This activity has resumed. Additionally, collaborators at Gifu continue to ship dried blood spots (DBS) and questionnaire data from individuals with MPS. To date, specimens from 114 MPS patients have been received and questionnaire data from 111 of those patients have been received. A portion of this specimen set willbe forwarded to Saint Louis University according to the active protocol.
- The research study team at Nemours does not obtain consent. The samples that are received from the collaborting sites are de-identified.
- The amendment application to the approved protocol to add and remove research team members was approved as submitted.
- To continue, the research requires IRB review and approval on an annual basis. April 27, 2016 is the last day that research may be conducted. The Principal Investigator is responsible for the timely submission of the continuing review application. Please post this date on your research calendar. Please be reminded that applications for continuing review need to be submitted at least 2 weeks ahead of the expiration date to give sufficient lead time for IRB review.

Reviewed/approved documents in this submission:

- Amendment/Modification - Amendment to add KS and remove EY (UPDATED: 04/15/2015)

- Continuing Review/Progress Report - Continuing Review Application (UPDATED: 04/17/2015)
- Cover Sheet - Cover memo (UPDATED: 04/15/2015)
- Investigator Agreement - Investigator Agreement_KS (UPDATED: 04/15/2015)

Investigator Agreement: As the PI, you have agreed to assure that this research is conducted in compliance with Nemours policy and all applicable federal regulations and [ICH standards], which also includes the following:

- All research must be conducted in accordance with this approved submission. Any revision to approved materials must be approved by the IRB prior to initiation.
- Remember that informed consent/parental permission is a process beginning with a description of the study and insurance of participant understanding followed by a signed consent form. Informed consent must continue throughout the study via a dialogue between the researcher and research participant. Federal regulations require each participant receive a copy of the signed consent document.
- All serious and unexpected adverse events and unanticipated problems affecting participants must be reported promptly to the IRB according to NOHSP policy.
- All non-compliance issues or complaints regarding this study must be reported to the Director, NOHSP.
- All research records must be retained for a minimum of three years.
- A Closure Report must be submitted to the IRB when this protocol is completed.

If you have any questions, please contact Camille Varacchi at AI duPont Hospital for Children 1600 Rockland Road, ARB Room 160-A, Wilmington, Delaware 19803 at (302) 651-6807 or Camille.Varacchi@nemours.org. Please include your study title and reference number in all correspondence with this office.

Nemours Office of Human Subjects Protection
Nemours/Alfred I. duPont Hospital for Children
1600 Rockland Road
Wilmington, DE 19803
Fax: 302-651-4683
Office: 302-298-7613
MEMORANDUM

DATE:	May 3, 2016
TO:	Robert Mason, PhD
FROM:	Nemours IRB 1
STUDY TITLE:	[281498-15] Newborn screening and biomarkers for mucopolysaccharidoses - Aim 2: Control vs mucopolysaccharidosis patients
IRB #:	281498
SUBMISSION TYPE:	Continuing Review/Progress Report
ACTION:	APPROVED
APPROVAL DATE:	May 3, 2016
EXPIRATION DATE:	May 2, 2017

Thank you for your submission of Continuing Review/Progress Report materials for this research study. Your submission received Expedited Review based on the applicable federal regulation and meets all DHHS [and FDA] criteria for approval. The above-referenced research study is approved.

The IRB has determined that:

- This is "Research not involving greater than minimal risk per 45CFR46.404 and 21CFR50.51".
- The research study team at Nemours does not obtain consent. The samples that are received from the collaborating sites are de-identifed.
- To continue, the research requires IRB review and approval on an annual basis. May 2, 2017 is the last day that research may be conducted. The Principal Investigator is responsible for the timely submission of the continuing review application. Please post this date on your research calendar. For submissions that can be reviewed by expedited review, please be reminded that applications for continuing review need to be submitted at least 4 weeks ahead of the expiration date to give sufficient lead time for IRB review.

Reviewed/approved documents in this submission:

- Continuing Review/Progress Report - CR application (UPDATED: 04/27/2016)

Investigator Agreement: As the PI, you have agreed to assure that this research is conducted in compliance with Nemours policy and all applicable federal regulations and [ICH standards], which also includes the following:

- All research must be conducted in accordance with this approved submission. Any revision to approved materials must be approved by the IRB prior to initiation.
- Remember that informed consent/parental permission is a process beginning with a description of the study and insurance of participant understanding followed by a signed consent form. Informed consent must continue throughout the study via a dialogue between the researcher and research participant. Federal regulations require each participant receive a copy of the signed consent document.

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- A Closure Report must be submitted to the IRB when this protocol is completed.

If you have any questions, please contact Camille Varacchi at AI duPont Hospital for Children 1600 Rockland Road, ARB Room 160-A, Wilmington, Delaware 19803 at (302) 651-6807 or Camille.Varacchi@nemours.org. Please include your study title and reference number in all correspondence with this office.