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Kingma, S.D.K.

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MPS I: Early diagnosis, and treatment of bone disease

Sandra D.K. Kingma

Everything should be made as simple as possible, but not simpler - Albert Einstein
MPS I: EARLY DIAGNOSIS, AND TREATMENT OF BONE DISEASE

Sandra Kingma
MPS I: EARLY DIAGNOSIS, AND TREATMENT OF BONE DISEASE

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ter overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op woensdag 16 september 2015, te 14:00 uur

door
Sandra Deborah Karina Kingma
geboren te Zaanstad
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Chapter
1

General introduction
HISTORY

In 1917 and 1919, Charles Hunter and Gertrud Hurler published, independently of each other, their observations on patients with disproportional dwarfism, skull deformities and hearing deficits. These were considered to be the first descriptions of Mucopolysaccharidosis (MPS) type I (Hurler syndrome) and II (Hunter syndrome). Later, it appeared that these conditions had been described previously by John Thomson in 1900. Subsequently, MPS I and MPS II, which were at that time not recognized as different diseases, have been described under various names in the early 20th century. Ellis et al. introduced the name ‘gargoylism’ in 1936, because of the resemblance to the figures on gothic churches. In 1946, Arne Nja reported on 2 different types of gargoylism: the autosomal recessive type (frequently presenting with corneal opacities) and the sex-linked type (only males affected, absence of corneal opacities). In 1956, Victor McKusick named the autosomal recessive disorder Hurler syndrome, and the X-linked disorder Hunter syndrome, because in contrast to Hurler, corneal opacities were not observed by Hunter.

When these two disorders were first described, their underlying defects were completely unknown. Initially, gargoylism was considered to be a lipid storage disease, based on the appearance of histological sections prepared by standard fixation procedures. In 1952, Brante isolated mucopolysaccharides from livers and meninges of Hurler patients and named the disease ‘mucopolysaccharidosis’. Subsequently, in 1957, these mucopolysaccharides were identified to be heparan sulfate (HS) and dermatan sulfate (DS). The recognition that patients with slightly different symptoms had also differences in the amounts of HS, DS, keratan sulfate (KS) and chondroitin sulfate (CS) in urine, confirmed the existence of different MPSs.
Around that time, Victor McKusick numbered Hurler syndrome, as the ‘prototype disease’ MPS I, and numbered the known variants subsequently: MPS II for Hunter, MPS III for Sanfilippo, MPS IV for Morquio and finally, MPS V for Scheie syndrome. MPS V Scheie, which was first described in 1962, was later demonstrated to be a less severe version of MPS I Hurler.

Meanwhile, the cell organelles ‘lysosomes’ had been discovered by Christian de Duve. Almost 10 years later, the concept of an ‘inborn lysosomal disease’ as a result of enzyme deficiency was proposed and using electron microscopy, enlarged lysosomes were demonstrated in MPS I liver tissue (figure 2). Reuben Matalon and co-authors were the first to demonstrate that MPS I is caused by deficiency of the lysosomal hydrolase α-L-iduronidase. Finally, in 1990, Scott et al. showed that the gene coding for α-L-iduronidase, IDUA, is located on chromosome 4p16.3.

The concept of enzyme replacement therapy (ERT, replacing the deficient enzyme by infusion) for lysosomal storage disorders (LSDs) was introduced for the first time by Christian de Duve in 1964, with the following comment: “In our pathogenic speculations and in our therapeutic attempts, it may be well to keep in mind that any substance which is taken up intracellularly in an endocytic process is likely to end up within lysosomes. This obviously opens up many possibilities for interaction, including replacement therapy.” Elizabeth Neufeld and her group established the basis of ERT and haematopoietic stem cell transplantation (HSCT) by the demonstration that co-cultured fibroblasts from patients with different LSDs mutually corrected each other. This study was the rationale for Hobbs and co-workers to perform HSCT in a Hurler patient in 1980. Subsequently, HSCT has become the treatment of choice for severely affected MPS I (Hurler) patients. HSCT is currently the only treatment that can

**Figure 2.** First electron microscopy picture of liver tissue of a MPS I patient. Modified from: Van Hoof F, Hers HG. Ultrastructure of the hepatic cells in Hurler’s disease (Gargoylism). C R Hebd Seances Acad Sci 1964.
effectively prevent neurological disease in MPS I patients, if performed at an early age. The first clinical study on the effect of ERT in MPS I was performed in the late 1990s and recombinant ERT (Aldurazyme®) gained marketing approval in 2003. ERT, which does not cross the blood-brain barrier, is currently used to treat and prevent non-neurological manifestations of MPS I.

BIOCHEMICAL BASIS

MPS I is biochemically characterized by the accumulation of undegraded HS and DS in lysosomes and the retention and excretion of these molecules in the extracellular matrix (ECM) and blood and increased excretion via the urine.

Glycosaminoglycan synthesis and structure

Glycosaminoglycans (GAGs) are linear polysaccharides comprised of repeating disaccharide units, linked to core proteins to form complexes called proteoglycans. The structure and modifications of the GAG chain are depicted in figure 3. After translocation of the protein components of proteoglycans into the endoplasmatic reticulum, a xylosyltransferase adds xylose to a serine residue of the protein core, which subsequently is transported to the Golgi apparatus. Next, 2 galactose residues are added in the cis/m medial-Golgi and a glucuronic acid is added in the trans-Golgi, which is the final location for all subsequent steps of GAG synthesis. The addition of the fifth saccharide determines whether the GAG chains become HS or DS/CS. HS is composed of a N-acetylglucosamine followed by disaccharide units of glucuronic acid and N-acetylgalactosamine. Subsequent steps include elongation of the chain, sulfation and epimerization of glucuronic acid into iduronic acid. DS and CS are composed of N-acetylgalactosamine followed by disaccharide units of glucuronic acid.
and N-acetylgalactosamine. Subsequent steps include elongation of the chain, sulfation, and in DS, epimerization of glucuronic acid into iduronic acid. There are 3 types of KS proteoglycans, the type depends on their linker region. In KS-I (abundant in cornea), KS chains are N-linked via a complex of 2 N-acetylglucosamines and 2 mannoses to asparaginyl residues. In KS-II (present in cartilage), KS chains are O-linked via GalNac to serine/threonine residues. In KS-III (present in brain), KS chains are linked to the protein core via mannose O-linked to serine. The KS chains are formed by repeating galactose and N-acetylglucosamine residues, which both can be sulfated.

**GAG processing and turnover**

When modification of GAGs in the Golgi apparatus is complete, most proteoglycans are distributed to the ECM or cellular membrane (figure 4). Proteoglycans are secreted directly into the ECM, or are shedded from the cell surface through proteolytic cleavage of the core protein. Proteoglycans that are internalized by endocytosis are targeted to the lysosome for degradation.

First, the core protein is degraded and the GAG chains are partially cleaved by heparinases, chondroitinases and hyaluronidase. This is followed by a well ordered sequential degradation, dependent on the structure of the monosaccharide on the non-reducing end of the GAG chain (figure 5). N- and O-sulfated groups must be removed from the monosaccharide on the non-reducing end, before the monosaccharide itself can be removed. Deficiencies in the enzymes responsible for these reactions lead to the accumulation of different GAGs and MPS subtypes (table 1).

**Functions of GAGs**

GAGs, as the carbohydrate part of proteoglycans, are a major component of the ECM of all organs. Due to the negative charge of GAGs, a hydrated matrix between cells is formed. In cartilage for instance, proteoglycans provide a matrix that is capable of absorbing compression by water desorption and resorption. GAGs also regulate the organization...
of basement membranes by providing a scaffold for cell migration, proliferation and differentiation. In addition, GAGs interact with a variety of extracellular ligands, such as adhesion molecules, growth factors and chemokines, thereby regulating development and inflammation. At the cellular membrane, GAGs are required as co-receptors for proper interaction of proteins with their receptor.

**EPIDEMIOLOGY AND GENETICS**

The birth prevalence of MPS I is approximately 1 per 100,000 live births, but varies between different regions. The highest reported birth prevalence rate is in the Irish Republic (3.8 per 100,000 live births). Genotype-phenotype correlations are limited in MPS I. Mutations that are likely to impact protein length, such as nonsense, frameshift and splice-site mutations, almost invariably result in a severe (Hurler) phenotype. The most common MPS I causing mutations W402X and Q70X, for instance, are clearly associated with the severe (Hurler) phenotype, if present in a homozygous or compound heterozygous combination. Most missense mutations may allow for some residual enzyme activity and are associated with a more variable clinical phenotype. The mutation P533R, for instance, has been identified in patients with a mild, intermediate and severe phenotype. L218P, however, is

**Figure 5.** Stepwise GAG degradation. GlcA glucuronic acid, GlcNAc N-acetylglucosamine, GlcN glucosamine, GaINAc N-acetylgalactosamine, Gal galactosamine, IdoA iduronic acid.
always associated with the severe (Hurler) phenotype, but R89Q is associated with the more attenuated (Scheie) phenotype. Mutations that allow for some residual IDUA activity may be more susceptible to the effects of modifying polymorphisms in other genes. This might explain the poor genotype-phenotype correlations for some mutations. Also, many patients have at least one private mutation, which complicates phenotype prediction.

PATHOPHYSIOLOGY

GAG storage itself seems the direct cause of the enlargement of lysosomes, cells and various organs in MPS I patients. For instance, in the brain, GAG accumulation in connective tissue may cause narrowing of the dura and subsequent myelopathy. Also, thickening of the meninges may lead to lack of reabsorption of cerebrospinal fluid and hydrocephalus. Due to the extensive regulatory function of GAGs, accumulation also has several, poorly understood, secondary pathological effects.

Secondary storage

Accumulation of GAGs may lead to the secondary storage of other metabolites, such as gangliosides, cholesterol or GAGs not expected based on the enzyme deficiency. The cause of secondary accumulation is unknown, but is hypothesized to involve the disruptive effects of accumulated GAGs on the processes of GAG synthesis, degradation and cellular trafficking. Pereira et al. showed that cells from the spleen of MPS I mice had a lower lysosomal H+ and Ca2+ content, a higher activity of cysteine proteases and alteration of pH homeostasis, and suggested that accumulation of GAGs led to increased lysosomal membrane permeability, followed by disruption of cellular homeostasis and apoptotic cell death. The increase in lysosomal pH may result in defective functioning of lysosomal enzymes, leading to secondary storage.
Inflammation

GAGs have the potential to stimulate immune cell activation. Studies in brain from MPS I and III mice reported neuro-inflammation, characterized by microglial activation, astrocytosis and increases in pro-inflammatory cytokines. Also, inflammatory responses have been suggested to lead to oxidative stress responses, for instance the release of reactive oxygen or nitrogen species, which have been observed in several organs in the MPS I mouse. A cause of inflammation could be the activation of Toll-like receptor 4 by undegraded GAGs that are excreted into the ECM in the MPSs. This mechanism has been associated with the pathogenesis of MPS bone disease and aortic dilatation.

Autophagy

During autophagy, cell components that are destined for degradation are isolated from the cell in a vesicle with a double membrane, known as an autophagosome. The autophagosome fuses with a lysosome, and its contents are degraded. It has been shown that GAG storage leads to the accumulation of cholesterol in endolysosomal membranes, through a poorly understood mechanism. This results in impaired fusion of lysosomes with autophagosomes, and subsequent accumulation of autophagosomes, increased levels of autophagic proteins and apoptosis.

Elastogenesis

Impaired elastogenesis has been demonstrated in vitro in MPS I and is hypothesized to be due to the functional inactivation of elastin binding protein by accumulated DS. Defects in elastic fiber assembly may contribute to abnormalities in connective tissue in MPS I, such as musculoskeletal disease and blood vessel abnormalities.

Figure 6. Endochondral bone formation. PTHrP parathormone related protein, Ihh Indian hedgehog, BMP bone morphogenic protein, FGF fibroblast growth factor. Adapted from: Kronenberg. Developmental regulation of the growth plate. Nature 2003.
Growth factors

Long bones and vertebrae are formed by the process of endochondral ossification, in which a cartilage model is formed and gradually replaced by bone matrix. In the developing growth plate, chondrocytes proliferate and synthesize ECM, leading to increased bone length (figure 6). Subsequently, chondrocyte hypertrophy leads to blood vessel formation and the attraction of osteoblasts, and bone mineralization. Each of these steps is regulated by several growth factors. For example, Indian hedgehog (Ihh), which is synthesized by hypertrophic chondrocytes, stimulates the proliferation of chondrocytes, osteoblast activity and regulates its own activity by stimulating the production of parathormone related protein (PTHrP). PTHrP, which is produced by resting chondrocytes, stimulates proliferation of chondrocytes. When chondrocytes are no longer stimulated by PTHrP, due to increased distance to the source of PTHrP production, chondrocytes stop proliferating, differentiate into hypertrophic chondrocytes and produce Ihh. Other important growth factors in the developing growth plate are fibroblast growth factors (FGFs) and bone morphogenic proteins (BMPs), which have opposing effects on proliferation of chondrocytes, Ihh production and terminal differentiation (figure 6) 50.

GAGs play a major role in development by binding growth factors and thereby regulating proliferation, differentiation and cell death. Growth factors are transported through the ECM by translocating from one GAG binding site to another (figure 7). The distribution of growth factors is therefore regulated by the spatial organization of GAGs in the ECM 51. Because this process is dependent on the quantity and structure of GAGs, and partially degraded GAGs accumulate in the MPSs, altered interaction between accumulated GAGs and growth factors may contribute to disease manifestations. Indeed, GAGs from the medium of MPS I cells have been shown to perturb the interaction and function of FGF2 and BMP4 52,53. Also, bones from MPS I mice show disorganization of the columnar structure of chondrocytes in the growth plate and abnormal arrangement of bone trabeculae 54. Because growth factors regulate the development of all organs, this process is also suggested to contribute to neurological abnormalities in MPS I 39.
Osteoclast activity

Bone modeling involves activity of osteoblasts to produce bone at some sites, and osteoclast activity to resorb cartilage and bone at other sites. Both increased and decreased osteoclast activity can have major consequences for bone remodeling. The activity of cathepsin K, the main bone degrading enzyme within osteoclasts, is tightly regulated by GAGs. Cathepsin K activity is activated by CS and KS, but inhibited by HS and DS. In MPS I mice, excess HS and DS inhibit the activity of cathepsin K, leading to decreases in cathepsin K mediated cartilage degradation and a delay in endochondral bone formation. In contrast, the activity of other enzymes abundant in osteoclasts, such as matrix metalloproteinases (MMPs), are enhanced in the MPSs.

Disrupted balance

The different mechanisms described above all influence each other, which makes the pathophysiological processes very complicated. For instance, inflammatory processes may activate cathepsin K and MMPs, leading to increased cartilage and bone degradation, and subsequent disruption of endochondral bone formation and osteopenia. Also, disturbed development and inflammation of joints may lead to joint deformation and mechanical abnormalities. In turn, however, joint deformation and mechanical abnormalities may trigger or increase inflammatory processes.

CLINICAL SIGNS AND SYMPTOMS

MPS I (figure 8) is characterized by a continuous spectrum of disease manifestations, but has traditionally been divided into three categories based on the severity of the disease: the severe Hurler, the intermediate Hurler-Scheie and the attenuated Scheie phenotype. These clinical phenotypes are all characterized by progressive multi-organ disease, but are
distinct in the age of disease onset and degree of disease progression. Patients with the severe Hurler phenotype often appear normal at birth, but develop symptoms in the first months of life. Patients present with a combination of heterogeneous manifestations, which are dominated by coarse facial features, progressive cognitive impairment and frequent upper and lower respiratory infections. Other symptoms include a large tongue, obstructive sleep apnea, hydrocephalus, carpal tunnel syndrome, hearing loss, umbilical and inguinal hernias and hepatosplenomegaly. Cardiac disease is common and includes cardiac valve disease (in particular mitral or aortic valve regurgitation), cardiomyopathy, coronary artery occlusion and conduction abnormalities. Ocular manifestations may include corneal clouding, glaucoma, retinopathy and optic atrophy.

Dysostosis multiplex is the constellation of radiographic abnormalities resulting from defective intramembranous and endochondral bone formation in MPS I. Musculoskeletal manifestations include growth arrest, progressive loss of range of joint motion with contractures, kyphosis, scoliosis, hip dysplasia and hypoplastic vertebral bodies resulting in spinal cord compression. Untreated patients usually die in the first decade of life due to cardiorespiratory complications.

Patients with the attenuated Scheie phenotype develop their first symptoms around the age of 5 years and demonstrate slower progression of the above mentioned symptoms, with normal mental development and survival into adulthood. Patients with an intermediate Hurler-Scheie phenotype develop symptoms around 1-2 years old, have no or mild cognitive impairment and if untreated, die in adolescence or early childhood.
DIAGNOSTIC APPROACH

The first steps in the diagnostic workup of MPS I patients are usually the measurement of undegraded GAGs in urine using a dimethylmethylene blue (DMB) based spectrophotometric assay, followed by electrophoresis, which enables the separation of different GAGs. Definite diagnosis is established by the measurement of IDUA activity and mutation analysis. Prenatal screening for at risk couples includes enzyme or mutation analysis on chorionic villi or amniocytes.

The combination of lack of disease awareness, non-specific symptoms and rarity of MPS I, often results in diagnostic delay. The diagnostic delay, which is on average 4 months for the severe Hurler, 2.5 years for the intermediate Hurler-Scheie and 5 years for the attenuated Scheie phenotype, has not decreased over the last decade. Early diagnosis by newborn screening (NBS) is possible by measuring IDUA activity or GAGs in bloodspots. NBS for MPS I is already initiated in some countries and will be initiated in the Netherlands.

There are no validated techniques available yet for early predicting the phenotype of patients diagnosed by NBS. These will be needed in the scope of NBS to guide decisions on the appropriate treatment strategy before the onset of symptoms.

TREATMENT AND MANAGEMENT

Current therapeutic options for MPS I are HSCT, i.e. the transplantation of haematopoietic stem cells that synthesize active IDUA, and ERT, the infusion of active IDUA.

HSCT significantly alters the natural course of MPS I and is currently the only therapy that prevents neurocognitive decline. There are, however, some major limitations. Despite successful engraftment, progression of retinal degeneration, thickening of cardiac valves and progression of musculoskeletal disease appears to be common. The efficacy of HSCT has been shown to depend on the age at transplantation, therefore, very early HSCT may increase the efficacy, perhaps even in difficult-to-treat organs. The use of HSCT is also limited by the risk of graft failure and transplantation related morbidity and mortality, for instance by graft versus host disease, pulmonary complications and infections. Studies that use improved guidelines on chemotherapeutic conditioning and stem cell sources, however, very recently reported survival rates of 95%.

ERT with recombinant IDUA (laronidase; Aldurazyme®) significantly alters the course of most MPS I symptoms, but the infused enzyme does not cross the blood-brain barrier. Therefore, ERT is indicated for the treatment of non-neurological manifestations in patients for whom no cognitive benefit is expected from HSCT, such as patients with a more attenuated phenotype or patients with irreversible neurological deficits. The same as for
CHAPTER 1

HSCT, ERT does not effectively alter the course of valvular heart disease or skeletal disease, but its efficacy may improve if treatment is initiated earlier in life 24,75.

OUTLINE OF THE THESIS

To optimize outcome of MPS I patients, early recognition of the clinical phenotype to guide decisions on therapeutic strategies are needed. Also, treatment of MPS I bone disease is currently not effective and lack of knowledge on pathophysiological mechanisms limits the development of new therapeutics. This thesis comprises studies on early diagnosis of MPS I, and pathophysiology and treatment of MPS I bone disease.

Chapter 2 provides an overview of the epidemiology, diagnostics and challenges of screening for LSDs. In Chapter 3, we describe an algorithm that can be used to predict the disease phenotype of MPS I patients at a very young age. Such an algorithm may enable the initiation of the proper treatment before the onset of symptoms and irreversible organ damage in patients diagnosed by NBS. In Chapter 4, a study on the secondary pathological effects of GAG accumulation in fibroblasts from MPS I and III patients is described. Chapter 5 describes alterations in growth factors and GAGs in MPS I bones, which is likely involved in the development of MPS I bone disease. Information on these secondary pathological effects may help to identify new biomarkers and also identify new treatment strategies that may efficiently treat MPS I bone disease. Finally, in Chapter 6 and 7, adverse effects of genistein are described in MPS I cells and mice. Because genistein has been promoted as a treatment for MPS III and as a plant-extract, is available over the counter, this information may be essential to discourage the use of genistein in MPS I patients outside a controlled setting such as a clinical trial.
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General introduction


Chapter
Epidemiology and diagnosis of lysosomal storage disorders; challenges of screening

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CHAPTER 2

ABSTRACT
The lysosomal storage disorders (LSDs) are a group of genetic disorders resulting from defective lysosomal metabolism and subsequent accumulation of substrates. Patients present with a large phenotypic spectrum of disease manifestations that are generally not specific for LSDs, leading to considerable diagnostic delay and missed cases. Introduction of new disease modifying therapies for LSDs has made early diagnosis a priority. Increased awareness, but particularly the introduction of screening programs allow for early diagnosis and timely initiation of treatment. This review will provide insight into the epidemiology and diagnostic process for LSDs. In addition, challenges for carrier screening, high-risk screening and newborn population screening for LSDs are discussed.
INTRODUCTION

The lysosomal storage disorders (LSDs) comprise a heterogenic group of more than 50 genetic disorders caused by progressive accumulation of specific substrates due to deficiency of hydrolytic enzymes, non-enzymatic lysosomal proteins or non-lysosomal proteins involved in lysosomal biogenesis. A wide range of disease manifestations can occur, including hydrops foetalis, neurocognitive decline, dysmorphia, hepatosplenomegaly and musculoskeletal abnormalities. Most LSDs are characterized by a broad phenotypic spectrum and may present from very early in life to late in adulthood. Due to the rarity of the diseases and the heterogeneity of disease manifestations, which are generally not specific for LSDs, lengthy diagnostic delays and missed cases are common. In this review, epidemiological studies that studied a large panel of LSDs, current diagnostic workup of patients suspected of LSDs and subsequent challenges for implementation of screening are discussed.

EPIDEMIOLOGY

Information about the incidence of LSDs is relatively limited. The results of the 6 largest epidemiological studies that studied birth prevalences of a relatively large panel of LSDs are presented in table 1. Birth prevalences of the neuronal ceroid lipofuscinoses (NCLs) and female Fabry carriers were only studied in some reports, and were therefore not included in this table. Combined birth prevalences of LSDs range from 7.5 per 100,000 in British Columbia to 23.5 per 100,000 live births in the United Arab Emirates (UAE) with the sphingolipidoses as the most prevalent group, followed by the mucopolysaccharidoses (MPSs). Social isolation, immigration and epidemiology

When discussing introduction of screening programs for LSDs, reliable epidemiological data are essential, as birth prevalences may differ considerably per population group. Striking differences in birth prevalences between countries can be observed (table 1) and these can, indeed, at least partially be explained by differences in immigration patterns or isolation, for instance due to geographical, lingual, ethnic or religious preferences or customs. For example, in persons from Ashkenazi Jewish ancestry, strikingly high prevalences of several genetic diseases occur, including some LSDs, which has led to the introduction of highly successful screening programs. The remarkable high birth prevalences of MPS VI, GM1 gangliosidosis and fucosidosis in the UAE are another example and primarily due to ethnic isolation and founder effects, which is illustrated by the observations that 95% of genotyped patients were homozygous for their LSD causing mutation and that, indeed, most patients were from the same tribes or blood-related. Birth prevalences for most of the LSDs are comparable between British Columbia, the Czech Republic, Australia and The Netherlands, with MPS I, Gaucher disease and metachromatic leukodystrophy (MLD) as the most prevalent LSDs (mean birth prevalences around 1/100,000 live births). The population of British Columbia and Australia are primarily of European and particularly British origin.
CHAPTER 2

suggesting a cause for the similarity in birth prevalences of LSDs. However, the increasing immigration rates from different countries and ethnic groups to Western countries, is likely to change the birth prevalences of genetic diseases, including LSDs, in the near future.

Clinical awareness and epidemiology

Extensive investigations in a region or population group and increased awareness may have major influences on epidemiological data in rare diseases, as only a few extra diagnosed cases were

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* Fabry disease: number of male cases per 100,000 live male and female births.

Table 1. Birth prevalences of LSDs reported in different countries: total number of cases within a certain period of time divided by the total number of births in the same period (Australia) or total number of diagnosed cases born within a certain period of time divided by the number of births in the same period (The Netherlands, British Columbia, Portugal, the Czech Republic, and United Arab Emirates), expressed as cases per 100,000 live births.
cases may have a considerable effect on calculated birth prevalences. This is suggested to be a partial explanation for the high birth prevalences observed in Northern Portugal, as other regions of Portugal were excluded from most of the epidemiological analysis.\textsuperscript{6,10}

However, birth prevalences reported in published epidemiological studies all date from before the start of newborn screening (NBS) pilot studies and likely underestimate the true prevalences of many of the LSDs. Indeed, NBS (pilot) studies done in Hungary, Austria, Taiwan, Italy and the states of New York and Washington, reported on average 5-80 times higher birth prevalences than previously reported.\textsuperscript{11–19} The increase is primarily due to recognition of more attenuated and/or later-onset forms of the diseases, as demonstrated by Spada et al., who reported that 10 out of 11 Fabry patients diagnosed by a NBS pilot study in a part of Italy were of the late-onset type.\textsuperscript{18}

**DIAGNOSIS OF LSD**

Although the clinical presentation of LSDs depends on the type, quantity and site of storage of undegraded material, there are a number of overlapping clinical features, that are, however, not specific for LSDs.\textsuperscript{20} LSD patients thus often present a diagnostic challenge, which, in combination with the rarity of the different disorders, may lead to significant diagnostic delay.\textsuperscript{21–23} The most prominent signs and symptoms that should lead to appropriate diagnostic studies for LSDs are probably loss of earlier acquired cognitive and motor skills and/or combinations of signs and symptoms affecting different organs or organ systems.\textsuperscript{2} The combination of neurological signs and symptoms with musculoskeletal and/or cardiac signs and symptoms, and/or umbilical or inguinal hernia and/or ophthalmologic features including corneal clouding and retinal changes with a ‘cherry red spot’ should not only lead to inclusion of LSDs in the differential diagnosis but may also lead to more specific investigations (table 2; based on references). Figure 1 (based on references) presents a schematic approach for the initial diagnostic workup of patients with dysmorphia, musculoskeletal manifestations and/or progressive cognitive impairment as key clinical characteristics. Table 2 presents other clinical signs and symptoms that may be used in the differential diagnosis of LSDs. However, as LSDs are characterized by a broad spectrum of clinical manifestations, phenotypic severity and age of onset, and occurrence of atypical clinical manifestations have been reported, figure 1 and table 2 should be used only for guidance and not as absolute criteria.

The first step in the diagnostic workup generally consists of urinary analyses for specific undegraded macromolecules (figure 1). Usually, quantitative analysis of glycosaminoglycans (GAGs) using a dye binding assay is followed by electrophoresis, enabling separation of different GAGs. Urine oligosaccharide screens involve separation of urine sugars by thin-layer or high-pressure liquid chromatography. In addition, free sialic acid concentration in urine
can be assessed by mass spectrometry. When a patient presents with musculoskeletal manifestations and/or progressive cognitive impairment, especially in combination with coarse facial features, these urinary tests can be used as a sensitive first diagnostic approach. However, metabolic screening of urine only covers distinct groups of LSDs and carries a risk of false negative results, especially in patients with attenuated phenotypes of MPS III or MPS IV. Therefore, if strongly suspected, normal urinary screens should still be followed by enzyme analysis.

Dysmorphic features, generally referred to as ‘coarse’, are a feature in a number of LSDs, in particular the more severe phenotypes of the MPSs and oligosaccharidoses. These coarse features include wide set eyes, a flattened wide nasal bridge, frontal bossing, enlarged gums
Lysosomal storage disorders; challenges of screening

and macroglossia, often in combination with thick and abundant hair. In mucolipidosis (ML) type II (I-cell disease), infantile sialic acid storage disease (SASD) and GM1 gangliosidosis, these features may already be observed in the newborn period.

A wide variety of neurological signs and symptoms may occur, either at presentation or during the course of the disease. Early symptoms can be ataxia, seizures, progressive cognitive and motor retardation. Pyramidal tract lesions and seizures may occur during the course of the disease in LSDs with progressive central nervous system disease. Only a few symptoms are sensitive and specific, such as vertical supranuclear gaze palsy, which is an early feature of Niemann Pick type C (NPC) disease. Several of the LSDs are clinically

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**Figure 1.** Algorithm for the diagnostic process of patients with several LSDs presenting with coarse facial features and/or musculoskeletal abnormalities (dysostosis multiplex and/or limited range of motion of large joints) and/or progressive cognitive impairment. GAGs glycosaminoglycans, ML mucolipidosis, MLD metachromatic leukodystrophy, MPS Mucopolysaccharidosis, MSD multiple sulfatase deficiency, NCL neuronal ceroid lipofuscinosis, NPA/B Niemann Pick type A/B, NPC Niemann Pick type C, SASD sialic acid storage disease.
characterized by only neurological symptoms, including GM1 and GM2 gangliosidoses, MLD, NCL and Krabbe disease.\(^{20,42}\)

Bone disease is a feature of several LSDs, and the constellation of radiographic abnormalities resulting from defective endochondral and intramembranous bone formation observed in the MPSs, ML II/III and galactosialidosis, is collectively referred to as ‘dysostosis multiplex’.\(^{45}\) In addition, patients with the attenuated phenotypes of the MPSs, oligosaccharidoses and ML II/III frequently present with limitation of motion range in large joints, perceived as joint stiffness (figure 1). Other types of bone disease observed in LSDs are osteonecrosis with pain crises in Gaucher disease, rickets in patients with cystinosis, skull deformities and osteolysis in cathepsin K deficiency (pycnodysostosis), and painful joint stiffness and swelling in Farber disease.\(^{37,45,48,49}\) Measurement of specific enzyme activity, generally followed by mutation analysis, is required to obtain a definitive diagnosis in LSDs. In some disorders, however, enzyme activity cannot be used for diagnosis. In NPC, diagnosis relies on a specific filipin staining assay in cultured skin fibroblasts in combination with mutation analysis.\(^{38}\) In patients suspected of Fabry disease, diagnosis based on enzyme testing and mutation analysis can be inconclusive, as a number of genetic variants of unknown significance resulting in partially deficient enzyme activity, may erroneously lead to a conclusive diagnosis.\(^{50}\) Although Fabry disease is an X-linked disorder, females may suffer from significant symptoms,\(^{51}\) however, diagnosis in females is even more complicated as the activity of the involved enzyme is often borderline or normal.\(^{41}\) The additional use of biomarkers and algorithms designed for the diagnosis of Fabry disease may help to improve the diagnostic approach in Fabry disease.\(^{50}\) Finally, deficiency of activator proteins or saposins, specifically required to assist a range of lysosomal enzymes, cannot be directly detected by enzymatic testing, and should be considered in patients highly suspected of a specific LSD but with normal enzyme activity in lymphocytes.\(^{35,43}\)

A number of additional studies, including magnetic resonance imaging (MRI) of the brain, cardiac ultrasound studies and ophthalmological examination may assist in the diagnostic workup (table 2).

**CHALLENGES OF SCREENING**

Early diagnosis, which is essential to allow timely initiation of disease modifying therapy, may be achieved through increased awareness among clinicians, as well as the general public. Although many ‘awareness campaigns’ have been initiated, especially for disorders for which enzyme replacement therapy (ERT) has become available, there are no data to support the effectiveness of such a strategy. Even more, studies failed to show a decrease in diagnostic delay for Pompe disease or MPS I over the last decade.\(^ {21,22}\) This is probably due to the fact that physicians not specialized in LSDs will generally see none or only very
few LSDs during their working career as the overall prevalence of many disorders varies between <1:1,000,000 and 1:20,000 live births (table 1). An alternative strategy is diagnosis by screening.

**Carrier screening**

Genetic drift and endogamy have led in certain population groups to a high risk for carriership for a limited number of mutations leading to high prevalence of specific genetic diseases. The purpose of carrier screening is to inform couples about the risk for genetic disease in offspring and to help decision-making on marriage and reproduction as well as to allow specific prenatal testing. A highly successful example is carrier screening for Tay-Sachs (GM2 gangliosidosis) in the Ashkenazi Jewish population, which resulted in a dramatic decrease in birth prevalence of this disorder. Carrier testing for other diseases, including ML IV, Niemann Pick type A/B (NPA/B) and Gaucher disease, have been introduced in screening panels for people of Ashkenazi Jewish ancestry. However, there are ethical considerations that need to be addressed when introducing carrier screening in high risk populations. An Israeli carrier screening program for Gaucher disease demonstrated that 84% of the identified couples were at risk for offspring with mild or even asymptomatic Gaucher disease. They observed that screening for some mutations does not necessarily identify children requiring treatment, but can rather lead to questionable pregnancy terminations. This illustrates the importance of reliable data on natural history, genotype-phenotype correlations and epidemiology in different population groups before introducing carrier screening.

The rapid decrease in costs of next generation sequencing (NGS) and the improvement in coverage and thus in reliability of screening, paves the way for introducing carrier screening for a number of genetic diseases, including LSDs, in the general population by screening potential parents before conception (preconceptional screening). If both parents are identified to be carriers for mutations in the same gene, they may opt for prenatal testing or preimplantation genetic diagnosis. If such a technique becomes widely available, it might considerably change the prevalence of a number of severe LSDs. However, such screening approaches are highly controversial, and several ethical issues need to be addressed before preconception screening should be made available as a screening option.

**High-risk screening**

High-risk screening is performed to identify patients with low prevalence diseases based on the presence of a specific clinical sign or symptom. Almost all high-risk screening studies in LSDs have been performed for Fabry disease, as relatively high frequencies of Fabry disease may be detected among patients presenting with cardiomyopathy, early cryptogenic stroke or kidney failure of unknown cause. There are, however, two major...
issues that need attention when considering high-risk screening for LSDs. Firstly, as patients will already exhibit disease signs and/or symptoms, this approach may lead to lower efficacy of treatment as irreversible organ damage may already be present. In Fabry patients, for instance, ERT is significantly less effective in patients with decreased renal function. Secondly, a recent systematic review showed that high-risk screening for Fabry disease resulted in the identification of individuals with genetic variants of unknown significance. High-risk screening may therefore lead to erroneously labelling of individuals as having a genetic disease and even to initiation of an ineffective, invasive and costly therapy.

Newborn screening

In the early 1960s, large scale newborn population screening began with Robert Guthrie’s pioneering studies on presymptomatic identification of phenylketonuria in dried blood spots. In 1968, Wilson and Jungner described a set of criteria for population screening in a report from the World Health Organization. These criteria include the availability of suitable tests and treatment, agreed policy on whom to treat, and adequate understanding of natural history. Wilson and Jungner’s principles are still the directive for the decision to

Table 3. Newborn screening studies in dried blood spots for LSDs published between 2003 and 2014, that have included >10,000 newborns. Birth prevalences are expressed as cases per 100,000 live births. MPS Mucopolysaccharidosis, NPA/B Niemann Pick type A/B, US United States. * Only male newborns were screened.
include diseases in screening programs and over the past decades most developed countries have expanded their NBS programs. Because of the invariably progressive nature of LSDs and because diagnostic and therapeutic strategies have significantly improved, several LSDs have become attractive candidates for inclusion in NBS programs. Especially in the disorders for which adequate disease modifying treatment is available, diagnosis before the onset of irreversible clinical manifestations through NBS may greatly improve (event-free) survival. This is the case for several neuronopathic LSDs, such as MPS I and Krabbe disease, in which early haematopoietic stem cell transplantation (HSCT) may prevent or delay irreversible neurological damage and for disorders in which early initiation of ERT may be lifesaving such as in infantile Pompe disease, or prevent irreversible cardiovascular or renal disease, such as in Fabry disease.

The only LSD that is currently nominated by the Secretary of Health and Human Services in the United States (US) to be included in the Recommended Uniform Screening Panel, is Pompe disease. However, five states in the US have recently mandated NBS for several LSDs and NBS for Krabbe disease was already initiated in the state of New York in 2006. Some other countries have started (pilot) NBS programs for a number of LSDs and in Taiwan, Pompe and Fabry disease are now parts of the NBS program. Table 3 shows the results on large scale (>10,000 dried blood spots (DBS)) NBS (pilot) studies published in the last decade (2003-2014). Not unexpectedly, striking differences have been found between birth prevalences in these studies and earlier studies (table 1). It is evident that with the expansion of NBS programs for different LSDs, a number of methodological and ethical considerations still need to be addressed.

Laboratory techniques

A wide range of laboratory tests that may be used for diagnosis of LSDs within the scope of NBS programs has been reported over the recent years. Most assays are based on the measurement of enzyme activity using artificial substrates in DBS, either by tandem mass spectrometry (MS/MS), microplate fluorometry or digital microfluidic fluorometry. Alternative approaches are by measurement of accumulating substrates in blood or urine. For NBS programs, these assays need to be multiplexed and adapted for high-throughput screening. In the (near) future, NGS of the involved genes, applying techniques allowing full coverage of all exomes such as the single molecule molecular inversion probes technique, may replace screening based on enzyme activities or metabolite concentrations, as NGS as screening technique may ultimately proof to be more reliable, easier to multiplex and less expensive. Despite this tremendous technical progress, several challenges remain in selecting and improving appropriate screening methods and sensitivity and specificity of the different techniques will be further studied in ongoing and future NBS (pilot) studies.

Costs of screening, follow up and treatment
The economic impact of expanding NBS panels with LSDs needs attention. Due to the major advances in high-throughput screening technology, including the introduction of NGS in the future, the costs of the screening process itself will probably not be prohibitive, even in lower-income countries. When trying to assess costs of screening, it is important to also take into account the costs of long-term follow up of patients identified by screening of whom not all will develop symptoms early in life, as well as the costs of disease modifying therapy. Therapies for LSDs are often very expensive, especially ERT. It appears logical that only those diseases for which access to treatment and reimbursement for treatment can be guaranteed, should be included in NBS programs.

‘Patients in waiting’ and genetic variants of unknown significance

One of the major concerns for inclusion of LSDs in NBS programs is the identification of individuals with attenuated or late-onset phenotypes as well as the detection of individuals with genetic variants resulting in decreased enzyme activity but with unknown clinical significance. The phenotypic spectrum of most LSDs is remarkably broad and ranges from patients with severe expression of the disease with early start of symptoms, who may benefit from early start of treatment, to individuals who remain completely asymptomatic until senescence and in whom early introduction of invasive, potentially dangerous and costly medication may be harmful and will unnecessarily increase medical costs. Prediction of the phenotype, in order to avoid overtreatment of patients with attenuated forms of a disease after identification by NBS, might be done by genotyping. This approach is used, for instance, in the screening program for Krabbe disease, initiated in 2006 in the state of New York, in order to identify patients at risk for infantile Krabbe disease that may benefit from early HSCT. However, genotype-phenotype correlations are not always sufficiently reliable in LSDs and other methods that allow very early differentiation between phenotypes need to be developed. This might be done by (combining) data on the genetic, enzymatic, clinical and biomarker data at the time of detection by metabolic screening, and these strategies should preferably be developed before inclusion of a disease in a NBS program.

In addition, those individuals that have been detected by NBS but are classified as ‘attenuated’ and therefore do not need immediate intervention (e.g. ERT or HSCT), need regular follow up in order to allow early diagnosis of symptoms and timely initiation of therapy. The time between diagnosis of the potential disease causing enzymatic deficiency in the newborn period and the development of symptoms may, however, be extremely long for some diseases (e.g. Pompe and Fabry disease), even up to a normal lifetime. This may lead to the phenomenon known as ‘patients in waiting’, a term cornered by Timmermans and Buchbinder in 2010 and discussed by Kwon and Steiner in relation to a report on an early diagnosed presymptomatic patient with Pompe disease. Being a ‘patient in waiting’ for a potentially severe, life threatening disorder for which there is disease modifying treatment...
available may lead to complex psychosocial problems, as well as to costs of long-term follow up programs.

In addition to the identification of individuals with ‘very attenuated’ phenotypes who may become ‘patients in waiting’, NBS for LSDs may also result in identification of significant numbers of individuals with genetic variants of unknown significance, many of which will not convey a risk for clinical disease. This is particularly true for Fabry disease. NBS pilot studies for Fabry disease led to the identification of a remarkably large number (tables 1 and 3), depending on the study/population \(^{13,15–19,64}\). This is for a large part due to the identification of patients with late-onset cardiac variants of Fabry disease, but also to the identification of individuals with genetic variants of unknown significance, some of which may never lead to clinical disease \(^{50}\). There is, therefore, an urgent need for protocols and algorithms, which allow separation of individuals with, or at risk for, disease from those with neutral variants and, again, these protocols should preferably be developed before introduction of a disease in a NBS program. In addition, clear guidelines on the management of presymptomatic individuals with LSDs detected by NBS need to be developed, such as the recently published guideline of the American College of Medical Genetics (ACMG) Work Group \(^{41}\).

**Acceptable and effective therapy**

One of the essential criteria for NBS is the availability of effective treatment \(^{60}\). While the currently available disease modifying treatment options for LSDs in general significantly ameliorate the course of the disease, effectiveness varies considerably between patients and there can still be significant residual disease. For example, although early, presymptomatic, HSCT for Krabbe disease greatly increases (event-free) survival, most patients still develop progressive neurological manifestations, despite successful engraftment \(^{78}\). In addition, ERT in infantile Pompe disease, even when started early, may not always prevent ventilator-free survival or lead to independent walking, partially due to the development of antibodies against the infused enzyme \(^{79,80}\). Although immunomodulation may significantly improve the outcome of those patients \(^{81}\), further studies are needed to establish the long-term efficacy of this approach. Antibody formation has also been shown to affect treatment efficacy in a number of other ERTs \(^{82–84}\). It is therefore paramount that parents are informed not only about the benefits of treatment but also about the limitations in order to be able to make balanced decisions.

The challenges reported for introduction of LSDs in NBS programs illustrate that several fundamental issues still need to be addressed. Firstly, increased knowledge on natural history, studies on genotype-phenotype correlation and the development of diagnostic algorithms allowing separation of severe, attenuated and genetic variants with no risk for developing clinical disease are urgently needed for decision-making strategies in the context
of NBS. Secondly, knowledge on the long-term outcome of disease modifying treatments is needed to be able to better balance costs and benefits and to improve information to parents. Although it poses ample challenges, NBS is probably the only way to learn about the natural history of genotypic variants, whom to treat how and when, and to learn more about epidemiology of the diseases, and, last but not least, significantly improve outcome of these devastating disorders.

CONCLUSION

The LSDs are a group of genetic disorders resulting from defective lysosomal metabolism and subsequent accumulation of substrates. Patients present with a large phenotypic spectrum of disease manifestations that are generally not specific for LSDs, leading to lengthy diagnostic delays and missed cases. Introduction of new disease modifying therapies for LSDs have made early diagnosis a priority. Increased awareness, but particularly the introduction of different screening programs allows for early diagnosis and treatment, prenatal counseling and prevention of long and burdensome diagnostic odysseys. Before introduction or expansion of screening programs for LSDs, however, different methodological and ethical challenges need to be addressed. Firstly, as the prevalence of different LSDs differs considerably between population groups, reliable epidemiological data are needed to assess the potential benefits of screening for LSDs in different population groups. Secondly, as NBS will identify all forms of diseases, natural history studies on particularly late-onset diseases and the separation from genetic variants of unknown significance are needed. Thirdly, studies on phenotypic prediction, e.g. genotype-phenotype correlation and biomarkers or algorithms, are urgently needed to predict disease manifestations and therapeutic efficacy, preferably very early in life. Fourthly, studies on long-term efficacy of disease modifying treatments are needed, and in addition, consensus on which outcomes justify implementation of screening. Fifthly, guidelines for diagnostic confirmation and management of presymptomatic individuals are essential. Although implementing screening programs results in ample challenges, outcomes of current newborn (pilot) screening programs will likely contribute in meeting these challenges.

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REFERENCES


Chapter
An algorithm to predict phenotypic severity in mucopolysaccharidosis type I in the first month of life

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CHAPTER 3

ABSTRACT
Mucopolysaccharidosis type I (MPS I) is a progressive multisystem lysosomal storage disorder caused by deficiency of the enzyme α-L-iduronidase (IDUA). Patients present with a continuous spectrum of disease severity, and the most severely affected patients (Hurler phenotype; MPS I-H) develop progressive cognitive impairment. The treatment of choice for MPS I-H patients is haematopoietic stem cell transplantation, while patients with the more attenuated phenotypes benefit from enzyme replacement therapy. The potential of newborn screening (NBS) for MPS I is currently studied in many countries. NBS for MPS I, however, necessitates early assessment of the phenotype, in order to decide on the appropriate treatment. In this study, we developed an algorithm to predict phenotypic severity in newborn MPS I patients. Thirty patients were included in this study. Genotypes were collected from all patients and all patients were phenotypically categorized at an age of >18 months based on the clinical course of the disease. In 18 patients, IDUA activity in fibroblast cultures was measured using an optimized IDUA assay. Clinical characteristics from the first month of life were collected from 23 patients. Homozygosity or compound heterozygosity for specific mutations which are associated with MPS I-H, discriminated a subset of patients with MPS I-H from patients with more attenuated phenotypes (specificity 100%, sensitivity 88%). Next, we found that analysis of IDUA activity in fibroblasts allowed identification of patients affected by MPS I-H. Therefore, residual IDUA activity in fibroblasts was introduced as second step in the algorithm. Patients with an IDUA activity of <0.32 nmol x mg⁻¹ x hr⁻¹ invariably were MPS I-H patients, while an IDUA activity of >0.66 nmol x mg⁻¹ x hr⁻¹ was only observed in more attenuated patients. Patients with an intermediate IDUA activity could be further classified by the presence of differentiating clinical characteristics, resulting in a model with 100% sensitivity and specificity for this cohort of patients. Using genetic, biochemical and clinical characteristics, all potentially available in the newborn period, an algorithm was developed to predict the MPS I phenotype, allowing timely initiation of the optimal treatment strategy after introduction of NBS.
INTRODUCTION

Mucopolysaccharidosis type I (MPS I, OMIM 252800) is a progressive multisystem lysosomal storage disorder (LSD) caused by a deficiency of the lysosomal hydrolase α-L-iduronidase (IDUA, [Genbank NG_008103]), resulting in the accumulation of the glycosaminoglycans (GAGs) heparan sulfate (HS) and dermatan sulfate (DS) in virtually all body tissues 1. MPS I encompasses a wide phenotypic spectrum, with at the severe end the Hurler phenotype (MPS I-H), which is the most prevalent phenotype, characterized by progressive central nervous system disease in addition to the most prominent somatic manifestations: severe musculoskeletal, pulmonary and cardiac disease, inguinal and umbilical hernias and corneal clouding, all resulting in a significantly reduced life expectancy if left untreated 2–5. Patients with the intermediate Hurler-Scheie phenotype (MPS I-H/S) are generally reported as having only mild or no cognitive impairment but relatively severe somatic symptoms that limit life expectancy to the 2nd or 3rd decade in the absence of treatment, while the attenuated Scheie phenotype (MPS I-S) is characterized by relatively milder somatic manifestations and a near normal life expectancy 5,6.

Two disease modifying treatment options are currently available in MPS I: haematopoietic stem cell transplantation (HSCT) and intravenous enzyme replacement therapy (ERT) 7. HSCT can stabilize neurocognitive function, significantly ameliorate the course of several of the somatic symptoms and improve overall survival 8–11. HSCT is the preferred treatment strategy for patients with a presumed MPS I-H phenotype who are diagnosed before the age of approximately 2.5 years 7. HSCT may also be considered in patients with MPS I-H/S who display progressive neurocognitive involvement 7. However, although outcomes have improved considerably 12, HSCT still carries a considerable risk for procedure-related morbidity and mortality. Weekly ERT with recombinant IDUA (Laronidase®) is, therefore, the preferred treatment for patients with the more attenuated phenotypes (MPS I-S and, in general, MPS I-H/S) and ERT was shown to improve respiratory and cardiac symptoms of MPS I and some of the skeletal and joint manifestations, reduce hepatosplenomegaly, and improve the overall quality of life 13–18.

As early initiation of the optimal treatment, either HSCT or ERT, is highly likely to improve clinical outcomes 8,9,11,19, early diagnosis is essential. However, the variable clinical expression and the nonspecific signs and symptoms, in combination with the rarity of the disorder, often lead to a long diagnostic delay 3. Population newborn screening (NBS), using dried blood spots for detection of MPS I 20–23, is probably the best approach to identify patients at a very young age, thus allowing timely initiation of treatment. The feasibility of inclusion of MPS I in NBS programs is currently studied in several countries 24–26. Early diagnosis of MPS I through NBS, however, requires early prediction of the phenotype in each MPS I patient to guide decisions on the optimal treatment strategy. To date, assessment of the phenotype is
generally based on signs and symptoms at clinical presentation and age of disease onset \(^{15}\), as genotype is often not informative \(^{27,28}\). A recent study, initiated by our group, revealed a lack of consensus between experts on the assessment of phenotypic severity using a scale from 0 to 10, based solely on signs and symptoms at presentation \(^{29}\). This may be even more complicated within the scope of a NBS program, as patients may still lack many of the characteristic symptoms on which phenotyping is currently based. In addition to clinical and genetic characteristics, biochemical predictors have been sought to distinguish between phenotypes, but studies so far have been unsuccessful \(^{30,31}\), except for one study by Fuller \textit{et al.} \(^{32}\). The assay reported in this latter study, however, is rather complex and requires the availability of specific antibodies which renders it difficult to implement in other laboratories.

As NBS for MPS I may be implemented in several countries within the near future, there is an urgent need for a tool which allows reliable prediction of the phenotype within the first months of life. Here we present an algorithm for early determination of phenotypic severity in patients with MPS I diagnosed through NBS, combining mutation analysis, determination of residual enzyme activity in cultured skin fibroblasts and clinical characteristics that are apparent within the first month of life. This algorithm may allow separation of those MPS I patients who will benefit from HSCT at an early age from those that will optimally benefit from an early start of ERT.

**MATERIALS AND METHODS**

Outline of the prediction algorithm

We decided to design our algorithm on the separation of two distinct phenotypic categories based on the indications for treatment: patients with MPS I-H, who will benefit from early HSCT and more attenuated non-MPS I-H patients (MPS I-H/S and S), who will benefit from early start of ERT and for whom HSCT is, in general, not considered the optimal treatment strategy \(^{7}\). To this aim, we collected data that can all be assessed within the first month of life and might be related to the phenotypic severity: genotype, residual enzymatic activity and GAG storage in cultured skin fibroblasts and clinical signs and symptoms that may become apparent in the first month of life.

Patients

Thirty patients with MPS I, who were diagnosed and treated in the Academic Medical Centre, Amsterdam, the Netherlands, were included. All patients were classified into three categories (MPS I-H, MPS I-H/S and MPS I-S) by one of the authors (FAW), a clinician experienced in the diagnosis and treatment of LSDs including MPS I. Classification was based on the clinical signs and symptoms at diagnosis and the clinical course of the disease, and not on genotype, biochemical variables or clinical signs in early life.
CHAPTER 3 An algorithm to early predict phenotypic severity in MPS I

Mutation analysis

Mutation analysis had been performed in all 30 patients by standard procedures within the scope of the normal diagnostic workup at our centre. Missense, nonsense, splice site mutations, insertions and deletions were identified. Based on literature, potentially discriminating genotypes were identified.

Residual IDUA activity

Fibroblasts

Fibroblast cultures were available from 18 of the 30 patients included in this study. Informed consent for the use of fibroblasts for these studies was obtained from all patients or parents.

Cell culture

To remove bovine IDUA activity from the Fetal Bovine Serum (FBS), FBS was inactivated by incubation at 56°C for 30 minutes before use. Patient and control fibroblasts were grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% inactivated FBS and 100 U x ml⁻¹ penicillin, 100 µg x ml⁻¹ streptomycin and 250 µg x ml⁻¹ amphotericin in a humidified atmosphere containing 5% CO₂ at 37°C. Fresh medium was added every 2 weeks. After culture for 1, 2, 4, 6, 8 or 10 weeks postconfluency, the medium was removed, cell layers were washed with phosphate buffered saline (PBS) and harvested. Cell pellets were washed once with PBS, twice with 0.9% NaCl and stored at -20°C until analysis.

IDUA activity analysis

The generally used method to measure residual IDUA activity, using 4-methylumbelliferyl-α-L-iduronide (Glycosynth Ltd., Warrington, Cheshire, England) as a substrate, was optimized by varying the quantity of cell lysate, time of incubation and amount of substrate in order to accurately determine very low enzyme activities in MPS I patient fibroblasts.

Cells were resuspended in PBS and disrupted by sonification using a Vibra Cell sonicator (Sonics & Materials Inc., Newtown, CT, USA). Protein concentration was measured in whole cell lysates as described by Lowry et al. 44. 20 µl of cell lysate was added to 1 mM 4-methylumbelliferyl-α-L-iduronide in 0.1 M sodium formiate buffer, pH 3.25 in a final volume of 60 µl and a final protein concentration of 0.5 mg x ml⁻¹. After 2 hours of incubation at 37°C, the reaction was stopped by addition of 1440 µl 0.2 M sodium carbonate/ glycine buffer, pH 10.5. Released 4-methylumbelliferone was measured fluorometrically with an excitation wavelength of 366 nm and an emission wavelength of 442 nm using a Perkin Elmer LS45 fluorescence spectrometer (Perkin Elmer, Waltham, MA, USA). IDUA activity in each sample was calculated using a calibration curve of 4-methylumbelliferone (Glycosynth Ltd., Warrington, Cheshire, England). All enzyme activity assays were performed in
duplicate and repeated at least once in independent cell cultures. Earlier experiments in our laboratory, using control fibroblasts, showed an intra-assay variation of 1.4% and an inter-assay variation of 18.5%. To control for this relatively large inter-assay variation, with each performed IDUA assay, IDUA activity was simultaneously determined in at least 4 other previously analyzed cell lines, to make sure that the results from different experiments could be reliably compared.

**GAG analysis in fibroblasts**

Levels of HS and DS were determined as described previously with minor modifications. GAGs in 25 μg of fibroblast lysate (prepared as described for the IDUA activity analysis) were enzymatically digested into disaccharides and as a final deproteination step samples were loaded on an Amicon Ultra 10 kD centrifugal filter (EMD Millipore, Billerica, MA, USA) (instead of a 30kD filter), and centrifuged at 14,000 g for 30 minutes on 25°C.

**Clinical characteristics**

There are currently only few data published on clinical signs and symptoms in MPS I patients at birth or within the first month of life. Therefore, we decided to study the absence or presence of 14 clinical signs and symptoms (table 1) which are reported as early presenting

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>Number of patients in whom information was available</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory manifestations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Signs and symptoms of upper respiratory tract obstruction</td>
<td>19</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hernia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Umbilical hernia</td>
<td>18</td>
<td>0.6</td>
</tr>
<tr>
<td>Inguinal hernia</td>
<td>19</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Organomegaly</td>
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<td></td>
</tr>
<tr>
<td>Hepatomegaly</td>
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<td>X</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>1</td>
<td>X</td>
</tr>
<tr>
<td>Musculoskeletal manifestations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stiff joints/ contractures</td>
<td>2</td>
<td>X</td>
</tr>
<tr>
<td>Kyphosis</td>
<td>1</td>
<td>X</td>
</tr>
<tr>
<td>Scoliosis</td>
<td>1</td>
<td>X</td>
</tr>
<tr>
<td>Hip dysplasia</td>
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<td>X</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hearing impairment</td>
<td>3</td>
<td>X</td>
</tr>
<tr>
<td>Hydrocephalus</td>
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<td>0</td>
<td>X</td>
</tr>
<tr>
<td>Macrocephaly</td>
<td>1</td>
<td>X</td>
</tr>
<tr>
<td>Corneal clouding</td>
<td>0</td>
<td>X</td>
</tr>
</tbody>
</table>

**Table 1.** Clinical characteristics scored in the patient cohort. X insufficient data available for statistical analysis.
An algorithm to early predict phenotypic severity in MPS I

Symptoms in MPS I develop in life, such as developmental delay and stunted growth, these were not included in our study. If information on certain clinical signs was not available in the charts, parents of the patients were contacted by telephone and additional information was added to the information retrieved from the charts. All characteristics were scored as absent, present, or data not available or excluded based on prematurity. 1,3,4 siblings, 2 homozygous twins.

### Table 2. Patient characteristics, H Hurler, H/S Hurler/Scheie, S Scheie, + present, - absent, X data not available or excluded based on prematurity. 1,3,4 siblings, 2 homozygous twins.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Phenoype</th>
<th>Gestational age (weeks + days)</th>
<th>Mutation allele 1</th>
<th>Mutation allele 2</th>
<th>IDUA activity (nmol x mg^-1 x hr^-1)</th>
<th>Upper respiratory tract involvement</th>
<th>Ingual hernia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H</td>
<td>37+0</td>
<td>p.W402X</td>
<td>p.W402X</td>
<td>0.31</td>
<td>X</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>H</td>
<td>38+1</td>
<td>p.W402X</td>
<td>p.Q70X</td>
<td>X</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>H</td>
<td>40+0</td>
<td>p.W402X</td>
<td>p.Q70X</td>
<td>X</td>
<td>-</td>
<td>X</td>
</tr>
<tr>
<td>4</td>
<td>H</td>
<td>39+6</td>
<td>p.W402X</td>
<td>p.W402X</td>
<td>0.25</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>H</td>
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</tr>
<tr>
<td>6</td>
<td>H</td>
<td>33+6</td>
<td>p.Q70X</td>
<td>p.L218P</td>
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<td>X</td>
<td>X</td>
</tr>
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<td>p.L218P</td>
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<td>X</td>
</tr>
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<td>p.L218P</td>
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<td>+</td>
<td>X</td>
</tr>
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<td>9</td>
<td>H</td>
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<td>p.Q70X</td>
<td>p.L218P</td>
<td>X</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>H</td>
<td>40+0</td>
<td>p.L218P</td>
<td>p.L218P</td>
<td>X</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>H</td>
<td>38+3</td>
<td>p.W402X</td>
<td>p.L218P</td>
<td>0.43</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>H</td>
<td>41+1</td>
<td>p.A367E</td>
<td>c.1650del117</td>
<td>X</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>H</td>
<td>38+1</td>
<td>c.494-1G&gt;A</td>
<td>c.494-1G&gt;A</td>
<td>0.23</td>
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<td>+</td>
</tr>
<tr>
<td>16</td>
<td>H</td>
<td>X</td>
<td>p.H425fs</td>
<td>p.H425fs</td>
<td>0.32</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>17</td>
<td>H</td>
<td>40+0</td>
<td>p.W402X</td>
<td>p.W402X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>18</td>
<td>H/S</td>
<td>40+0</td>
<td>p.W402X</td>
<td>p.R505G</td>
<td>0.77</td>
<td>+</td>
<td>-</td>
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<td>p.N348K</td>
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<td>-</td>
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<tr>
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<td>p.W402X</td>
<td>p.N348K</td>
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<td>-</td>
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<tr>
<td>21</td>
<td>H/S</td>
<td>41+2</td>
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<td>p.D315Y</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>22</td>
<td>H/S</td>
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<td>p.P533R</td>
<td>p.P533R</td>
<td>X</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>H/S</td>
<td>37+1</td>
<td>p.P533R</td>
<td>p.P533R</td>
<td>2.43</td>
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<td>-</td>
</tr>
<tr>
<td>24</td>
<td>S</td>
<td>40+0</td>
<td>p.W402X</td>
<td>p.R383H</td>
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<td>-</td>
</tr>
<tr>
<td>25</td>
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<td>p.R383H</td>
<td>1.17</td>
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<td>X</td>
</tr>
<tr>
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<td>p.R383H</td>
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<td>X</td>
<td>X</td>
</tr>
<tr>
<td>28</td>
<td>S</td>
<td>40+5</td>
<td>c.474-2A&gt;G</td>
<td>p.R383H</td>
<td>X</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>29</td>
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<td>40+5</td>
<td>c.474-2A&gt;G</td>
<td>p.R383H</td>
<td>1.61</td>
<td>-</td>
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<tr>
<td>30</td>
<td>S</td>
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<td>c.474-2A&gt;G</td>
<td>p.R383H</td>
<td>X</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
available (table 2). Clinical characteristics from patients born before 37 weeks of gestational age were excluded, as symptoms related to prematurity, such as respiratory complications or increased occurrence of inguinal hernia may be confounding. Characteristics that showed a significant difference between MPS I-H and MPS I-non Hurler patients were considered distinguishing and considered for the prediction algorithm.

Statistics
Statistical analysis was performed using the SPSS Statistics software version 19 (IBM Corp., Armonk, NY, USA). Nonparametric ranking statistics (Mann-Whitney-U tests) were used to analyze the relationship between the assigned MPS I phenotypes (MPS I-H versus non-MPS I-H) and residual IDUA activity and GAG levels in fibroblasts. The most efficient cut-off values to discriminate between MPS I phenotypes based on IDUA activity were identified using receiver operating characteristic (ROC) curve analysis. True positive rates (sensitivity) were plotted against false positive rates (1-specificity) for all classification points, and p-values were calculated for the area under the curve. Differences between the phenotypic groups (MPS I-H versus non-MPS I-H) in the frequency of specific mutations or clinical characteristics were assessed either by Fisher’s exact test (dichotomous variables) or Mann-Whitney-U test (numeric variable). A three-step algorithm was designed and discrimination of the three phases in the flow chart was assessed separately by calculating sensitivity and specificity. This was also done for the algorithm as a whole, as a way to perform internal validation. All p-values were based on two-sided testing and differences with p values <0.05 were considered statistically significant.

RESULTS
For all 30 patients with MPS I included in this study, information on at least one potentially predictive criterium (genetic, biochemical or clinical) was available.

Mutation analysis
Based on the literature, a list of 26 mutations, which have been shown to reliably predict a Hurler phenotype when patients are homozygous or compound heterozygous for these mutations, was constructed (table 3). In our cohort, the association of these mutations with MPS I-H could be confirmed for the mutations p.Q70X, p.W402X, p.L218P, c.134del12 and c.494-1G>A. As mutations that have been associated with the more attenuated phenotypes may be more susceptible to the effects of modifying polymorphisms in other genes, these mutations were not included in our algorithm. Using the list of predictive mutations in table 3 in our group of 30 patients, a specificity of 100% for prediction of the MPS I-H phenotype and a sensitivity of 88% was calculated. The list of predictive mutations was integrated as first step in the prediction algorithm.
Biochemical analyses

Residual IDUA activity

Figure 1 shows that IDUA enzyme activity was linear up to at least 120 minutes of incubation time (fig. 1A) and 0.5 mg x ml\(^{-1}\) final protein concentration (fig. 1B). Based on these findings, we selected these conditions for subsequent studies. Substrate titrations were performed (fig. 1C) and although maximal enzyme activity was not reached, subsequent activity measurements were performed using a final substrate concentration of 1 mM. This concentration resulted in a 45% increase in activity, as compared to the commonly used substrate concentration \(^{42,43}\).

IDUA activity was determined in human skin fibroblast cell lines from the 18 MPS I patients (table 2 and fig. 2) of whom cell lines were available. Analyses were performed a week after the cells had reached confluency. Residual IDUA activity in MPS I cell lines ranged from 0.23-2.43 nmol x mg\(^{-1}\) x hr\(^{-1}\) (table 2), which is less than 2.5% of the activity found in control fibroblasts (control range: 101-270 nmol x mg\(^{-1}\) x hr\(^{-1}\)). MPS I-H fibroblasts could be completely discriminated from the MPS I-S fibroblasts (\(p<0.01\)) based on IDUA activity. Most MPS I-H/S fibroblasts had an intermediate IDUA activity (fig. 2). The diagnostic accuracy of the IDUA assay in differentiating MPS I-H from non-H MPS I patients showed an area under the ROC curve of 0.951 (\(p<0.001\), fig. 3A), indicating a good discrimination. Two cut-off values were calculated, resulting in three categories of enzyme activity: an IDUA activity of <0.32 nmol x mg\(^{-1}\) x hr\(^{-1}\) identified MPS I-H fibroblasts with a specificity of 100% (sensitivity 56%), as shown in figure 3B. This was regarded as the lower threshold, as only MPS I-H patients were found below this level of activity. Furthermore, 100% sensitivity (specificity 89%) was reached at a cut-off value of 0.66 nmol x mg\(^{-1}\) x hr\(^{-1}\) enzyme activity to discriminate MPS I-H fibroblasts from cell lines of non-H MPS I patients.

<table>
<thead>
<tr>
<th>Nonsense</th>
<th>Other</th>
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<tbody>
<tr>
<td>p.W41X</td>
<td>p.G51D</td>
</tr>
<tr>
<td>p.Y64X</td>
<td>p.134del12(20,24)</td>
</tr>
<tr>
<td>p.Q60X</td>
<td>p.G208V</td>
</tr>
<tr>
<td>p.Q63X</td>
<td>p.L218P</td>
</tr>
<tr>
<td>p.Q70X</td>
<td>p.A327P</td>
</tr>
<tr>
<td>p.P201X</td>
<td>p.T366P</td>
</tr>
<tr>
<td>p.E274X</td>
<td>c.494-1G&gt;A</td>
</tr>
<tr>
<td>p.Q310X</td>
<td>c.704insS</td>
</tr>
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<tr>
<td>p.W626X</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Mutations described as MPS I-Hurler associated in literature.

Figure 1. IDUA activity analysis. (A) Time dependence, using 1 mM substrate and 0.17 mg x ml\(^{-1}\) protein, (B) Protein dependence, using 1 mM substrate and 60 min incubation, (C) Substrate titration, using 0.17 mg x ml\(^{-1}\) protein and 60 min incubation.
Subsequently, this was set as the upper threshold; no MPS I-H fibroblasts had an enzyme activity higher than 0.66 nmol x mg\(^{-1}\) x hr\(^{-1}\). The same sensitivity for discrimination of phenotypes was obtained when cells were cultured for 2, 4, 8 or 10 weeks postconfluency. With increasing culture time, however, residual enzyme activity in all fibroblast cell lines decreased, as compared to cells cultured for 1 week postconfluency (results not shown).

**HS and DS levels in fibroblasts**

No significant differences were seen between MPS I-H fibroblasts and non-MPS I-H cells in total HS and DS or in the levels of individual disaccharides (results not shown).

**Clinical characteristics**

Information on clinical signs and symptoms in the first month of life was available for 23 patients (table 2). 3 patients, however, were excluded from the analysis because they were born at a gestational age <37 weeks. A significant difference between the incidence of signs and symptoms of upper respiratory tract obstruction (\(p=0.005\)) and inguinal hernia (\(p=0.033\)) was found between MPS I-H patients and non-MPS I-H patients.

**Prediction algorithm**

Mutation analysis was integrated as the first step in the prediction algorithm (specificity 100%, sensitivity 88%) and IDUA activity was chosen as the second step. A cut off value of <0.32 nmol x mg\(^{-1}\) x hr\(^{-1}\) was used to identify MPS I-H patients. An IDUA activity of >0.66 nmol x mg\(^{-1}\) x hr\(^{-1}\) identified non-MPS I-H patients. Patients with IDUA activity between 0.32-0.66 nmol x mg\(^{-1}\) x hr\(^{-1}\) were further classified by the presence of either one of the associated clinical characteristics (sensitivity 100%, specificity 100%). This resulted in a sensitivity and specificity of 100% for the complete prediction algorithm. The flow chart for the prediction algorithm is shown in figure 4.
DISCUSSION

Here we present an algorithm, based on the combination of mutation analysis, residual IDUA activity and clinical signs and symptoms during the first month of life, which may allow early, sensitive and specific prediction of the phenotype in MPS I patients diagnosed through NBS. Such an algorithm can be essential as the decision to implement NBS for MPS I will depend, at least in a number of countries, on the feasibility to decide on the optimal treatment strategy at an early age. NBS for MPS I is of high interest as early initiation of treatment, i.e. either HSCT for patients who will develop a MPS I-H phenotype and ERT for the non-MPS I-H patients, likely improves the disease outcome \(^8,9,12,19,51\), and early diagnosis on clinical recognition can be very difficult.

To date, more than 200 different mutations in the IDUA gene have been reported \(^{52}\), and this genetic heterogeneity partially explains the phenotypic variability in MPS I. For most of the mutations no clear genotype-phenotype correlation is known. However, some mutations have been found to reliably predict a severe disease phenotype \(^{10,27,33-41}\). This was confirmed in our cohort for the mutations p.Q70X, p.W402X, p.L218P, c.134del12 and c.494-1G>A. Therefore, mutation analysis was included as the first step in the algorithm to predict MPS I phenotype. Several missense mutations, such as the p.R383H and p.R89Q mutations, are generally reported in association with more attenuated disease \(^{30,37,50}\). We did not include these latter mutations in our algorithm, however, because the effect of attenuated mutations might vary due to novel combinations of mutations, polymorphisms in other genes or environmental factors \(^{50}\). Other mutations present in our cohort were also not incorporated in the algorithm because of functional heterogeneity (e.g. the same mutations seem to have a different effect on phenotypic severity) in earlier studies, such as

![Figure 3. ROC curve analysis. (A) ROC curve of IDUA activity for discrimination between MPS I-H and non MPS I-H. (B) Sensitivity and specificity for IDUA activity cut-off levels to discriminate between MPS I-H and non MPS I-H. Dashed lines represent chosen cut-off levels of 0.32 and 0.66 nmol x mg\(^{-1}\) x hr\(^{-1}\) IDUA activity.](image)
the mutations p.P533R and c.474-2A>G. Studies on genotype-phenotype correlations in large cohorts, focusing on allelic combinations of rarer mutations, could further improve the predictive power of this first step in our algorithm. Currently, rapid mutation analysis of the IDUA gene may not be available to all centers diagnosing MPS I. However, the fast technological advancements for gene sequencing will result in more universal access to mutation analysis, allowing reliable results within 4 weeks after diagnosis for most patients and applicability of the algorithm presented in this study.

It is highly likely that the introduction of NBS for MPS I will result in the identification of many novel mutations with unknown phenotypes. Therefore, a tool for prediction of phenotypic severity within the scope of NBS needs to include other variables. We found that the concentrations of HS and DS and of the individual disaccharides in cultured fibroblasts did not correlate with the phenotype. In contrast, Fuller et al. demonstrated that the levels of specific HS and DS derived trisaccharides in patient fibroblasts could discriminate between MPS I patients with and without neurological involvement. In that study, only levels of short chain HS and DS oligosaccharides were measured, while the HS and DS derived disaccharides detected in our study originate predominantly from relatively larger HS and DS chains. Possibly, fibroblasts from patients with neurological involvement store more

**Figure 4.** Algorithm for assessment of phenotypic severity in MPS I patients. Mutation analysis, residual IDUA activity and clinical characteristics present before the age of 1 month are combined, ¹Measured as described in this article, ²Upper airway obstruction included the following symptoms: excessive snoring during sleep, continuously runny nose, obstructive sleep apneas, feeding difficulties due to obstructed nasal breathing.
short GAGs chains, as compared to patients without neurological symptoms, but similar levels of larger HS and DS oligosaccharides, which hinders discrimination between these phenotypes using our GAG analysis.

Analyses of IDUA activity in fibroblasts or leukocytes is generally used as the confirmatory step in MPS I diagnosis. However, the most commonly used method, though sensitive for diagnostics, is not sensitive enough to reliably discriminate between the different MPS I phenotypes. A study in a cohort of 13 MPS I patients, where the IDUA protein was first captured using antibodies followed by enzymatic studies, showed clear discrimination between patients with and without neurological involvement. This method, however, makes use of specific anti-IDUA antibodies which are not commercially available, making this assay difficult to implement in other laboratories. In addition, specific putative mutations might result in a loss of epitopes, obstructing capture of the protein and thus interfering with the analysis. For this reason, we optimized the 4-methylumbelliferyl-α-L-iduronide IDUA activity assay to provide a method that may be more generally applicable. A higher concentration of substrate combined with a longer incubation time and increased amount of protein, resulted in accurate measurement of very low enzyme activities, as seen in MPS I patients. Interestingly, a very narrow range of low IDUA activities is responsible for a broad range of clinical presentations in MPS I patients, as IDUA activity in all MPS I fibroblasts was less than 2.5% of the activity measured in healthy control fibroblasts. Despite this small range of IDUA activities, cut-off values could be calculated using ROC curve analysis to differentiate between MPS I Hurler and non-Hurler fibroblasts.

Measurement of residual IDUA activity could not fully differentiate between phenotypes of patients with an activity in the range of 0.32-0.66 nmol x mg⁻¹ x hr⁻¹, as one MPS I-H/S cell line had an IDUA activity in this range. Although HSCT may be considered in some MPS I-H/S patients with neurocognitive involvement, this is not common practice. Therefore, the algorithm was improved by inclusion of potentially discriminating clinical characteristics early in life.

Of the 14 clinical characteristics studied, the presence of two were found to differ significantly between MPS I-H and non-MPS I-H patients: presence of inguinal hernia and the presence of signs and symptoms of upper airway obstruction. Including clinical characteristics in the algorithm resulted in complete differentiation between MPS I-H patients and patients with more attenuated phenotypes. Another clinical characteristic that may differentiate between MPS I-H patients and more attenuated patients is probably the severity of dysostosis multiplex, a collection of radiographic abnormalities resulting from defective endochondral and membranous growth throughout the body seen in the mucopolysaccharidoses. Especially thoraco-lumbar kyphosis before the age of one month, might be a very sensitive and specific symptom for MPS I-H. However, early kyphosis is
often initially not recognized by parents and caregivers and could therefore not be included in this model, which was based on retrospective analysis of clinical data.

Our study has some limitations. Firstly, due to the ultra-orphan nature of the disease, the proposed algorithm is validated in only a relatively small number of patients (n=30). Validation in other cohorts of patients needs to be performed to further determine its value. Secondly, our study includes a retrospective analysis of signs and symptoms during the first month of life. This may result in a recall bias, as both parents and investigators knew the phenotype of the patients. To address this, only characteristics that could be clearly distinguished and are often well documented in the newborn period were used for this algorithm. Thirdly, the prevalence of mutations firmly associated with certain phenotypes differs between regions around the world. Therefore, positive and negative predicting values of the proposed algorithm may differ between countries and this needs to be further investigated. Also, as new mutations will be detected once NBS for MPS I has been introduced, a prediction algorithm including mutation analysis needs to be continuously adjusted and improved. Likewise, NBS will allow for further investigation on the predictive value of certain clinical signs such as early kyphosis, which could not be included in this study.

As a result of future studies, the algorithm might be adapted to also differentiate between MPS I-H/S patients with and without neurocognitive involvement. The improved outcome of HSCT, in combination with increasing knowledge on the risk for neurocognitive decline in a subset of MPS I-H/S patients, may result in a shift in treatment protocols, with HSCT as treatment of choice for this group of patients.

With the phenotypic prediction algorithm presented here, we hope to provide the basis for a tool to reliably predict phenotype in the majority of MPS I patients diagnosed through NBS. Prospective studies could result in inclusion of additional predictive factors and improvement of the prediction algorithm.

CONCLUSION

Using genetic, biochemical and clinical characteristics, which can all be studied within the first month of life, an algorithm was developed for accurate prediction of the phenotype at an early age in MPS I patients. Such an algorithm allows timely initiation of the optimal treatment strategy, thus improving disease outcome. With the future launch of NBS programs for MPS I, patients will not have developed all characteristic signs and symptoms currently used for assessment of the phenotype, making a prediction algorithm for early assessment of phenotypic severity indispensable.

ACKNOWLEDGEMENTS

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Chapter
Secondary storage and alterations in composition and distribution of glycosaminoglycans in MPS I and III fibroblasts

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CHAPTER 4

ABSTRACT

The mucopolysaccharidoses (MPSs) are caused by the deficiency of lysosomal glycosaminoglycan (GAG) degrading enzymes and subsequent GAG accumulation. Clinical manifestations may include shortened lifespan, neurological deterioration, multi-organ failure and bone dysplasia. Differences in clinical phenotype between different MPSs with the same accumulating GAGs, and phenotypic differences within the same type of MPS and the same disease causing mutations, suggest the contribution of secondary processes involved in the pathophysiology. We studied secondary GAG storage, extracellular GAG distribution and gene-expression of enzymes involved in GAG synthesis in fibroblasts (FBs) from MPS I (heparan sulfate (HS) and dermatan sulfate (DS) accumulation) and MPS III (HS accumulation) patients. We demonstrate secondary accumulation of chondroitin sulfate (CS) in both MPS I and III FBs and secondary accumulation of DS in MPS III FBs. Also, we observed differences in the fractions of HS-derived disaccharides and CS-derived disaccharides between MPS I and MPS III FBs. In addition, we observed alterations in the extracellular distribution of sulfated HS domains in MPS I FBs and decreased abundance in MPS III FBs, as compared to healthy control FBs. Finally, gene-expression levels of HS, DS and CS synthesis enzymes were increased in MPS I and MPS III FBs, as compared to healthy control FBs. These secondary effects may contribute to clinical heterogeneity and poor genotype-phenotype correlations in MPS patients. In the future, treatments that aim to decrease synthesis or increase degradation of GAGs not typically associated with a certain MPS subtype might assist in altering the clinical course of MPS patients.
INTRODUCTION

The mucopolysaccharidoses (MPSs) comprise a group of heterogenic lysosomal storage disorders, characterized by glycosaminoglycan (GAG) accumulation. Each MPS subtype is caused by the deficiency of a single lysosomal enzyme required for the degradation of the GAGs heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfate (CS), keratan sulfate (KS) or hyaluronan. GAG accumulation results in cellular damage which affects multiple organ systems. Clinical manifestations may include reduced lifespan, multi-organ failure, bone dysplasia and cognitive impairment [1]. Disease severity is thought to depend on the degree of enzyme deficiency and the storage product. Differences in phenotype between the MPSs in which the same GAGs accumulate or even between patients with the same disease causing mutations, however, remain largely unexplained. This indicates that there may be secondary biochemical and cellular pathways involved in the pathogenesis of these diseases, initially triggered by the accumulation of non-degraded or partially degraded GAGs [2]. Such processes include inflammation, autophagy, growth factor signaling defects and the storage of secondary metabolites [3]. Secondary metabolites may include gangliosides, which have been reported to accumulate in several MPSs [4,5] or GAGs not typically associated with a certain MPS subtype. The cause of secondary accumulation remains unknown, but probably involves the disruptive effects of storage products on lysosomal enzymes and cellular trafficking [4,6,7]. Also, changes in gene-expression of GAG synthesis enzymes have been suggested to contribute to secondary storage and disease manifestations [8–9].

In order to better understand the pathogenesis of the MPSs, which is needed for the identification of new therapeutic targets, it is essential to understand the complex cascade of events leading to secondary substrate accumulation. As far as we know, no studies have been reported in MPS I or MPS III on increased GAG synthesis as a cause of secondary GAG storage, nor on alterations in GAG content and distribution in the extracellular matrix (ECM).

In order to explore the potential role of secondary GAG synthesis and storage in the MPSs, we studied secondary GAG storage and GAG composition in fibroblasts (FBs) from MPS I (OMIM 607014, HS and DS accumulation) and MPS IIIA, B and C (OMIM 252900, 252920, 252930, HS accumulation) patients. In addition, we performed immunohistochemistry to study GAG distribution in the ECM. Finally, we studied gene-expression of HS, DS and CS synthesis enzymes.

MATERIALS AND METHODS

Chemicals and materials

Primers were from Biolegio or Sigma Aldrich. Goat serum was from Dako. The antibodies for specific HS (HS4C3, EV3C3, HS4E4, NS4F5, LKIV69), DS (LKN1, GD3A12), CS domains (IO3H10,
GD3G7) and anti-VSV antibody were a kind gift from dr. van Kuppevelt (Radboud University Medical Center, Nijmegen, The Netherlands). Brightvision DPVB-AP kit was from Immunologic and SIGMA Fast 3,3-diaminobenzidine (DAB) tablets and DPX mounting medium were from Sigma. Phosphate buffered saline (PBS), \( \text{H}_2\text{O}_2 \), paraformaldehyde, eosin and haematoxylin were of analytical grade.

Cell culture

Informed consent for the use of FBs was obtained from all patients or parents. Cell culture was performed as described earlier. Experiments were performed 12 days after plating.

GAG analysis

GAG levels were determined by measuring HS, DS and CS-derived disaccharides using HPLC-MS/MS, as described previously, with a minor modification: 40μg of FB lysate was used. As D0a4 was the only detectable DS-derived disaccharide, only this value is given. Significant differences in GAG levels or fraction of ≥25% were considered biologically relevant.

Immunohistochemistry

FBs were grown on coverslips. All steps of the immunohistochemical procedures were carried out at room temperature unless otherwise stated and coverslips were washed three times with PBS for 5 minutes between each step. FBs were fixed in 4% w/v paraformaldehyde in PBS for 15 minutes. Subsequently, coverslips were incubated for 3 minutes with 3% v/v \( \text{H}_2\text{O}_2 \) to eliminate endogenous peroxidase activity. Coverslips were blocked with 3% v/v goat serum for 1 hour and incubated overnight at 4°C with antibodies against specific HS, DS and CS domains, diluted 1:10 in block solution. Next, coverslips were incubated with a
mouse anti-VSV antibody (1:10 dilution) for 1 hour, with post-antibody block (BrightVision Kit) for 15 minutes and subsequently with HRP GaM/R (Brightvision Kit) for 30 minutes. Staining was visualized with DAB substrate. Finally, coverslips were stored in PBS at 4°C for at least 1 day, dehydrated, and mounted in DPX. Cell morphology was analyzed with routine hematoxylin/eosin (HE) staining.

Quantitative real-time PCR
RNA isolation and qPCR analysis was performed as described earlier. Primer sequences are described in Table 1. Significant differences in gene expression of ≥2 fold gene expression were considered biologically relevant.

Statistical analysis
Statistical analysis was performed using nonparametric tests: Kruskal Wallis tests for comparison between more than 2 groups and two-tailed Mann-Whitney-U tests for comparison between 2 groups and post-hoc analysis. SPSS Statistics software version 21 (IBM Corp.) was used. Significance was assumed where p values were <0.05.

RESULTS
GAG analysis

GAG levels
As expected, HS levels in MPS I, MPS IIIA, MPS IIIB and MPS IIIC FBs were increased (10, 19, 14 and 16 fold respectively, p<0.05), as compared to healthy control FBs (fig. 1A). DS levels were 11 fold (p<0.05) increased in MPS I FBs, but also 6 fold (p<0.05) and 4 fold (p<0.05) increased in MPS IIIA and MPS IIIC FBs, respectively, as compared to healthy control FBs (fig. 1B). Surprisingly, CS levels were 6 fold (p<0.05), 6 fold (p<0.05) and 4 fold (p<0.05) increased in MPS I, MPS IIIA and MPS IIIC FBs, respectively, as compared to healthy control (fig. 1C). FBs from all MPS IIIB patients followed the same trends, but due to large variation between cell lines, changes were not significant. KS was not detectable in any of the studied FBs (results not shown).

Kruskal Wallis tests were performed to study differences between 3 groups: healthy control, MPS I and MPS III FBs. Because MPS IIIA, MPS IIIB and MPS IIIC are characterized by HS accumulation and the same clinical manifestations, and because the values were not significantly different between the MPS III subtypes, we combined these groups together for the Kruskal Wallis tests. Total DS levels were significantly different between healthy control, MPS I and MPS III FBs (p<0.01).
GAG fractions

No biologically relevant differences (>25%) in fractions of the HS-derived disaccharides D0A0, D0S0, D0A6-D2A0 and D0S6-D2S0 were observed between healthy control and MPS FBs (results not shown). As compared to healthy controls, fractions of the highly sulfated HS-derived disaccharide D2S6 were 64% ($p<0.05$) and 109% ($p<0.05$) increased in MPS IIIA, and MPS IIIC FBs, respectively (fig. 1D). No difference in D2S6-fraction between
healthy controls and MPS I FBs were observed. The fractions of CS-derived disaccharides D0a0 (fig. 1E) and D0a6 (fig. 1F) were not significantly different in MPS FBs, as compared to healthy control FBs.

Kruskal Wallis tests and subsequent post-hoc analysis revealed significant differences in fractions of CS-derived disaccharides D0a0 \((p<0.05)\) and D0a6 \((p<0.05)\) between MPS I and MPS III FBs \((p<0.05)\). Because the fraction of HS-derived disaccharide D2S6 was significantly different between MPS III subtypes (increased in MPS IIIC FBs as compared to MPS IIIA and B, \(p<0.05)\), no Kruskal Wallis test was performed to study differences between healthy control, MPS I and all types of MPS III FBs. However, D2S6 fraction was significantly higher in MPS IIIA and MPS IIIC FBs \((p<0.05)\), as compared to MPS I FBs.

**GAG staining**

Staining with antibodies against specific HS, DS and CS domains was performed to study GAG content and distribution in the ECM. Using the anti-HS antibody LKIV69, which binds to 2-O-, and N-sulfated HS domains \(^{16}\), differences between healthy controls, MPS I and MPS III FBs were observed (fig. 2). In healthy control FBs (fig. 2A), a typical pattern of HS staining could be observed in the ECM, which was present in ridges in a fishnet pattern. In MPS I FBs (fig. 2B), HS staining was distributed equally and evenly in the ECM. HS staining was less abundant in the ECM of MPS IIIA (fig. 2C), MPS IIIB (fig. 2D) and MPS IIIC (fig. 2E) FBs, as compared to healthy control and MPS I FBs. We observed no differences in morphology or growth patterns between healthy control, MPS I and MPS III FBs (fig. 2F-J).

*Figure 2.* Immunostaining of healthy control (A), MPS I (B), MPS IIIA (C), MPS IIIB (D) and MPS IIIC (E) FBs with the anti-HS antibody LKIV69. HE staining of healthy control (F), MPS I (G), MPS IIIA (H), MPS IIIB (I) and MPS IIIC (J) FBs. For each group, the experiment was performed in 3 different cell lines, 1 representative figure per group is shown.
Gene expression of enzymes involved in GAG synthesis

Gene expression of enzymes involved in GAG synthesis was measured using RT-qPCR (table 2). Expression levels of the following genes involved in HS synthesis were significantly different (p<0.05) in the MPSs, as compared to healthy control: EXT2 (MPS IIIC 3 fold), HS6ST1 (MPS I 4 fold, MPS IIIC 4 fold), NDST1 (MPS I 13 fold, MPS IIIA 8 fold, MPS IIIC 6 fold) and NDST2 (MPS I 7 fold, MPS IIIC 3 fold). Expression levels of the DS synthesis gene CHST14 was 5 fold increased in MPS I, 5 fold in MPS IIIA and 4 fold in MPS IIIC FBs (p<0.05). There was a significant increase (p<0.05) in the following genes involved in both DS and CS synthesis: CHPF2 (MPS I 10 fold, MPS IIIC 4 fold), CHST12 (MPS I 4 fold, MPS IIIC 3 fold) and CHSY1 (MPS I 5 fold, MPS IIIC 3 fold). Expression of the KS synthesis gene CHST2 was 13 fold increased in MPS IIIC FBs (p<0.05). Expression levels of other KS synthesis genes were below detection level in FBs. FBs from all MPS IIIB patients followed the same trends, but due to large variation between cell lines, changes were not significant.

DISCUSSION

The MPSs are characterized by clinical heterogeneity and, for most disease causing mutations, poor genotype-phenotype correlations. In order to better understand the pathogenesis of the MPSs and to identify new therapeutic targets, further studies on the processes that may contribute to these striking differences between patients with the same accumulating substrates are warranted. We used cultured FBs from MPS I and MPS III patients to study

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Table 2. Percentage change ± standard deviation in mRNA expression of GAG synthesis enzymes. Expression levels in healthy control FBs are set at 100%. All values were normalized against the housekeeping gene PPIB. All measurements were performed in duplicate in 3 different cell lines, *p <0.05.
Secondary storage in MPS I and III fibroblasts

several potential factors in the disease pathogenesis of the MPSs: secondary storage of GAGs, alterations in the composition and distribution of GAGs and gene-expression of enzymes involved in GAG synthesis.

We demonstrate secondary storage of CS in MPS I, MPS IIIA and MPS IIIC FBs. We also confirm the results of Lamanna et al. 7 on secondary DS storage in MPS IIIA and MPS IIIC FBs which was, thus far, the only report on secondary GAG storage in MPS FBs. Secondary elevations of different GAGs in urine and plasma from MPS patients have been reported more frequently 19,22,23.

Interestingly, we observed differences in the fractions of GAG derived disaccharides between FBs from MPS I and MPS III patients. The fractions of the 2-, 6-O-, N-sulfated HS-derived disaccharide D2S6 and CS-derived disaccharide D0a6 were increased in MPS III FBs, as compared to MPS I FBs. In contrast, the unsulfated CS-derived disaccharide D0a0 was increased in MPS I FBs, as compared to MPS III FBs. Earlier studies have shown increased sulfation of HS in brain from MPS I and particularly MPS III mice, and it was suggested that this may contribute to the severity of neuropathology 8,24.

GAGs regulate many biological processes, such as inflammation and growth factor signaling, by interaction with a number of proteins present in the ECM. The function of GAGs is dependent on its extracellular distribution, structure and sulfation pattern 25. However, very little is known about extracellular GAG composition and distribution in the MPSs. To study whether MPS FBs exhibit abnormalities in GAG content or distribution in the ECM, FBs were stained with antibodies against specific HS, DS and CS domains. We show altered distribution of 2-O-, and N-sulfated HS-domains in the ECM of MPS I FBs, and decreased abundance in the ECM of MPS III FBs. These results seem in contrast to our GAG analysis results, in which we observed no difference in the fraction of the 2-O-, N-sulfated sulfated D2SO and a higher fraction of 2-, 6-O-, N-sulfated D2S6 in MPS III FBs. GAG fractions, however, may be different between the intracellular and the extracellular compartment. Also, the results of McGlynn et al. 4, which suggest that GAG accumulation leads to defective cellular trafficking of secondary metabolites, may explain the differences that we observed in GAG composition and storage between the intracellular and extracellular compartment.

To investigate whether GAG synthesis is a contributing factor to secondary storage and differences in GAG composition in FBs, we studied gene-expression of several GAG synthesis enzymes. We observed increased gene expression of HS6ST1 (6-O-sulfation), NDST1 (N-sulfation) and NDST2 (N-sulfation) in MPS I and MPS IIIC FBs, which are all necessary for the synthesis of the HS-derived disaccharide D2S6. Also, we observed an increase in expression of genes involved in DS and CS synthesis. We did not observe any decreases
in gene-expression of GAG synthesis enzymes. These results suggest that increased GAG synthesis may indeed contribute to the secondary GAG storage and abnormalities in GAG composition that we observed in MPS FBs. Accumulated GAGs are known to induce pro-inflammatory responses \textsuperscript{26}, which may in turn stimulate GAG synthesis \textsuperscript{27}. In addition, because GAGs interact with growth factors, alterations in GAG distribution may lead to alterations in growth factor distribution and signaling, including growth factors that regulate GAG synthesis. 2-O- and N-sulfated HS domains, for instance, are known to bind growth factors \textsuperscript{16}.

An important limitation of this study was the considerable difference in GAG levels and gene-expression between MPS IIIB cell lines, due to variation in 1 cell line. Therefore, future studies should be initiated, studying more cell lines. Also, in addition to GAG synthesis, GAG degradation and trafficking may be studied for a more complete overview of the contribution of several pathways on secondary GAG storage and alterations of GAG distribution. Finally, \textit{in vitro} studies in other cell types and \textit{in vivo} studies are needed to confirm that secondary GAG storage and alterations in GAG composition and distribution may contribute to disease manifestations in the MPSs.

CONCLUSION

In conclusion, our studies show, for the first time, secondary storage of CS, abnormalities in HS composition and distribution and increased gene expression of GAG synthesis enzymes in FBs from MPS I and III patients. These secondary pathological effects may influence disease progression and contribute to clinical heterogeneity and poor genotype-phenotype correlations in MPS patients. In the future, therapies that increase degradation or decrease synthesis of GAGs not typically associated with a MPS subtype, may improve the efficacy of current disease modifying treatments.

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Chapter
Altered interaction and distribution of glycosaminoglycans and growth factors in Mucopolysaccharidosis type I bone disease

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CHAPTER 5

ABSTRACT
The mucopolysaccharidoses (MPSs) comprise a group of lysosomal storage disorders characterized by deficient enzymatic degradation and subsequent accumulation of glycosaminoglycans (GAGs). Progressive bone and joint disease are a major cause of morbidity in the MPSs, and current therapeutic strategies have limited effect on these symptoms. By elucidating the pathophysiological mechanisms underlying bone disease, new therapeutic targets may be identified. Longitudinal growth is regulated by the interaction between GAGs and various growth factors. Because GAGs accumulate in the MPSs, we hypothesized that altered interaction between growth factors and GAGs contributes to the pathogenesis of MPS bone disease. In this study, binding between GAGs from MPS I chondrocytes to fibroblast growth factor 2 (FGF2) was not significantly different from binding of FGF2 to GAGs from control chondrocytes. FGF2 signaling, however, was increased in MPS I chondrocytes after incubation with FGF2, as compared to control chondrocytes. Using a bone culture system, we demonstrated decreased growth of WT mouse bones after incubation with FGF2, but no effect on growth of MPS I bones. However, MPS I bones showed decreased growth in the presence of GAGs from MPS I chondrocytes. Finally, we demonstrate alterations in GAG distribution in MPS I chondrocytes, and alterations in GAG, FGF2 and Indian hedgehog distribution in growth plates from MPS I mice. In summary, our results suggest that altered interaction and distribution of growth factors and accumulated GAGs contributes to the pathogenesis of MPS bone disease. In the future, targeting growth factor regulation or the interaction between certain growth factors and GAGs might be a promising therapeutic strategy for MPS bone disease.
INTRODUCTION

The mucopolysaccharidoses (MPSs) comprise a group of lysosomal storage disorders, each characterized by the deficiency of a single lysosomal enzyme required for glycosaminoglycan (GAG) degradation. This leads to accumulation of the GAGs heparan sulfate (HS), dermatan sulfate (DS), chondroitin sulfate (CS) and keratan sulfate (KS) in lysosomes and in the extracellular matrix (ECM). Accumulation of these products leads to progressive cellular dysfunction, resulting in central nervous system disease, multi-organ failure and reduced lifespan \(^1\). In addition, skeletal dysplasia, generally referred to as dysostosis multiplex, is a striking feature of most of the MPSs and a major cause of morbidity. Patients may show progressive loss of range of joint motion with contractures, growth arrest, kyphosis, scoliosis, hip dysplasia and hypoplastic vertebral bodies resulting in spinal cord compression \(^1,2\). This musculoskeletal disease frequently leads to orthopaedic surgeries such as cervical decompression, femoral osteotomy and hip replacements. These procedures carry significant risks due to airway compromise, cardiac disease and cervical instability often present in the MPSs \(^2,3\).

Current available therapies for the MPSs are enzyme replacement therapy (ERT) and haematopoietic stem cell transplantation (HSCT) \(^1\). Potential future therapeutic options include gene therapy, substrate reduction therapy and anti-inflammatory therapy \(^4\). Current therapies, however, have limited effects on bone disease which may be at least partially due to the fact that treatment is usually started after the onset of irreversible bone lesions, which may already be present before birth \(^5\). In addition, the effect of both HSCT and ERT is limited due to the inability of the relatively large lysosomal enzymes to traffic through the poorly vascularized cartilage to target cells \(^6\). Also, cartilage cells are derived from mesenchymal stem cells, which are not transplanted in sufficient amounts by HSCT \(^7\). Finally, the pathophysiological cascades initiated by accumulated GAGs are still poorly understood, limiting the development of new therapeutic strategies.

Longitudinal growth of long bones results from the complex developmental process of endochondral bone formation. During this process, mesenchymal cells differentiate into chondrocytes, which proliferate and produce extracellular matrix (ECM). Chondrocytes undergo the process of hypertrophy, attract blood vessels and stimulate perichondral cells to differentiate into osteoblasts, followed by mineralization. These processes are dependent on the production and distribution of multiple growth factors, such as Indian hedgehog (Ihh), Fibroblast Growth Factors (FGFs) and Bone Morphogenic Proteins (BMPs) \(^8\). Proteoglycans, complexes of protein-attached GAGs, are a major class of ECM molecules, and play a pivotal role in regulating growth factor signaling \(^9\). Upon release from producing cells, growth factors may be transported across several cell diameters via interaction with GAGs on the cell surface or in the ECM, which creates a gradient of growth factors through
the developing growth plate. At the cellular membrane, HS proteoglycans are required as co-receptors for proper interaction of growth factors with their receptors\textsuperscript{10,11}. Because the MPSs are characterized by the accumulation of non-degraded or partially degraded GAGs, we hypothesized that altered interaction between growth factors and GAGs contributes to MPS bone disease. We used MPS I chondrocytes and MPS I mouse bones, in which HS and DS accumulate, as a model for MPS bone disease, to study binding between GAGs and FGF2, FGF2 signaling, the influence of FGF2 on bone growth, and GAG and growth factor distribution.

MATERIALS AND METHODS

Chemicals and materials

Minimal Essential Medium (MEM), L-glutamine and Non Essential Amino Acids (NEAA) were from Life Technologies. Diethylaminoethyl (DEAE) sepharose fast was from Pharmacia and 3Kd centrifugal filters from Millipore. Proteinase K (Tritrachium album) and heparin were from Sigma. FGF2 protein for ELISA experiments and western blot experiments were from e-Biosciences. Anti-FGF2 antibody for ELISA experiments were from Novus Biologicals (catalog number NB100-78224). GAG binding plates were from Iduron. Streptavidin-HRP was from R&D systems. Tetramethyl Benzidine Liquid Substrate (TMB) was from MP Biomedicals. P-p44/42 MAPK (phosphorylated ERK, pERK, catalog number 43713) antibody was obtained from Cell Signaling Technologies and total MAPK-1 (ERK, catalog number m5670) and β-actin antibodies were from Sigma Aldrich. All secondary antibodies were from Westburg B.V. BGJb medium was from Life Technologies. Penicillin, streptomycin and amphotericin were from Lonza. FGF2 protein for bone growth experiments was from either Novus Biologicals or e-Biosciences. Anti-FGF2 antibody for immunohistochemistry was from Abcam (catalog number ab8880). Anti-Ihh antibody was from LifeSpan Biosciences (catalog number C40514). Primers were from Sigma Aldrich. Goat serum, Rabbit Immunoglobulin and Envision System HRP Kit were from Dako. The VSV-tagged (Vesicular Stomatitis Virus tagged) antibodies for specific HS (HS4C3, EV3C3, HS4E4, NS4F5, LKIV69), DS (LKN1, GD3A12), CS domains (IO3H10, GD3G7)\textsuperscript{12–19}, and anti-VSV antibody were a kind gift from dr. van Kuppevelt (Radboud University Medical Center, Nijmegen, The Netherlands). Brightvision DPVB-AP kit was from Immunologic and SIGMA Fast 3,3-diaminobenzidine (DAB) tablets and DPX mounting medium were fromSigma. Ammoniumacetate, sodiumchloride, triton-X-100, tween-20, Nonfat dried milk powder, phosphate buffered saline (PBS), sulfuric acid, paraformaldehyde, eosin and hematoxylin were of analytical grade.

Experiments with human cell lines

Cell culture

Informed consent for the use of fibroblasts was obtained from all patients or parents.
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Chondrogenic differentiation of fibroblasts was performed essentially as described earlier, with minor modifications for FGF2 signaling experiments. Instead of DMEM, MEM supplemented with 1% L-glutamine and 1% NEAA was used. In addition, 4 days before the completion of chondrogenic differentiation (3 weeks after plating), the medium was replaced by serum-free medium. For all cell experiments, MPS I Hurler (severe phenotype) cell lines were used.

**Isolation of GAGs**

All steps were carried out at room temperature (RT) unless otherwise stated. Columns containing 3mL of DEAE resin were washed with wash buffer containing 20mM ammonium acetate pH6, 0.11mM sodium chloride and 1g/L Triton-X-100. Next, culture medium of healthy control and MPS I chondrocytes was loaded on a column, washed with wash buffer and eluted with 20mM ammonium acetate pH6 and 1mM sodium chloride. The elution fractions were applied onto a 3kD filter and centrifuged at 14,000g for 30 minutes. The filter was washed twice with MilliQ by centrifuging at 14,000g for 3 minutes. Finally, to elute the GAGs from the filter, 150µl MilliQ was applied to the filter, and the filter was inverted and centrifuged at 1,000g for 2 minutes. The GAG content of the elution fractions was analyzed with HPLC-MS/MS, as previously described. Before use in bone growth experiments, isolated GAGs were incubated with 2.5mg/ml (final concentration) proteinase K for 15 minutes at 37°C, to remove remaining protein. The reaction was stopped by the addition of a 10% volume of 200mM w/v phenylmethanesulfonyl fluoride (PMSF) in propanol and samples were boiled for 3 minutes and another 10% of the total volume of the sample of PMSF solution was added.

**Growth factor ELISA**

GAG binding plates were coated overnight at RT with 0.4µg/ml GAGs isolated from the medium of healthy control or MPS I chondrocytes, diluted in standard assay buffer (SAB) consisting of 50mM sodium acetate, 100mM sodium chloride and 0.2% Tween-20. Between each of the following steps, the plate was washed three times with SAB and all subsequent steps were carried out at 37°C unless otherwise stated. Plates were blocked with 1% w/v nonfat dried milk powder and 0.1% v/v Tween-20 in PBS and subsequent dilutions were made in blocking solution. Plates were incubated with 1µg/ml of FGF2 for 2 hours, followed by incubation with anti-FGF2 antibody (diluted 1:500) for 1 hour. Next, the plates were incubated with streptavidin-HRP (diluted 1:200). Color development was performed with TMB at RT and the reaction was stopped by adding 1.8M sulfuric acid. Absorbance at 450nm was measured and concentrations were calculated in the arbitrary unit of percent binding capacity of HS, relative to a standard curve of heparin.
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Immunohistochemistry

Chondrocytes were grown on coverslips. All steps of the immunohistochemical procedures were carried out at RT unless otherwise stated and coverslips were washed three times with PBS for 5 minutes between each step. Fibroblasts were fixed in 4% w/v paraformaldehyde in PBS for 15 minutes. Subsequently, coverslips were incubated for 3 minutes with 3% v/v H$_2$O$_2$ to eliminate endogenous peroxidase activity. Coverslips were blocked with 3% v/v goat serum for 1 hour and incubated overnight at 4°C with antibodies against specific HS, DS and CS domains, diluted 1:10 in block solution. To check the specificity of the staining, negative controls (slides incubated with 5% v/v goat serum instead of the primary antibody) were included in each experiment. Next, coverslips were incubated with a mouse anti-VSV antibody (1:10 dilution) for 1 hour, with post-antibody block (BrightVision Kit) for 15 minutes and subsequently with HRP goat anti-mouse/rabbit (Brightvision Kit) for 30 minutes. Staining was visualized with DAB substrate (SIGMA-Fast). Finally, coverslips were stored in PBS at 4°C for at least 1 day, dehydrated, and mounted in DPX.

FGF2 signaling

To examine FGF2 signaling in control and MPS I cells, chondrocytes were incubated for 10 minutes with 3.75ng/ml FGF at 37°C. At this concentration phosphorylation of the second messenger phosphorylated ERK (pERK) is induced to 50% of the maximal level in control chondrocytes (results not shown). Next, cells were harvested and levels of total and phosphorylated ERK were analyzed by western blot analysis as described earlier 20, with the following antibody dilutions: pERK 1:2,000, ERK 1:10,000, actin 1:10,000, IRDye 800 goat anti-rabbit 1:10,000, IRDye 680 donkey anti-mouse; 1:5,000.

Experiments with mouse bones

Animal experiments

MPS I mice (B6.129-Idua$^{tm}$ClkJ 21) were purchased from Jackson Laboratory and maintained as a heterozygote line on an inbred C57BL/6J background at the Academic Medical Centre, Amsterdam, The Netherlands. The mice were housed at 21 ± 1°C, 40–50% humidity, on a 12 hours light-dark cycle, with ad libitum access to regular food pellets and water. Genotypes were identified by PCR using a protocol provided by Jackson Laboratory, using the primers: 5’-GGAACCTTTGAGACTTGGGAATGAACCAG-3’ (common forward), 5’-CATGTAAATAGGGGTATCCTTGAACTC-3’ (WT reverse) and 5’-GGATTGGGAAGACAATAGCAGGCATGCT-3’ (MPS I reverse). At the age of 6 days, 3 weeks or 11 weeks, mice were anesthetized with an intraperitoneal injection of 100mg/kg pentobarbital and euthanized by exsanguination via cardiac puncture. All experimental procedures involving animals were approved by the institutional review board for animal experiments at the Academic Medical Centre, University of Amsterdam (Amsterdam, The Netherlands).
CHAPTER 5 Altered interaction and distribution of GAGs and growth factors in MPS I

Bone growth experiments
Metatarsalia of 6 day old female WT and MPS I mice were collected and placed in BGJb medium containing 0.1% w/v BSA and 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin in a humidified atmosphere containing 5% CO₂ at 37°C. Pictures of bones were made at day 0 and 5, were anonymized for the researcher and bone length was determined using Adobe Illustrator. To enable comparisons, bone growth of a metatarsal was always compared with bone growth of the corresponding metatarsal from the other paw of the same animal. 2 ng/ml FGF2 was compared with untreated, 2 ng/ml FGF2 + 5 ng/ml healthy control or MPS I GAGs isolated from chondrocytes was compared with 2 ng/ml FGF2, and 2 ng/ml FGF2 + 5 ng/ml MPS I GAGs was compared with 2 ng/ml FGF2 + 5 ng/ml healthy control GAGs. Factor correction was used to remove the between-session variation, as described by Ruijter et al.²².

Immunohistochemistry
Femurs of 3 and 11 week old male WT and MPS I mice were collected and placed in 5% w/v paraformaldehyde in PBS. After 1 day, femurs were decalcified in a buffer containing 41.3 g/L disodium-ethylenediaminetetraacetic acid and 0.8% v/v formalin, for approximately 6 weeks. Next, bones were embedded in paraffin by routine procedures and 4.5 μm transversal sections were cut. After dewaxing and dehydration, sections were treated with 1% w/v proteinase K for 7 minutes at 37°C. GAGs in bones were stained as described for chondrocytes, with slight modifications. The antibody dilutions were as follows: LKN1 1:5, HS4C3 1:10, EV3C3/HS4E4/NS4F5 1:20, LKIV69/IO3H10/GD3A12 1:40, GD3G7 1:80 and a routine counterstain with hematoxylin was performed. For the FGF2 and Ihh immunohistochemistry, the same protocol was used with a few modifications. The H₂O₂ step was performed with peroxidase block from the Envision kit and blocking with a 1:3 dilution of goat serum. After incubation with the primary antibody against FGF2 in a 1:200 dilution or Ihh in a 1:50 dilution, sections were incubated for 1 hour with the labeled polymer HRP goat anti-rabbit (Envision kit), followed by visualization using DAB substrate (Envision kit). To check the specificity of the staining, negative controls (slides incubated with matched non-immune IgG instead of the primary antibody) were included in each experiment.

To study morphology, a routine hematoxylin/eosin (HE) staining was performed on femurs of 3 and 11 week old mice.

Statistical analysis
Statistical analysis was performed using SPSS Statistics software. Data were analyzed using two-tailed Mann-Whitney-U tests for nonparametric analysis. Significance was assumed where p values were less than 0.05.
RESULTS

FGF2 binding of MPS I GAGs

To study the binding capacity of GAGs to FGF2, a GAG binding plate was coated with GAGs isolated from the medium of healthy control chondrocytes and GAGs from the medium of MPS I chondrocytes. As expected, medium of MPS I chondrocytes contained 2.5 fold more GAGs, compared to medium of control chondrocytes (results not shown). When equal quantities of GAGs were coated to the plates, there was a trend, which was not significant ($p=0.13$) towards lower binding capacity of MPS I GAGs, as compared to healthy control GAGs (fig. 1).

Figure 1. Binding of FGF2 to GAGs isolated from the medium of healthy control and MPS I chondrocytes, expressed as % of the binding capacity of the same amount of coated heparin. All values are mean ± standard deviation of 3 different cell lines. All analyses were repeated at least once in independent cell cultures, with similar results. Each sample was analyzed in triplicate.

Figure 2. Immunostaining of healthy control (A) and MPS I chondrocytes (B) with the anti-HS antibody LKIV69, healthy control (C) and MPS I chondrocytes (D) with the anti-HS antibody HS4C3, and healthy control (E) and MPS I chondrocytes (F) with the anti-DS antibody GD3A12. For each group, the experiment was performed in 3 different cell lines, 1 representative figure per group is shown.
GAG distribution in MPS I chondrocytes

Staining with antibodies against specific HS, DS and CS domains was performed to study GAGs content and distribution in the ECM of healthy control and MPS I chondrocytes. Using the anti-HS antibody LKIV69 which binds to 2-0-, and N-sulfated HS domains \(^{18}\), an even distribution of GAGs in the ECM of healthy control chondrocytes was observed (fig. 2A). In MPS I chondrocytes, this antibody showed a more focal GAG distribution (fig. 2B). Similar changes were observed using the anti-HS antibody HS4C3 (fig. 2C, D), which binds to 3-O-sulfated glucosamine domains \(^{19}\), and the anti-DS antibody GD3A12 (fig. 2E, F) which binds to disaccharides composed of iduronic acid and 4-O-sulfated galactosamine, and possibly also to 2-O-sulfated DS domains \(^{17}\). No differences between cell types were observed with the other antibodies (results not shown).

FGF2 signaling in MPS I chondrocytes

To study differences in FGF2 signaling, healthy control and MPS I chondrocytes were incubated with FGF2 and protein levels of the second messenger pERK were measured,
which were normalized for β-actin and total ERK levels in each sample. FGF2 incubation led to a mild but significant \( p<0.05 \) increase in pERK protein levels in MPS I chondrocytes (fig. 3), as compared to healthy control chondrocytes.

**Ex vivo growth in MPS I bones**

To study the effect of FGF2 on bone growth, metatarsalia from WT and MPS I mice were cultured and growth was measured. The coefficient of variation (%CV) of bone growth measurements was 0.3% (results not shown). After 5 days of incubation, bone growth slowed to a plateau phase (results not shown), therefore, bones were incubated for 5 days in subsequent experiments. Growth of MPS I bones (16±6%) was significantly decreased \( p<0.01 \), as compared to WT bones (22±3.5%, results not shown).

After FGF2 incubation, growth was significantly different between WT and MPS I bones \( p<0.01 \), as expected, FGF2 incubation led to decreased growth of WT bones \( p<0.01 \), but FGF2 incubation had no effect on growth of MPS I bones (fig. 4A).

Next, the additional effect of GAGs isolated from the medium of healthy control and MPS I chondrocytes was analyzed. When incubation with FGF2 and MPS I GAGs was compared to incubation with FGF2 and healthy control GAGs, there was a significant difference in growth between WT and MPS I bones \( p<0.01 \), as compared to healthy control GAGs \( p<0.05 \), however, MPS I bones showed decreased growth in the presence of MPS I GAGs \( p<0.05 \), as compared to healthy control GAGs.

To confirm this, incubation with FGF2 was compared to incubation with FGF2 and control GAGs, or FGF2 and MPS I GAGs in a separate experiment. Incubation with control GAGs, in addition to FGF2, did not significantly alter growth of healthy control or MPS I bones (results not shown). Incubation with MPS I GAGs, however, significantly decreased growth of MPS I bones \( p<0.01 \), as compared to incubation with only FGF2, but did not affect WT bone growth (results not shown).

**Structural abnormalities in MPS I growth plates**

Femur length of 11 week old MPS I mice was 4% decreased \( p<0.05 \), as compared to WT mice (results not shown). As earlier reported \(^{23}\), growth plates of MPS I mice exhibit several structural abnormalities, as compared to growth plates of WT mice (fig. 5). In growth plates of 3 week old WT mice, large proliferating zones and smaller hypertrophic zones of chondrocytes were observed (fig. 5A). These zones were of equal size in MPS I growth plates (fig. 5B). With a higher magnification, long rows of proliferating chondrocytes and a heterogeneous aspect of the hypertrophic chondrocytes were observed in WT growth
plates (fig. 5C). In MPS I growth plates, however, the rows of proliferating chondrocytes were shorter and more chaotically distributed (fig. 5D). In addition, the large hypertrophic zone was densely packed with chondrocytes with a square and swollen aspect (fig. 5D). In growth plates of 11 week old MPS I mice, the hypertrophic zones still showed an increased amount of large hypertrophic chondrocytes (fig. 5F), as compared to WT growth plates (fig. 5E).

GAG distribution in MPS I growth plates

To study whether growth plates of MPS I mice exhibit alterations in GAG distribution, as was observed for MPS I chondrocytes (fig. 2), staining with antibodies against specific HS, DS and CS domains was performed. Using the anti-HS antibody LKIV69 (fig. 1A, B), which binds to 2-\(O\)-, and \(N\)-sulfated HS domains, brown GAG staining was observed in the ECM of growth plates of 3 week old WT mice (fig. 6A), but not in the ECM of MPS I growth plates (fig. 6B). In addition, the use of the anti-HS antibody HS4C3 revealed a higher abundance of 3-\(O\)-sulfated glucosamine domains in growth plates of 11 week old MPS I mice (fig. 6D), as compared to WT growth plates (fig. 6C).
Growth factor distribution in MPS I growth plates

DAB staining using an anti-FGF2 antibody was performed to analyze the distribution of FGF2 in growth plates of 3 week old WT and MPS I mice. FGF2 staining was clearly present in chondrocytes and in the ECM of the proliferating and hypertrophic zone of the WT growth plate (fig. 7A). In the proliferating zone of MPS I growth plates, however, a low abundance of FGF2 and a clearly visible blue nuclear counterstaining was observed (fig. 7B).

To study whether MPS I growth plates also exhibited alterations in other growth factors, DAB staining using an anti-Ihh antibody was performed. In the WT growth plate, most Ihh staining was present in the hypertrophic and prehypertrophic zone, with clearly visible nuclear counterstaining in the proliferating zone (fig. 7C). In the MPS I growth plate, however, most of the Ihh staining was present in the proliferating and prehypertrophic zone (fig. 7D).
DISCUSSION

Skeletal disease is one of the most prevalent and incapacitating disease manifestations in patients suffering from the MPSs and frequently results in the need for multiple surgical interventions. Current therapeutic strategies have a limited effect on the progression of MPS bone disease. The pathophysiological processes initiated by accumulated GAGs are still poorly understood, which limits the development of new therapeutic strategies. In this study, we demonstrate alterations in the interaction and distribution of GAGs and growth factors in chondrocytes from MPS I patients and growth plates of MPS I mice. This suggests that these pathophysiological processes contribute to MPS bone disease and we hypothesize that growth factor regulation may be a future therapeutic target.

Figure 7. Immunostaining of WT (A) and MPS I (B) growth plates using an anti-FGF2 antibody and staining of WT (C) and MPS I (D) growth plates with an anti-Ihh antibody in 3 week old mice. For FGF2 staining, the arrows show brown DAB staining in nuclei of proliferating chondrocytes of WT (A) and absence of brown DAB staining, but clearer blue counterstaining in the nuclei of proliferating chondrocytes in MPS I (B) growth plates. For Ihh staining, the arrows show clear blue counterstaining of nuclei of proliferating chondrocytes in WT (C) and brown DAB staining in nuclei of proliferating chondrocytes in the MPS I growth plate (D). For each group, the experiment was performed in bones from 4 different mice, 1 representative figure per group is shown. P is the proliferating zone and H the hypertrophic zone of chondrocytes.
The influence of GAGs on growth factor regulation and signaling is complex and depends on the growth factor itself, on the binding capacity of GAGs for growth factors, and on the quantity, location (ECM, cell membrane, intracellular) structure (sulfation) and distribution of GAGs. Increased GAG levels in the ECM, for instance, may enhance the distribution of certain growth factors to their target cells, or on the other hand, may cause a delay in the distribution of other growth factors.

Because previous studies showed that GAGs with abnormal sulfation patterns accumulate in MPS cells and tissues, we investigated whether this results in altered interaction and distribution of GAGs and growth factors, as this may contribute to MPS I bone disease.

Pan et al. showed that binding of FGF2 to GAGs from the culture medium of human multipotent stem cells was decreased in MPS I. The medium of MPS I cells, however, probably contained an increased amount of GAGs, as compared to the medium of healthy control cells, which may have influenced the results. Using the same amounts of GAGs that were isolated from the conditioned medium of chondrocytes, we observed a trend, although not significant, towards lower FGF2 binding capacity of MPS I GAGs, as compared to healthy control GAGs. A small decrease in growth factor binding capacity of GAGs may, especially in the presence of increased quantities of these GAGs and abnormal GAG structure or distribution, significantly alter growth factor function. For instance, a decrease in growth factor binding may alter growth factor distribution, and may also change the rate of inactivation of growth factors, as it has been shown that growth factors bound by GAGs are protected against inactivation.

Next, to investigate whether MPS I chondrocytes exhibit abnormalities in GAG content or distribution, chondrocytes were stained using antibodies against specific HS, DS, and CS domains. We observed altered distribution of 2-O-, 3-O-, and N-sulfated HS-domains and DS-disaccharides composed of iduronic acid and 4-O-sulfated galactosamine residues in the ECM of MPS I chondrocytes.

As alterations in GAG distribution may cause alterations in growth factor signaling, FGF2 signaling was studied by analyzing the second messenger pERK. After FGF2 incubation, pERK levels were increased in MPS I chondrocytes, as compared to control chondrocytes. Possible causes of this effect include GAG accumulation in the ECM of MPS I chondrocytes, which may, despite the small decrease in binding capacity of MPS I GAGs, lead to increased binding of FGF2, protection from inactivation, and increased distribution to target cells. We cannot exclude that MPS I cells are more sensitive to the effects of FGF2, and MPS I chondrocytes show no differences in gene-expression of FGF-receptors (unpublished results).
To study the effect of FGF2 on bone growth, metatarsals from WT and MPS I mice were incubated with FGF2. FGF2 treatment led to decreased growth of WT bones, which was expected, because FGF2 is an anti-proliferative growth factor in growth plates. With the concentration used in our experiments, FGF2 did not affect growth of MPS I mouse bones. An explanation may be that, due to the altered distribution of GAGs in MPS I bones, FGF2 is not distributed effectively and cannot induce its anti-proliferative effect. Also, due to accumulation of GAGs in the ECM, FGF2 may be bound and delayed the ECM. On the other hand, decreased binding of FGF2 to MPS I GAGs may result in increased degradation of FGF2, and a loss of its anti-proliferative effect.

Next, we studied the additional effect of GAGs isolated from the medium of healthy control or MPS I chondrocytes. Incubation of MPS I bones with medium containing MPS I GAGs, in the presence of FGF2, led to decreased bone growth, as compared to medium that contained control GAGs. In contrast, incubation of WT bones with MPS I GAGs, in the presence of FGF2, led to increased bone growth, or more correctly, decreased growth inhibition, as compared to incubation with control GAGs. Taken together, these results show that WT and MPS I bones react differently on treatment with FGF2 and externally added GAGs. The observed differences in growth patterns, however, are difficult to explain based on only alterations in FGF2 binding, signaling or distribution. Our results, therefore, suggest that externally added GAGs may also impact other growth factors that are present in the bone itself, or growth factors that are excreted in the medium by bone cells. Earlier reports on the effects of incubation of MPS I multipotent stem cells with growth factors showed decreased BMP4 signaling and a decreased effect of FGF2 treatment. These results are in contrast to our observation of increased FGF2 signaling in MPS I chondrocytes, but are in agreement with our observed decrease in FGF2-induced growth inhibition of MPS I mouse bones. Bone growth and growth factor signaling depend on many factors, such as growth factor activity and distribution, GAG distribution in the ECM and on the cell membrane and growth factor receptor expression. Likely, these factors, which were not all analyzed in our study, contribute to the differences in the effect of FGF2 that are observed in different studies.

We confirmed our results on altered distribution of 2-O-, 3-O-, and N-sulfated HS-domains in MPS I chondrocytes in MPS I mouse bones. Because these GAG domains are known to interact with growth factors, growth factor distribution was studied. The distribution of FGF2 in the developing growth plate of MPS I mice was altered, as compared to WT growth plates. Also, distribution of another essential growth factor, Ihh, suggests that alterations in GAG quantity, structure and distribution may cause alterations in the function and distribution of multiple growth factors, which may significantly contribute to the pathophysiological mechanisms underlying MPS bone disease.
Previous studies have shown GAG accumulation in the ECM of MPS cells, but distribution of different GAG domains and growth factors, have, to our knowledge not been described earlier. Our results, however, are in agreement with the results of Heppner et al. on abnormalities in gene expression of ECM proteins in MPS I mouse bones, and their suggestion that ECM disruption significantly contributes to the pathogenesis of MPS I bone disease.

There are a number of promising future therapeutic strategies for MPS bone disease, such as anti-inflammatory therapy to prevent the secondary inflammation processes induced by accumulating GAGs. Also, as chondrocytes are derived from mesenchymal cells, mesenchymal stem cell transplantation may increase the therapeutic efficacy of current HSCT protocols. Our results suggest that targeting growth factor regulation or the interaction between growth factors and GAGs may also be a promising therapeutic strategy for the prevention of MPS bone disease. Growth factor therapy, such as monoclonal antibodies against growth factor receptors is currently used as a therapy for some forms of cancer. These therapies, however, are focused on achieving cell death, and may have severe adverse effects. For the prevention of MPS bone disease, only drugs with an excellent safety profile that can be used for a long term period at and a very young age, are acceptable. Furthermore, the role of different growth factors and the contribution of different types of GAGs in the pathogenesis of MPS bone disease first need to be elucidated. Also, because growth factors regulate growth and development of all tissues, the interaction between growth factors and GAGs in other tissues than bone should be studied in the different MPSs.

CONCLUSION
Our results suggest that altered interaction and distribution of accumulated GAGs and growth factors contributes to the pathogenesis of MPS bone disease. In the future, targeting growth factor regulation or the interaction between certain growth factors and GAGs might be a therapeutic strategy for MPS bone disease.

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We thank Ronald Wanders and Rene Leen for technical assistance and helpful discussions. We thank Marie-Louise Kingma and Jan Ruijter for help with statistical analysis. We thank Toin van Kuppevelt for kindly providing the GAG antibodies. This work was funded by the foundation ‘Steun Emma Kinderziekenhuis AMC’ and the ‘WE Foundation’.

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Chapter
Genistein increases glycosaminoglycan levels in Mucopolysaccharidosis type I cell models

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ABSTRACT
Mucopolysaccharidosis type I (MPS I) is a lysosomal storage disorder characterized by diminished degradation of the glycosaminoglycans (GAGs) heparan sulfate and dermatan sulfate, which results in the accumulation of these GAGs and subsequent cellular dysfunction. Patients present with a variety of symptoms, including severe skeletal disease. Genistein has been shown previously to inhibit GAG synthesis in MPS fibroblasts, presumably through inhibition of tyrosine kinase activity of the epidermal growth factor receptor (EGFR). To determine the potentials of genistein for the treatment of skeletal disease, MPS I fibroblasts were induced into chondrocytes and osteoblasts and treated with genistein. Surprisingly, whereas tyrosine phosphorylation levels (as a measure for tyrosine kinase inhibition) were decreased in all treated cell lines, there was a 1.3 and 1.6 fold increase in GAG levels in MPS I chondrocytes and fibroblasts, respectively (p<0.05). Sulfate incorporation in treated MPS I fibroblasts was 2.6 fold increased (p<0.05), indicating increased GAG synthesis despite tyrosine kinase inhibition. This suggests that GAG synthesis is not exclusively regulated through the tyrosine kinase activity of the EGFR. We hypothesize that the differences in outcomes between studies on the effect of genistein in MPS are caused by the different effects of genistein on different growth factor signaling pathways, which regulate GAG synthesis. More studies are needed to elucidate the precise signaling pathways which are affected by genistein and alter GAG metabolism in order to evaluate the therapeutic potential of genistein for MPS patients.
INTRODUCTION
The mucopolysaccharidoses (MPSs) comprise a group of lysosomal storage diseases, each caused by a single enzyme deficiency, leading to diminished glycosaminoglycan (GAG) degradation. Consequently, GAGs accumulate in lysosomes, resulting in progressive cellular and organ dysfunction. MPS type I (OMIM 252800), is caused by deficiency of the hydrolase α-L-iduronidase (IDUA, EC 3.2.1.76), resulting in impaired heparan sulfate (HS) and dermatan sulfate (DS) degradation and subsequent GAGs accumulation. This leads to progressive cardiac and pulmonary disease, inguinal and umbilical hernia, corneal clouding and severe musculoskeletal disease. In addition, patients with the severe (Hurler) phenotype also suffer from progressive central nervous system (CNS) disease, significantly limiting life expectancy 1.

The constellation of radiographic abnormalities resulting from defective intramembranous and endochondral bone formation is collectively referred to as dysostosis multiplex. These skeletal changes may lead to progressive loss of joint motion with contractures, growth arrest, kyphosis, scoliosis, hip dysplasia and hypoplastic vertebral bodies resulting in atlanto-axial instability and spinal cord compression 1,2. Current therapeutic strategies, such as haematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT), effectively treat many features of MPS I, but have limited effects on bone disease 3–5. Efficacy of HSCT is limited because cartilage cells are derived from mesenchymal stem cells and these are not transplanted in sufficient amounts by HSCT 6. In addition, circulating enzymes do not easily reach growth plates and other cartilaginous tissue due to the relative avascularity of cartilage. Finally, the relatively large molecular weight of lysosomal hydrolases hampers easy diffusion through the matrix to target cells, which limits the efficacy of both HSCT and ERT on cartilaginous tissues 3,4,7,8.

An alternative strategy for the treatment of bone disease in MPS I might be substrate reduction therapy which aims to reduce GAG synthesis, thus decreasing the accumulation of undegradable material. Such an approach may prevent or halt the pathophysiological cascades initiated by the accumulating GAGs, which involve inflammatory processes, dysregulation of osteoclastogenesis and apoptosis of chondrocytes, all resulting in abnormal bone formation and growth 9. Genistein, which is an isoflavone naturally occurring in soy and several other plants, inhibits the activity of tyrosine kinase receptors including the epidermal growth factor receptor (EGFR) 10. Previous studies demonstrated that genistein inhibits GAG synthesis in MPS fibroblasts (FBs), presumably through this mechanism 11,12. Genistein is well tolerated, appears to be safe also in high doses 13 and can reach bone tissue 14. In this study, we aimed to investigate the effects of genistein on GAG accumulation in induced chondrocytes (ICs) and osteoblasts (IOs) of MPS I patients in order to determine the potential in the treatment of MPS I related bone disease.
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MATERIALS AND METHODS

Chemicals

Dulbecco’s Modified Eagle’s Medium (DMEM), penicillin, streptomycin and amphotericin were obtained from Lonza (Basel, Switzerland). Fetal bovine serum (FBS) was from Bodinco B.V. (Alkmaar, The Netherlands). Bovine serum albumin (BSA), phosphatase inhibitors, insulin, dexamethasone, alcian blue and nuclear fast red were from Sigma Aldrich (St. Louis, MO, USA). Genistein aglycone (>98% pure) was from Sigma Aldrich or received as a kind gift from Axcentua (Huddinge, Sweden). Recombinant TGF-β was from MT-diagnostics (Metten-Leur, The Netherlands). Complete mini protease inhibitor cocktail, first-strand cDNA synthesis kit and LC480 SYBR Green Master mix was from Roche Applied Science (Indianapolis, IN, USA). 1-step Para-nitrophenyl-phospate (PNPP) solution was from Thermo scientific (Waltham, MA, USA). Para-nitrophenol was from Merck Schuchardt (München, Germany). CellTiter 96® Aqueous One Solution Reagent was from Promega (Mannheim, Germany). TRIzol was from Invitrogen (Bleiswijk, The Netherlands). Primers were from Biolegio (Nijmegen, The Netherlands) or Sigma Aldrich. H$_{35}$SO$_4$ was from Hartmann Analytic GmbH (Huissen, The Netherlands). Antibodies against phosphorylated tyrosin (pTyr), LAMP-1 and tubulin (mouse) were from Cell signaling technologies (Danvers, MA, USA), tubulin (rabbit) from Sigma Aldrich and all secondary antibodies from Westburg B.V. (Leusden, The Netherlands). Paraformaldehyde, glutaraldehyde, Triton-X-100, sodium thiosulphate, silver nitrate, eosin, hematoxylin, ascorbate-2-phosphate and glycerol-2-phosphate were of analytical grade.

Cell culture

Informed consent for the use of FBs was obtained from all patients or parents. FBs were grown in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100μg/ml streptomycin and 250μg/ml amphotericin in a humidified atmosphere containing 5% CO$_2$ at 37°C.

Induction of differentiation

Chondrogenic and osteogenic differentiation of FBs was performed essentially as described earlier $^{15}$, with minor modifications. Osteogenic induction medium contained 5% FBS instead of 10%. One day after plating cells, induction medium was added and cells were maintained in induction medium for 3 weeks. Medium was changed twice a week.

Chondrogenic induction was verified by analyzing cell morphology, proteoglycan production (alcian blue staining) and gene-expression of chondrogenic markers. Osteogenic induction was verified by analysis of cell morphology, calcium deposition (von Kossa staining), alkaline phosphatase (ALP) activity and gene-expression of an osteogenic marker.
Isoflavone treatment

After 2 weeks of induction, induction medium of ICs and IOs was supplemented with 50μM of genistein dissolved in DMSO (final DMSO concentration in medium was 0.1%). Unsupplemented cells were incubated with induction medium containing only 0.1% DMSO. Cells were harvested after one week of genistein treatment. In the experiments with undifferentiated FBs, cells were allowed to attach after which genistein was added to the medium. FBs were treated for 2, 4 or 7 days with 50μM genistein or 2 days with 25μM genistein.

Cell viability

For all the conditions tested, cell viability was measured using CellTiter 96° Aqueous One Solution Reagent (containing MTS tetrazolium). The quantity of produced formazan was measured at 485nm and is directly proportional to the number of metabolically active cells in culture. All measurements were performed in triplicate.

Histology

Cell morphology was analyzed with routine hematoxylin/eosin (HE) staining. Alcian blue staining was used to detect proteoglycans and von Kossa staining to detect calcified extracellular matrix (ECM). Cells were fixed in 4% glutaraldehyde, washed three times with phosphate buffered saline (PBS), and for alcian blue staining, stained 15 minutes with 10g/L alcian blue in 3% acetic acid and counterstained for 9 minutes using nuclear fast red. For von Kossa staining, fixed cells were stained for 90 minutes with 50g/L silver nitrate solution under a 40 watt light, and treated for 9 minutes with 50g/L sodium thiosulphate.

Alkaline phosphatase activity

To confirm osteogenic differentiation, ALP activity was measured. Cells were detached with a cell scraper in 9g/L NaCl solution containing a cocktail of protease inhibitors, and disrupted

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Table 1. Primer sequences used for RT-qPCR
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Genistein increases glycosaminoglycan levels in MPS I cell models

by sonification using a Vibra Cell sonicator (Sonics & Materials Inc., Newtown, CT, USA). Protein concentration was measured in whole cell lysates as earlier described. 50μl of cell lysate was added to 100μl of PNPP solution, to a final protein concentration of 0.1 mg/ml. After 10 minutes of incubation at 37°C, the reaction was stopped by addition of 100μl of 1M NaOH. The absorbance of released para-nitrophenol was measured at a wavelength of 405nm. ALP activity was calculated using a calibration curve of para-nitrophenol. All assays were performed in duplicate.

Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent. cDNA was produced using a first-strand DNA synthesis kit. qPCR analysis for chondrogenic markers (ACAN, COL2A1, COL10A1, SOX9), the osteogenic marker SPARC (osteonectin) and genes involved in HS synthesis (XYLT1, EXT1, EXT2, NDST1, NDST2, HS2ST1, HS6ST1) was performed using LC480 SYBR Green Master mix. Melting curve analysis and gel electrophoresis were carried out to confirm the generation of a single product. Duplicate analyses were performed for all samples. Data were analyzed using linear regression calculations as earlier described. To adjust for variations in the amount of RNA, all values were normalized against the housekeeping gene PPIB (cyclophilin B). Primer sequences are described in table 1.

GAG analysis

The effect of genistein on GAG levels in FBs, ICs and IOs was determined by measuring HS and DS-derived disaccharides using HPLC-MS/MS, as described previously, with one minor modification: instead of 25μg, 12.5-50μg of protein was used. In all experiments, the effects of genistein were similar for the different disaccharides, therefore only values of the sum of all GAG derived disaccharides are given.

Sulfate incorporation

GAG synthesis was approximated by monitoring 35S incorporation using an earlier described, modified protocol. FBs were plated and the next day the medium was supplemented with 50μM genistein or only DMSO. After 48 hours of incubation, half of the medium was removed and fresh medium containing 50μM genistein and 20μCi/ml of H₂³⁵SO₄ was added. 24 hours later, cells were washed 6 times with PBS and lysed in PBS containing a cocktail of protease inhibitors and 25g/L Triton-X-100. 35S incorporation was measured in a scintillation counter. Counts were corrected for protein concentration measured in aliquots of the same cell lysates. 35S incorporation experiments were performed in at least 2 independent cultures.

Western blot analysis

Cells were detached with a cell scraper in a 9g/L NaCl solution containing protease and
phosphatase inhibitors, and 5g/L Triton-X-100. Cells were disrupted by sonification and protein concentration was measured. 40μg of protein was loaded onto a 10% SDS-polyacrylamide gel for detection of pTyr or 50μg for detection of LAMP-1, and after electrophoresis, transferred onto a nitrocellulose membrane. Membranes were blocked in 50g/L BSA, 0.1% Tween-20 in Tris buffered saline, pH 7.4, for detection of pTyr or 30g/L BSA, 0.1% Tween-20 in PBS for the detection of LAMP-1. Expression levels were normalized for the housekeeping protein tubulin, detected on the same membrane. Antibodies were diluted as follows: pTyr; 1:400, tubulin (rabbit); 1:1000, LAMP-1; 1:1,000, tubulin (mouse); 1:5,000 solution, IRDye 800 goat anti-mouse; 1:5,000, IRDye 680 goat anti-rabbit; 1:5,000, IRDye 800 goat anti-rabbit; 1:10,000, IRDye 680 donkey anti-mouse; 1:5,000.

Statistical analysis
Statistical analysis was performed using Students t-test. Significance was assumed where \( p \) values were less than 0.05.

RESULTS

Evaluation of chondrogenic and osteogenic induction
After induction, HE staining revealed that both ICs and IOs lost the elongated shape typical of FBs (fig. 1A-C). In addition, while FBs grew neatly in a monolayer (fig. 1A), ICs were forming ridges with empty spaces in between (fig. 1B). In contrast, IOs grew in multiple layers with high cellular density (fig. 1C). Alcian blue staining revealed the ECM of ICs to be rich in proteoglycans (fig. 1D-E). Furthermore, ICs had a 275 fold, 53 fold, 25 fold and 48 fold increase in gene expression of ACAN, COL2A1, COL10A1 and SOX9, respectively, as compared to FBs (fig. 1F). Von Kossa staining revealed a calcified ECM around IOs (fig. 1G-H). Furthermore, there was a 3 fold increase in gene expression of SPARC (fig. 1I) and a 27 fold increase in ALP activity (fig. 1J), as compared to FBs. These results suggest successful transdifferentiation of FBs into ICs and IOs.

Cell viability
At the end of each experiment, cell viability was analyzed. In ICs and IOs, cell viability was more than 80% in genistein-treated cells compared to untreated cells (results not shown). Cell viability was more than 90%, 70% and 60% in FBs treated for 2, 4 and 7 days, respectively (results not shown).

GAG analysis
As expected, analysis of GAG levels revealed significantly higher levels in MPS I cells, as compared to healthy control cells (fig. 2). There was no effect of genistein on GAG levels in
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healthy control ICs (fig. 2A). In contrast, a significant increase of GAG levels was observed when MPS I ICs were treated with genistein (fig. 2A). In IOs, there was no significant difference in GAG levels between genistein-treated and untreated cells (fig. 2B). MPS I FBs treated for either 2, 4, or 7 days with 50μM genistein, showed significantly higher GAG levels compared to untreated MPS I FBs (fig. 2C-E). When MPS I FBs were treated for 2 days with 25μM genistein, there was no effect of genistein on GAG levels (results not shown). To exclude that the unexpected increases in GAG levels were due to the source of genistein, FBs were treated for 7 days with 50μM of genistein from another manufacturer (Axcentua). Again, no decrease in GAG levels after genistein treatment was observed in either control or MPS I FBs (results not shown).

Figure 1. Transdifferentiation of FBs. HE staining of FBs (A), ICs (B) and IOs (C). Alcian blue staining of FBs (D) and ICs (E), GAGs are stained blue. Fold change in mRNA expression of chondrogenic markers in ICs (F), expression levels in FBs are set at 1. Von Kossa staining of FBs (G) and IOs (H), deposited calcium is stained brown. Fold change in mRNA expression of the osteogenic marker osteonectin (SPARC) in IOs (I), expression levels in FBs are set at 1. Fold change in ALP activity in IOs (J), all values are mean ± standard deviation of 3 healthy control cell lines in independent experiments, activity in FBs is set at 1. Gene expression data: all values are mean ± standard deviation of replicates in one representative experiment in a control cell line (expression levels in fibroblasts were detectable but often below limit of quantification, therefore no standard deviation could be calculated between different experiments). All analyses were repeated at least once in independent cell cultures in another control cell line and MPS I cell line, with similar results.
Sulfate incorporation

GAG synthesis was approximated by monitoring $^{35}$S incorporation. In accordance with the increase in GAG levels measured by HPLC-MS/MS, sulfate incorporation was significantly increased (almost 3 fold) in MPS I FBs treated with genistein compared to untreated MPS I FBs (fig. 2F). There was no difference between treated and untreated healthy control FBs.

Lysosomal abundance

LAMP-1 protein levels were determined by western blot as a measure of lysosomal
Levels of phosphorylated tyrosine

To confirm that genistein inhibited tyrosine kinase activity, we determined global phosphorylated tyrosin (pTyr) levels in cells treated with genistein. Protein levels of pTyr differed between cell lines, however, in all cell types, pTyr levels were clearly decreased in genistein treated cells compared to their untreated controls (fig. 3). When data from the different cell lines were grouped and mean pTyr levels were calculated, a significant decrease in pTyr levels in MPS I ICs treated with genistein was observed, as compared to untreated controls. In MPS I IOs and healthy control FBs, a trend towards lower pTyr levels ($p=0.08$) was observed when data from different cell lines were grouped (results not shown). These results indicate that genistein inhibited tyrosine phosphorylation in these cell culture models.

Gene expression of enzymes involved in HS synthesis

The influence of genistein on gene expression of enzymes involved in HS synthesis was measured using RT-qPCR. Significant differences in gene expression of $\geq2$ fold gene expression were considered biologically relevant and significant differences of $\geq1.5$ fold were considered as a trend towards higher or lower gene expression. In control ICs treated
with genistein, there was a significant increase (fig. 4A) in NDST2 (2.4 fold, $p<0.05$) and HS2ST1 (2.3 fold, $p<0.05$). In MPS I ICs, a 2.3 fold increase ($p<0.01$) in HS6ST1 was observed, as compared to untreated ICs. There was a trend towards higher expression of most of the other HS synthesis genes in genistein treated ICs (fig. 4A). As compared to untreated ICs, EXT2 was 1.5 fold ($p<0.05$) increased in control ICs and NDST1, NDST2 and HS2ST1 were 1.5 fold ($p<0.05$), 1.5 fold ($p<0.01$) and 1.9 fold ($p<0.001$) increased, respectively, in MPS I ICs. There were no (biologically relevant) differences detected in FBs or IOs treated with genistein, as compared to untreated cells (fig. 5B-C).

**DISCUSSION**

Skeletal disease is one of the most prevalent and incapacitating disease manifestations of the MPSs and frequently results in the need for multiple orthopaedic surgeries $^{1,2}$. Disease modifying therapies for the management of MPS I (HSCT and ERT) have led to increased lifespan but have little effect on the progression of skeletal deformities $^{5,8,21}$. Therefore, therapeutic strategies targeting bone disease are urgently needed.

The isoflavone genistein has been investigated for its potential benefit in the treatment of CNS disease in MPS III, as *in vitro* studies showed that genistein may reduce GAG synthesis and thereby GAG accumulation $^{12,22-24}$. Indeed, an *in vivo* study revealed decreased GAG levels in the brain of MPS III mice treated with genistein $^{25}$. As prevention of GAG accumulation in cartilage and bones might halt the progression of skeletal disease in the MPSs, we set out to study the *in vitro* effects of genistein on ICs and IOs, derived from MPS I FBs. However, in remarkable contrast to previously published studies on the effects of genistein in MPS...
FBs 12,22–24, GAG levels significantly increased in MPS I ICs and FBs treated with genistein and remained unchanged in IOs. The discrepancy between our results and previously published data does not appear to be due to the source of genistein, as we observed similar responses using two different sources of genistein. Also differences in the methodology used for GAG analysis cannot explain this discrepancy as both HPLC-MS/MS analysis and sulfate incorporation measurements, which is the most frequently used test to measure GAG synthesis in in vitro studies on the effects of genistein in the MPSs, showed increased GAG synthesis or levels in MPS I FBs.

In vivo studies on the effects of genistein in MPS patients and animal models also show divergent results. When MPS III patients were treated with low dose soy extracts (containing genistein/genistin at a dose of 5-15mg/kg/day), some open-label studies showed positive effects 26,27, while in another study no effect on both biochemical and clinical parameters was observed 28. In a placebo controlled trial, a small but significant reduction in plasma and urinary GAGs was observed, with no effects on clinical variables 29. In a study in MPS III mice, a high dose of pure genistein (160mg/kg/day) for 9 months resulted in an impressive decrease in brain storage levels, decreased neuroinflammation and improvement of behavioural abnormalities 25. However, no decrease of urinary GAG levels or improvement in neurocognitive or disability scores were observed in MPS III patients who received a similar dose of 150mg/kg/day of pure genistein for at least 1 year 13.

The concentrations of genistein, used in our study, were higher or equal (50-25μM) to concentrations used in previous in vitro studies 12,22–24. In MPS III patients treated with low dose soy extracts (containing genistein and its glucuronide genistin at a total dose of 10mg/kg/day) 29, the total genistein/genistin concentration measured in plasma was 8.8mM, while the concentration of the biologically active form, genistein (the aglycone form), was 48μM (unpublished results). The concentration of genistein aglycone corresponds with the concentration used in our study.

The reduction in GAG levels, as observed in previously published in vitro studies and some of the in vivo studies, is considered a result of EGFR inhibition and subsequent downregulation of GAG synthesis 23. In our study, however, no downregulation of genes involved in HS synthesis was observed, in contrast, there was a trend towards upregulation of some of these genes, while the levels of phosphorylated tyrosine were lowered after genistein treatment. This indicates that genistein does not exclusively influences GAG levels through inhibition of phosphorylation of tyrosine residues of the EGFR.

Although maximal GAG synthesis requires the presence of either EGF or follicle-stimulating hormone 30, many other growth factors are also involved in the regulation of GAG production,
such as insulin-like growth factor 1 (IGF1) 31, platelet derived growth factor 32 and members of the TGF-β superfamily, including TGF-β1 32 and bone morphogenic proteins 33,34. The actions of these growth factors are essential for bone and cartilage metabolism 35 and genistein affects at least some of them. For instance, the IGF1 receptor is - among a large amount of other receptors - a tyrosine kinase receptor and expression levels have shown to be inhibited by genistein 36. In addition, TGF-β expression was shown to be decreased in cells treated with genistein 37, though enhancement of TGF-β secretion in the medium of cells has also been described 38.

Regulation of GAG synthesis is a very complicated process and numerous feedback mechanisms are likely to be involved. Small differences in cell culture conditions (e.g. composition of the culture medium and the source of fetal bovine serum) in in vitro studies, as well as genetic heterogeneity and external factors in patients and mouse models may contribute to the differences observed between studies.

CONCLUSION

Our study underscores the complexity of the regulation of GAG synthesis which may result in seemingly conflicting effects of isoflavones (genistein and other components of soy extracts) in in vitro and in vivo studies. We feel that our study shows that genistein should be used with caution in patients with the MPSs, as its effects are difficult to predict and may be adverse. Additional studies to elucidate the biochemical processes influenced by genistein in the MPSs, as well as placebo controlled clinical trials, are urgently needed to assess the scope of biochemical and clinical effects of genistein.

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Chapter
Adverse effects of genistein in a Mucopolysaccharidosis type I mouse model

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ABSTRACT

Mucopolysaccharidosis type I (MPS I) is a lysosomal storage disorder characterized by diminished degradation of the glycosaminoglycans (GAGs) heparan sulfate (HS) and dermatan sulfate (DS). Patients present with a variety of symptoms, including severe skeletal disease. Current therapeutic strategies have only limited effects on bone disease. The isoflavone genistein has been studied as a potential therapy for the mucopolysaccharidoses because of its putative ability to inhibit GAG synthesis and subsequent accumulation. Cell, animal and clinical studies, however, showed variable outcomes. To determine the effects of genistein on MPS I related bone disease, wild type (WT) and MPS I mice were fed a genistein supplemented diet (corresponding to a dose of approximately 160mg/kg/day) for 8 weeks. HS and DS levels in bone and plasma remained unchanged after genistein supplementation, while liver HS levels were decreased in genistein-fed MPS I mice as compared to untreated MPS I mice. Unexpectedly, genistein-fed mice exhibited significantly decreased body length and femur length. In addition, 60% of genistein-fed MPS I mice developed a scrotal hernia and/or scrotal hydrocele, manifestations which were absent in WT or untreated MPS I mice. In contrast to studies in MPS III mice, our study in MPS I mice demonstrates no beneficial but even potential adverse effects of genistein supplementation. Our results urge for a cautious approach on the use of genistein, at least in patients with MPS I.
INTRODUCTION

Mucopolysaccharidosis type I (MPS I, OMIM 252800) is a lysosomal storage disorder (LSD) caused by α-L-iduronidase (IDUA, EC 3.2.1.76) deficiency, resulting in impaired degradation and subsequent accumulation of the glycosaminoglycans (GAGs) heparan sulfate (HS) and dermatan sulfate (DS). Patients may present with cardiac and pulmonary disease, inguinal and umbilical hernia, corneal clouding and progressive central nervous system (CNS) disease which significantly limits life expectancy. In addition, skeletal dysplasia, generally referred to as dysostosis multiplex, is a striking feature and a major cause of morbidity. Patients show progressive loss of range of joint motion with contractures, growth arrest, kyphosis, scoliosis, hip dysplasia and hypoplastic vertebral bodies resulting in spinal cord compression. Current therapies, including haematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy, effectively treat many features of MPS I, but have limited effects on bone disease. Several factors contribute to this lack of effect. Firstly, the inability of the relatively large enzyme to traffic through the poorly vascularized matrix of growth plates and other cartilaginous tissue to target cells. Secondly, cartilage cells are derived from mesenchymal stem cells, which are not transplanted in sufficient amounts by HSCT. Thirdly, therapy is started after the onset of irreversible bone lesions, which may already exist before birth. An alternative treatment strategy for LSDs is substrate reduction therapy, which aims to reduce the synthesis of the accumulating material, thereby preventing or halting lysosomal storage. This approach has been used successfully in Gaucher disease and Niemann Pick disease type C.

The isoflavone genistein has several biological activities. It is an antioxidant, has estrogenic activity and inhibits the activity of tyrosine kinase receptors including the epidermal growth factor receptor (EGFR). Genistein has been shown to reduce GAG synthesis in MPS fibroblasts, at least partly via the latter mechanism. An in vivo study with a high dose of genistein in MPS IIIB mice, showed reduced GAG levels in brain and impressive amelioration of neurological symptoms. Although genistein appears to be well tolerated in high doses, adverse effects, which are associated with its potential antiproliferative and estrogenic actions, have been reported and may include hepatotoxicity and hormonal disbalance.

The only study on the effects of genistein on MPS related bone disease showed increased range of joint motion in genistein treated MPS II patients, suggesting that genistein at least reaches the surrounding connective and muscle tissue of the joints. Because genistein can reach bone tissue, we fed MPS I mice a high dose genistein diet to evaluate its potential for the treatment of MPS I related bone disease.
CHAPTER 7

MATERIALS AND METHODS

Animals

MPS I mice (B6.129-Iduagtm2Clk/J, \cite{19}) were purchased from Jackson Laboratory and maintained as a heterozygote line on an inbred C57BL/6J background at the Academic Medical Centre. The mice were housed at 21 ± 1°C, 40-50% humidity, on a 12 hours light-dark cycle, with ad libitum access to water and food. Genotypes were identified by PCR using a protocol provided by Jackson Laboratory.

An AIN93M diet \cite{20} with 3.7% sunflower oil and 0.3% rapeseed oil instead of 4% soy oil was produced (Research Diet Services B.V.). For the genistein-fed group, genistein aglycone (kind gift from Axcentua) was added to the diet in a concentration of 0.1% (w/w). Assuming a food intake of 0.16g food per gram of body weight per day, this diet results in a dose of 160mg/kg/day, which is similar to other studies on the effects of a high dose of genistein in MPS mice \cite{14,21}. One week before weaning, mice received the soy-free diet. At 3 weeks of age, male wildtype (WT) and MPS I mice were weaned on the soy-free diet or the genistein-supplemented diet (n=10 per group). Every week, mice were weighed and examined for general pathological manifestations. If scrotal abnormalities were observed, scrotal hydrocele and/or scrotal hernia were objectified by macroscopic inspection, transillumination and/or ultrasound by a skilled animal technician and macroscopic inspection of the scrotum and abdominal and pelvic cavity after sacrifice. At 11 weeks of age, mice were anesthetized with an intraperitoneal injection of 100mg/kg pentobarbital and euthanized by exsanguination via cardiac puncture. Body length and femur length were measured, and blood and tissues collected. All animal experiments were approved by the animal institutional review board at the Academic Medical Centre, University of Amsterdam.

Tissue processing

Blood was collected into EDTA tubes, kept on ice and centrifuged at 240g for 10 minutes. Plasma was collected and stored at -80°C until analysis. Mouse livers were snapfreezed in liquid nitrogen and stored at -80°C. Before analysis, mouse livers were homogenized in PBS and protein concentration was determined using Pierce® BCA Protein Assay Reagent A (Thermo Scientific) as described by the manufacturer. Mouse humerae were collected and placed in 0.9% NaCl containing Complete mini protease inhibitor cocktail (Roche Applied Science) at 4°C for >1 day. Next, soft tissue was removed and humerae were stored at -80°C. Before analysis, humerae were homogenized in PBS, sonificated twice for 15 seconds on 40 joules/watt/second using a Vibra Cell sonicator (Sonics & Materials Inc.) and centrifuged for 1 minute at 400g. Protein concentration of the supernatant was determined as described above.
GAG analysis
GAG levels in mouse plasma, liver and humerus homogenates were determined using HPLC-MS/MS, as described previously, with one modification for tissue samples: 12.5 μg protein of liver or humerus homogenates were used. Genistein supplementation did not alter HS or DS disaccharide composition, therefore only values of the most abundant HS derived disaccharide D0A0 and DS derived disaccharide D0a4 are given.

Genistein measurement
Genistein levels were determined by ultra-high performance liquid chromatography (UHPLC) with a protocol modified from Seppen et al. 40 μl of plasma or humerus homogenate was acidified with 5 μl 1M 4-morpholineethanesulfonic acid. Genistein was hydrolyzed by adding 2.5 μl β-glucuronidase (Sigma Aldrich) and 2.5 μl sulfatase (10 mg/ml in PBS, Sigma Aldrich) and incubated at 37°C for 20 hours. Protein was precipitated by adding 100 μl methanol and samples were centrifuged at 8000 g for 2 minutes. The supernatant was vaporized using a Techne® Dri-block® heater (Bibby Scientific) and the residue dissolved in 20% v/v acetonitrile. UHPLC was performed on a Dionex 3000 Ultimate UHPLC, equipped with a Polar advantage C18 column (Thermo Scientific), variable wavelength detector set at 260nm, 5 μl injection and a flow rate of 0.5ml/min. The following program was used with solvent A (A, 0.1% v/v formic acid in water) and solvent B (B, 0.1% v/v formic acid in acetonitrile): Start 30% B, t=5 55% B, t=5.1 100% B, t=6.1 30% B, t=10 30% B. Linear gradients were employed and t is in minutes. Chromeleon Dionex software (Thermo Scientific) was used to integrate the chromatograms and genistein level was calculated using a calibration curve of genistein dissolved in dimethyl sulfoxide, diluted in mouse plasma.

Morphology of testes
Morphology of testicular cells and structures were analyzed by routine hematoxylin/eosin (HE) staining.

Statistical analysis
Statistical analysis was performed using Mann-Whitney-U tests for nonparametric analysis with SPSS Statistics software version 21 (IBM Corp.). Significance was assumed where p values were <0.05.

RESULTS
Genistein decreases GAG levels in liver, but not in bone or plasma
As expected, analysis of GAG levels revealed significantly higher levels in MPS I plasma and
tissues \( (p<0.0001) \), as compared to WT (fig. 1). Genistein supplementation did not affect GAG levels in WT or MPS I bone or plasma (fig. 1A and B). HS levels in liver from genistein-fed MPS I mice were 25\% decreased \( (p<0.01) \), as compared to untreated mice. No difference in liver DS levels was observed between genistein-fed and untreated MPS I mice (fig. 1C).
Genistein was present in plasma, but undetectable in bone
Genistein levels in plasma of genistein-fed MPS I mice were 782nM ± 503. In the control group, genistein concentration in plasma was below the limit of quantification (data not shown). Genistein levels in bone were below detection level in all MPS I mice (data not shown).

Genistein causes decreased skeletal growth and scrotal hernia/hydrocele in MPS I mice
Total body weight of MPS I mice was 11% increased ($p<0.05$), as compared to WT mice (fig. 2A). Total body weight of genistein-fed MPS I mice was decreased by 16% ($p<0.001$) (fig. 2A), as compared to untreated MPS I mice. Following genistein supplementation, total body length was 4% ($p<0.05$) and 6% ($p<0.001$) decreased in WT and MPS I mice (fig. 2B), respectively, as compared to untreated mice. Femur length of MPS I mice was 4% decreased ($p<0.05$), as compared to WT mice (fig. 2C). Femur length of genistein-fed mice were 3% ($p<0.01$) and 7% ($p<0.001$) decreased in WT and MPS I mice (fig. 2C), respectively, compared to untreated mice. Surprisingly, 60% of the genistein-fed MPS I mice had an enlarged scrotum (fig. 3) with redness of the overlying skin and the mice showed a wide based gait. None of the WT or untreated MPS I mice showed scrotal abnormalities. The scrotal enlargements were observed to be due to either scrotal hernia and/or scrotal hydrocele. HE staining of

Figure 3. Scrotum of a MPS I mouse on control diet (A) and a MPS I mouse on genistein diet (B). 60% of MPS I mice with genistein had either scrotal hydrocele and/or scrotal hernia, as depicted in (B). All other mice had scrota as depicted in (A).
testes revealed no morphological changes of testicular cells and structures in genistein-fed or untreated mice (results not shown).

DISCUSSION

Skeletal disease is one of the most prevalent and incapacitating disease manifestations in patients suffering from the MPSs and frequently results in the need for multiple surgical interventions \(^1\text{-}^2\). Current disease modifying therapies for the management of MPS I ameliorate a number of clinical signs and symptoms, but have a limited effect on the progression of skeletal disease \(^3\text{-}^4\). Therefore, therapeutic strategies targeting bone disease are urgently needed.

The isoflavone genistein inhibits the activity of tyrosine kinase receptors, thereby modulating the expression of several genes, including some involved in GAG synthesis \(^12\text{-}^24\). Genistein is being investigated for its potential benefit in the treatment of CNS disease in MPS III, as it passes the blood-brain barrier and an \textit{in vivo} study showed that genistein may reduce GAG accumulation in the brain and corrects behavioural abnormalities in MPS III mice \(^14\text{-}^25\text{-}^26\). As prevention of GAG accumulation in cartilage and bone might halt the progression of skeletal disease in the MPSs, we investigated the effects of genistein on GAG accumulation in MPS I mice in order to evaluate its potential for the treatment of MPS I related bone disease.

In agreement with results of previously published studies on the effects of genistein in MPS mice \(^21\text{-}^27\), GAG levels were significantly decreased in liver of genistein-fed MPS I mice. Surprisingly, we observed no effect on GAGs in bone or plasma. The effect of genistein on GAG levels in plasma or bone of MPS III mice has not been described in previous studies \(^14\text{-}^21\text{-}^27\). In rats, genistein is known to reach bone in approximately 17 times lower quantities when compared to plasma levels, 2 hours after administration of 4mg/kg genistein \(^18\) (in the current study we used 160 mg/kg/day oral supplementation). Although plasma genistein concentrations in our study were comparable with, or even higher than the concentrations reported in other studies on the effects of genistein in mice \(^28\text{-}^29\), genistein concentrations in bone in our study were below detection level. These results suggest that the treatment duration and/or dosage of genistein may have been insufficient to treat bone. In our study, mice were treated from 3 weeks of age, for a period of 8 weeks as, at 11 weeks of age, the growth plate is almost closed and skeletal development almost completed. Therefore, we do not expect any additional effect on bone with a longer supplementation period. Earlier initiation of genistein supplementation is not feasible as mice are weaned at 3 weeks of age.

Our observation that genistein supplementation led to decreased body weight in mice was not surprising. Although not decreased previously in MPS III mice \(^14\text{-}^21\text{-}^27\), the effect of genistein on decreasing lipid deposition and body weight has been reported earlier in mice
and rats and may be due to the estrogenic effect of increasing lipoprotein lipase activity \(^{30}\), or decreased food intake \(^{23}\). Genistein-fed mice in our study, however, also exhibited decreased skeletal growth, including decreased femur length, which has not been described previously. In addition, 60% of genistein-fed MPS I mice developed a scrotal hernia and/or a scrotal hydrocele. The cause of the decrease in skeletal growth and the observed scrotal hernia/hydrocele is unclear. Increased levels of DS, an abundant GAG in connective tissue, may have contributed to the development of adverse effects, as our recently published study showed that genistein can increase HS and DS storage in MPS I chondrocytes and fibroblasts (but not in osteoblasts) \(^{31}\). In the present study, GAG levels were analyzed in complete bones and chondrocytes only make up a small portion of the entire bone. This might explain the fact that no increase in GAG levels was observed in bones of genistein treated MPS I mice, while these animals did show a decrease in femur length. Increased GAG storage in certain cell types might further stimulate the pathophysiological mechanisms causing bone and connective tissue disease in MPS I. In addition, patients with MPS I already frequently display hernia and hydrocele \(^{32}\). Genistein may thus lead to a more severe MPS I phenotype, at least in mice. Therefore, increasing the dose of genistein or prolonging the supplementation period, likely will not result in amelioration of bone disease in MPS I mice. The only study in patients on the effects of a high dose, i.e. 150 mg/kg/day, of (synthetic) genistein (at least one year treatment of 22 MPS III patients), concluded that genistein is safe, but did report elevation of liver enzymes, breast development in boys or young girls, menstrual irregularities, and bilateral deep vein thrombosis in a 20 year old woman with several other risk factors for thrombosis \(^{16}\). This study did not report on growth rate.

**CONCLUSION**

In conclusion, our study suggests that high doses of genistein might lead to decreased growth and increased incidence of scrotal hydrocele and hernia, at least in MPS I. These data, in combination with other potential adverse effects reported in previous studies \(^{15,16}\), support a cautious approach to the introduction of high doses of genistein in patients with various MPSs and underscores the need for well-designed and controlled clinical trials allowing the collection of all potential adverse effects over longer periods of time.

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Chapter
General discussion and future perspectives
General discussion and future perspectives

Over the last decades, improved understanding about the pathophysiology and natural history of Mucopolysaccharidosis type I (MPS I), in combination with the availability of disease-modifying treatments, has greatly improved survival of MPS I patients. However, as all patients still display significant residual disease despite therapy, it is clear that more work needs to be done in order to optimize treatment. This thesis comprises studies on two of the important challenges in MPS I: early diagnosis, and treatment of MPS I bone disease.

NEWBORN SCREENING

Early initiation of treatment is probably one of the most important factors that may improve clinical outcome in MPS I patients. Pre-symptomatic diagnosis and initiation of treatment, made possible by newborn screening (NBS), may result in the most optimal treatment outcome. Other advantages of diagnosis by NBS are its potential to prevent the often long and burdensome diagnostic odysseys in MPS I and to allow timely reproductive choices and prenatal counseling. NBS (pilot) programs for several lysosomal storage diseases (LSDs) have been initiated in a number of countries, including for MPS I in Taiwan and the United States (chapter 2). On April 14th 2015, the Dutch Minister of Health decided to include MPS I in the current neonatal screening program after recommendation from the Health Council of the Netherlands. There are, however, several issues that complicate the introduction of MPS I in a NBS program. Firstly, the optimal treatment strategy currently depends on the clinical phenotype, and therefore, early phenotypic prediction is essential for MPS I patients diagnosed by NBS. To date, phenotypic severity is often assessed on the basis of clinical signs and symptoms, when irreversible organ damage may already be present. Therefore, the development of tools or biomarkers that may accurately predict the phenotype before the onset of symptoms, is essential. Secondly, the effect of current treatment options on MPS I bone disease remain limited.

Early assessment of phenotype

Early prediction of phenotypic severity in MPS I patients can be complicated. Mutation analysis, enzyme activity and clinical characteristics of MPS I patients alone all have limited value in predicting disease severity. Although homozygosity or compound heterozygosity for nonsense mutations corresponds with a severe Hurler phenotype, most missense mutations are associated with a more variable clinical outcome. This variable clinical phenotype is due to several factors, which may include the involvement of secondary biochemical pathways in the pathogenesis of clinical signs and symptoms, such as secondary accumulation of gangliosides or glycosaminoglycans (GAGs) not typically associated with MPS I (chapter 4). Also, over 30 polymorphisms in the IDUA gene have been reported. Although by itself regarded as non-pathogenic, these polymorphisms may modify the severity of MPS I and contribute to the variability in IDUA activity seen in healthy subjects. Also, it seems likely that the introduction of NBS for MPS I will result in the identification of many novel...
mutations with unknown effect, which will further complicate phenotype prediction based on genotype. Assessment of phenotypic severity based on signs and symptoms at clinical presentation is difficult, and expert opinion on phenotypic severity appears to be remarkably variable. As newborns diagnosed by NBS will probably lack most of the characteristic symptoms, assessment of the phenotypic severity will be even more difficult.

We combined mutation analysis, enzyme activity measurements and clinical manifestations before the age of 4 weeks in an algorithm (chapter 3), which enables the prediction of phenotypic severity of newborn MPS I patients, and allows timely decisions on the optimal treatment strategy. Before applying this algorithm in a clinical setting, it needs further validation in other cohorts of patients. Also, studies on the effect of certain mutations, combinations of mutations and polymorphisms on IDUA protein levels and IDUA activity should be initiated to improve the predictive value of these mutations. Furthermore, clinical symptoms in an algorithm may be subjective, and studies aimed at objectifying clinical symptoms or modification of the algorithm to include for instance, predictive biomarkers, may be preferable. Finally, especially after introduction of MPS I in NBS panels, such an algorithm needs to be continuously adapted in order to incorporate novel mutations. The early initiation of treatment in patients detected through NBS, however, will completely change the natural course of the disease, which will no longer allow or halt studies on genotype-phenotype correlations.

After NBS implementation
In chapter 2, we describe challenges of NBS for LSDs in general. Findings from (pilot) programs and experience with the introduction of NBS for other inborn errors of metabolism have clearly shown that this will probably lead to the identification of individuals with very attenuated phenotypes and individuals with genetic variants of unknown significance resulting in lowered enzyme activity. Natural history studies on these groups of patients are urgently needed to learn who to treat and when to start treatment, thus avoiding overtreatment in patients who may remain asymptomatic until old age. Also, within the scope of NBS for MPS I, a longitudinal follow-up program for patients diagnosed by NBS is needed. This program should collect data on natural history and treatment responses but also clinical and biochemical data in the first months of life. This may provide essential information for early prediction of the disease severity, for improving our prediction algorithm, and to guide decisions on timing and choice of appropriate treatment strategies. For this aim, the MPS I registry (www.mpsiregistry.com), which is an international database that tracks outcomes of MPS I patients, might be helpful. However, the observational nature of the MPS I registry has led to incomplete datasets, which makes assessment of treatment outcomes in different patient groups difficult. A recent retrospective multicenter study on the outcome of haematopoietic stem cell transplantation (HSCT) in MPS I Hurler patients
showed that essential data on treatment efficacy can, in part, be obtained by international collaboration and rigorous analysis of available data. Such studies might also be pursued for the more attenuated phenotypes of MPS I, and on the outcome of patients on enzyme replacement therapy (ERT). An alternative approach is the use of new international, prospective, and mandatory databases. However, institution of such databases will face significant legal hurdles. In addition, financing the maintenance of these databases and systems for source data verification, may prove to be difficult.

### CHALLENGES IN TREATING BONE DISEASE

The current disease modifying therapeutic strategies for MPS I, which are HSCT and ERT, effectively treat many of the clinical signs and symptoms. With increased survival, however, disease manifestations that are refractory to treatment, such as MPS I bone disease, become more prominent. Therapeutic efficacy is greatly limited because irreversible bone lesions occur at a very early age, and may already be present before birth. In addition, large proteins such as lysosomal enzymes have difficulties in reaching target cells in the poorly vascularized cartilage. Studies on therapies for MPS I bone disease that aim to overcome these obstacles, are needed.

### Pathophysiology of bone disease

The pathophysiological cascades initiated by accumulating GAGs are largely unknown, which hampers the development of new therapeutic strategies. We studied (chapter 4 and 5) secondary pathological effects of GAG accumulation that likely contribute to MPS I bone disease. Our observation that distribution of GAGs and growth factors are altered in MPS I chondrocytes and MPS I mouse bones (chapter 5), suggest that targeting growth factor regulation may be a future therapeutic strategy for MPS I bone disease. Growth factor therapy, such as the use of monoclonal antibodies against growth factor receptors, is a standard and successful therapy for some forms of cancer. These therapies, however, aim to eliminate cells and the adverse effects are only acceptable for diseases that are otherwise life threatening. Furthermore, treatment in MPS I would probably be very long-term as compared to cancer treatment. Drugs with a better safety profile or with only a partial stimulating or inhibiting effect on certain growth factor receptors may be preferable for treatment or prevention of MPS I bone disease. The effects of such therapies may be studied in the future, however, the involvement of other growth factors in the pathophysiology of MPS I bone disease needs to be elucidated first.

### Early start of current therapies

**ERT**

Initiation of ERT very early in life may (partially) prevent MPS I bone disease. However, the
only evidence comes from case history studies on siblings started on ERT at different ages \(^8,9\). A study on ERT started directly after birth in MPS I mice, however, showed an improved effect on other difficult-to-treat organs such as blood vessels and heart valves, but no alteration of the course of MPS I bone disease \(^10\).

**HSCT**

A study on the long term effect of HSCT in patients engrafted between the ages of 9 months and 2.5 years old, showed that HSCT does not alter the natural history of MPS I bone disease \(^11\). A recent study on HSCT in neonatal MPS I mice, however, reported almost complete prevention of bone disease \(^12\). These results indicate that the upper age limit of 2.5 years for HSCT \(^13\), may be too high to prevent MPS I bone disease.

The efficacy of HSCT to ameliorate GAG storage has shown to be superior to the effects of ERT \(^14\), which is probably due to the fact that ERT is administered only weekly. In contrast, with HSCT, IDUA is produced continually by haematopoietic cells, which may lead to a better pharmacokinetic profile and increased penetration of hard-to-treat organs, such as cartilage. The success of reducing mortality after HSCT, with 95% overall survival, as described by Aldenhoven *et al.* \(^15\), opens up the possibility of treating more attenuated patients. If performed very early, this may prevent MPS I bone disease, which is a frequent and incapacitating manifestation in MPS I patients with all phenotypic severities, but will also improve other clinical manifestations. Also, HSCT is significantly less expensive than (life-long) ERT and ideally a once-only procedure, thus reducing costs and potentially improving the quality of life of patients.

**Emerging therapies**

*Modification of ERT*

Modification of conventional ERT, such as chemical alteration of the enzyme, leading to prolonged circulation \(^16\), or the targeting of the enzyme to a component of the bone matrix, which is studied in MPS VI and VII mice, may increase the effect of ERT on MPS I bone lesions \(^17,18\). Also, intra-articular ERT seems more efficacious than conventional ERT for MPS I bone disease in dogs \(^19\), but is, due to the many joints that are involved in MPS I bone disease, probably not a feasible approach in patients.

*Mesenchymal stem cell transplantation*

As cartilage cells are derived from mesenchymal stem cells, transplantation of mesenchymal stem cells in addition to haematopoietic stem cells, may improve the efficacy of current transplantation protocols to prevent MPS I bone disease. Koç *et al.* \(^20\) studied the potential of allogeneic mesenchymal stem cell (MSC) infusion in MPS I Hurler patients with a median
age of 15 years that previously received HSCT. They concluded that MSC infusion is safe and improved some of the residual MPS I bone disease. As MPS I bone disease is present very early in life, performing MSC transplantation at an early age, may significantly ameliorate MPS I bone disease.

**Gene therapy**

Gene therapy, which involves the transfer of a gene that encodes the deficient enzyme into cells of the body, is often considered as the ‘holy grail’ for the treatment of inborn errors of metabolism and studies in MPS I have been initiated. Preclinical studies on liver directed neonatal gene therapy have shown promising results for most MPS I manifestations. Skeletal disease, however, was ameliorated, but not prevented completely 21.

Gene therapy of autologous haematopoietic stem cells before transplantation is another promising approach. A study on the effect of this strategy in MPS I mice showed supranormal IDUA activity in haematopoietic stem cells and almost complete prevention of MPS I neurologic and bone disease 22. Recently, this therapeutic strategy has shown to be safe and successful in halting the progression of the neurodegenerative diseases X-adrenoleukodystrophy and metachromatic leukodystrophy 23,24.

**Small molecule therapies**

Small molecule therapies, aimed at better penetration of the poorly vascularized cartilage, which are currently under investigation for the MPSs, are substrate reduction therapy (genistein), chaperone therapy and stop codon read-through therapies.

Genistein, a natural occurring isoflavone, is the most investigated substrate reduction therapy for the MPSs and is available over the counter. The first report on the effect of genistein in decreasing GAG synthesis in MPS I, II and III fibroblasts 25, was quickly followed by a clinical trial in MPS III patients by the same group 26. Subsequently, a lot of different cell, animal and clinical studies followed, particularly for MPS III, and variable effects of genistein were observed (described in chapter 6 and 7). We investigated the effect of genistein on MPS I bone disease (chapter 6 and 7). Surprisingly, we observed increased GAG synthesis and storage in cultured MPS I chondrocytes and significant adverse effects in MPS I mice after genistein treatment. We hypothesize that this effect is due to the very unspecific effect of genistein on multiple pathways, such as growth factor signaling. These results discourage the use of genistein as a treatment for MPS I, and emphasize that genistein should be used with caution in the other MPSs. Currently, a double-blind, randomized, placebo controlled trial on the effect of a high dose of genistein in MPS III patients is executed in the United Kingdom, which will hopefully elucidate potential beneficial and adverse effects of genistein in MPS III patients 27.
Another emerging therapy is chaperone therapy, consisting of small molecules that have the potential to improve folding of misfolded proteins or protect misfolded proteins from degradation. However, patients with the severe Hurler phenotype frequently have nonsense mutations with very little residual enzyme. Therefore, improved folding or protection from degradation of such small amounts of protein will probably not have a clinically significant effect. However, for patients with a more attenuated phenotype, chaperone therapy may be an interesting option in the future.

Due to the high prevalence of nonsense mutations in the severe Hurler phenotype, stop codon read-through therapy could be a future therapy. Compounds such as chloramphenicol and aminoglycosides have the ability to suppress premature stop codon mutations and allow the protein to be fully translated. More recently, the nonsense suppression drug PTC124® (ataluren), which is currently used as an investigational drug in patients with cystic fibrosis and Duchenne muscular atrophy, has been put forward as a potential therapy for MPS I. It has been shown to reduce GAG storage in brain and liver of MPS I mice and clinical trials in MPS I patients are currently planned.

**Anti-inflammatory therapy**

A very promising therapeutic strategy for MPS I bone disease is the use of anti-inflammatory agents, which reduces secondary inflammation processes caused by GAG accumulation. Pentosan polysulfate sodium (PPS) is a FDA approved drug for interstitial cystitis and has anti-inflammatory and chondrogenic effects. Treatment of MPS VI rats with PPS led to reduced inflammation, partial rescue of the bone phenotype and improved mobility. The first clinical trial on weekly subcutaneous injections of PPS in patients with MPS I is currently planned.

Because of the broad pathophysiology of MPS I bone disease, it is likely that the most efficient approach is to combine different treatment modalities.

**FUTURE PERSPECTIVES**

It is clear that treatment for bone disease can only be effective if initiated at a very early stage of the disease. Therefore, implementing NBS for MPS I may prove indispensable. Clinical trials on the effect of very early (neonatal) HSCT and ERT to prevent MPS I bone disease are essential and should be initiated soon after implementation of NBS.

Because bone lesions may already be present before birth, starting treatment directly after birth might, however, not completely prevent MPS I bone disease. The possibility and efficacy of prenatal treatments, such as gene therapy and HSCT, are being investigated in MPS VII mice. Prenatal treatment, however, requires prenatal diagnosis. Prenatal
counselling, which is currently done by enzyme or molecular testing on chorionic villi or amniocytes, is usually only considered in families of MPS I patients \(^{37}\). Some studies on less invasive strategies for prenatal diagnosis of the MPSs have been performed, such as nuchal translucency for diagnosing MPS VII patients, and maybe even MPS II patients \(^{38-40}\).

Probably more applicable for MPS I, the detection of skeletal abnormalities that may already be present before birth, using sensitive imaging techniques for prenatal diagnostics, might be a future diagnostic approach. Another approach is preconception screening, to investigate whether the parents are carriers for MPS I causing mutations. Finally, fetal DNA and RNA can be detected in maternal blood from 5 weeks gestational age, thereby proving a method for early non-invasive prenatal diagnosis. Because maternal blood is a mixture of small amounts of fetal nucleic acids within a wide background of maternal nucleic acids, it is difficult to distinguish fetal nucleic acids from maternal nucleic acids. Therefore, current studies are based on the analysis of specific paternally inherited traits that are not present in the maternal genome \(^{41}\). In the future, however, single nucleotide polymorphisms (SNPs) may be used to distinguish fetal nucleic acids from maternal nucleic acids and to diagnose maternally inherited disorders \(^{42}\).

For an ultra-orphan disease as MPS I, collecting and sharing data is important to gain as much information as possible, complete studies as quickly as possible, and prevent unnecessary studies. Therefore, all the earlier mentioned efforts should be undertaken in a collaborative and international approach to ensure optimal research on future treatment of MPS I and prevention of MPS I bone disease.
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with rhIDUA is safe, well-tolerated, and reduces articular GAG storage in the canine model of mucopolysaccharidosis type I. Mol Genet Metab 2014;112:286-293.


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Chapter
Summary

Samenvatting
SUMMARY OF THE THESIS

Mucopolysaccharidosis type I (MPS I) is a lysosomal storage disorder (LSD) caused by the deficiency of the enzyme α-L-iduronidase (IDUA), which leads to diminished degradation of certain types of glycosaminoglycans (GAGs). Accumulation of the GAGs heparan sulfate (HS) and dermatan sulfate (DS) in lysosomes and in the extracellular matrix (ECM) leads to progressive multisystem disease. Patients present with a large spectrum of disease manifestations, with, in the severe Hurler phenotype, among other symptoms, shortened lifespan, mental retardation and severe skeletal disease. Current therapeutic strategies are haematopoietic stem cell transplantation (HSCT) for the Hurler phenotype and enzyme replacement therapy (ERT) for the more attenuated phenotypes (Hurler-Scheie and Scheie phenotype). Early diagnosis, which will be feasible by newborn screening (NBS), and timely start of treatment considerably improves clinical outcome in MPS I patients. This thesis comprises studies on two of the most important challenges in MPS I: 1) early prediction of the clinical phenotype to determine the appropriate treatment strategy, and 2) treatment of MPS I bone disease, for which there is currently no effective therapy available.

Chapter 2 provides an overview of epidemiological studies in a large panel of LSDs and the current diagnostic work-up of patients suspected of a LSD. In addition, challenges for carrier screening, high risk screening and NBS are discussed. We concluded that several methodological and ethical challenges need to be addressed for successful implementation of screening. This includes the acquisition of reliable epidemiological data to study the potential benefits of screening in different populations. Also, natural history studies on late-onset diseases and genetic variants of unknown significance and studies on phenotypic prediction are needed to guide decisions on optimal treatment. Finally, studies on long-term efficacy of disease modifying treatments and guidelines on management of presymptomatic individuals are necessary.

In chapter 3, we describe an algorithm that can be used to predict the phenotype (Hurler phenotype or a more attenuated phenotype) of MPS I patients at a very young age. As presymptomatic treatment may greatly improve clinical outcome and as the optimal treatment modality is related to the phenotype, timely phenotypic characterization of patients is essential. This algorithm comprises 3 steps. In the first step, the mutation(s) are analyzed and classified as strongly associated with a severe Hurler phenotype or not. Next, measurement of residual IDUA activity, using an improved protocol, further discriminated Hurler patients from more attenuated patients. Finally, the presence or absence of Hurler associated clinical characteristics (inguinal hernia and/or upper respiratory tract obstruction) before the age of 4 weeks, led to complete separation of Hurler patients from more attenuated patients. This algorithm may enable the initiation of proper treatment before the onset of symptoms and irreversible organ damage in patients diagnosed by NBS.
In chapter 4, a study on the secondary pathological effects of GAG accumulation in fibroblasts from MPS I and III patients is described. We demonstrated secondary accumulation of chondroitin sulfate (CS) in MPS I fibroblasts and CS and DS in MPS III fibroblasts. Also, we observed differences in the composition of GAGs and altered distribution of HS in the ECM of MPS I and III fibroblasts. These secondary pathological effects may contribute to clinical heterogeneity between patients and between the MPS subtypes. Future therapies that aim to decrease synthesis of GAGs not typically associated with the MPS type, may improve the efficacy of current therapies.

Chapter 5 describes a study on the interaction between growth factors and GAGs, a process that is essential for growth and development. We observed increased fibroblast growth factor 2 (FGF2) signaling and alterations in GAG distribution in MPS I chondrocytes. In addition, MPS I mouse bones exhibited altered growth after incubation with FGF2 and GAGs, and showed alterations in the distribution of GAGs and the growth factors FGF2 and Indian hedgehog in the growth plate. These results suggest that altered interaction and distribution of GAGs and growth factors likely contributes to MPS I bone disease, and we hypothesized that interference with growth factor biology may be a future therapeutic target for MPS I bone disease.

In chapter 6 and 7, adverse effects of genistein in MPS I are described. Genistein, a plant-extract that is available over the counter, has been promoted as a treatment for MPS III. We, however, showed increased GAG synthesis and storage in fibroblasts and chondrocytes from MPS I patients after genistein treatment (chapter 6). Also, genistein-fed MPS I mice exhibited decreased skeletal growth and developed scrotal hernia/hydrocele (chapter 7). Our results indicate that one should be very cautious with the use of genistein in MPS I patients outside a controlled setting such as a clinical trial.

Finally, in chapter 8, we provide a general discussion and future perspectives on NBS for MPS I and the treatment of MPS I bone disease. Within the scope of NBS for MPS I, validated tools, such as our algorithm or biomarkers that can accurately predict the MPS I phenotype, are needed. Furthermore, the introduction of a longitudinal follow-up program to track, among other items, outcomes of patients with genetic variants of unknown significance who are diagnosed by NBS, is important. Finally, because MPS I bone disease is extremely refractory to current therapies and because bone disease may already be present at birth, studies on the very early start of current and emerging therapeutic strategies and even studies on prenatal diagnosis and treatment are urgently needed.
SAMENVATTING VAN HET PROEFSCHRIFT
Mucopolysaccharidosis type I (MPS I) is een lysosomale stapelingsziekte (LSZ) die wordt veroorzaakt door een defect in het enzym α-L-iduronidase (IDUA) waardoor bepaalde glycosaminoglycanen (GAGs) niet afgebroken kunnen worden. Stapeling van de GAGs heparan sulfaat (HS) en dermatan sulfaat (DS) in lysosomen en in de extracellulaire matrix (ECM) leidt tot afwijkingen in meerdere orgaansystemen. Patiënten presenteren zich met een breed spectrum van ziekteverschijnselen, onder andere (bij de ernstigste vorm van de ziekte) met vroegtijdig overlijden, mentale retardatie en ernstige botafwijkingen. Hematopoietische stamceltransplantatie (HSCT) is de voorkeursbehandeling voor deze ernstig aangedane (Hurler fenotype) patiënten. Minder ernstig aangedane patiënten met het Hurler-Scheie of Scheie fenotype, worden bij voorkeur behandeld met intraveneuze enzymvervangende therapie (ET). Vroege diagnose, bijvoorbeeld door middel van neonatale screening, en vroegtijdige start van therapie geeft een significante verbetering van de klinische uitkomst in MPS I patiënten. Dit proefschrift beschrijft onderzoek naar twee van de belangrijkste uitdagingen die we tegenkomen bij de behandeling van MPS I: 1) voorspelling van het fenotype op zeer jonge leeftijd om vroegtijdig de juiste behandeling te kunnen starten, en 2) therapie voor botziekte bij MPS I, waarvoor op dit moment nog geen effectieve behandeling beschikbaar is.

Hoofdstuk 2 is een samenvatting van studies naar de epidemiologie van LSZs en het diagnostische proces bij verdachte op een LSZ. Tevens worden de uitdagingen die spelen bij screening op dragerschap, screening van hoog risico patiënten en neonatale screening besproken. Wij concludeerden dat er meerdere methodologische en ethische kwesties opgelost moeten worden voordat het maximale potentiële effect van screening behaald kan worden. Een voorbeeld is de noodzaak tot het verkrijgen van betrouwbare epidemiologische data om het effect van screening in verschillende populaties te bepalen. Voor optimale behandeling van LSZ patiënten zijn studies naar het natuurlijke beloop van milder aangedane patiënten, patiënten met een tot dusver onbekende mutatie en studies naar de mogelijkheden om het fenotype te voorspellen nodig. Tot slot moeten de lange termijn effecten van huidige therapieën bepaald worden en zijn richtlijnen nodig voor de behandeling van presymptomatische patiënten.

In hoofdstuk 3 beschrijven we een algoritme dat gebruikt kan worden om het toekomstige fenotype van MPS I patiënten te voorspellen. Het algoritme bestaat uit 3 stappen. In de eerste stap worden de ziekte veroorzakende mutaties geanalyseerd en geclasseerd als sterk of niet sterk geassocieerd met een ernstig Hurler fenotype. Als tweede stap wordt IDUA activiteit gemeten met een aangepast protocol geschikt voor zeer lage activiteiten, wat Hurler patiënten en patiënten met een minder ernstig fenotype verder differentieert. De laatste stap bestaat uit het vaststellen van de aan- of afwezigheid van een inguinale
hernia of hoge luchtwegobstructie voor de leeftijd van 4 weken, waardoor Hurler patiënten volledig gescheiden kunnen worden van minder ernstig aangedane patiënten. Vroege voorspelling van het fenotype door middel van dit algoritme maakt vroege behandeling van MPS I patiënten mogelijk waardoor irreversibele orgaanschade voorkomen kan worden.

Hoofdstuk 4 beschrijft een studie naar de secundaire pathologische gevolgen van GAG stapeling in fibroblasten van MPS I en III patiënten. Wij observeerden secundaire stapeling van chondroitine sulfaat (CS) in MPS I fibroblasten en CS en DS in MPS III fibroblasten. Tevens observeerden we veranderingen in de samenstelling van GAGs en veranderingen in de extracellulaire distributie van HS in MPS I en III fibroblasten. Deze secundaire effecten dragen vermoedelijk bij aan de sterke verschillen in fenotype tussen MPS patiënten. Medicijnen die de aanmaak van GAGs, die niet typisch geassocieerd zijn met het MPS subtype, remmen, kunnen een waardevolle uitbreiding zijn op huidige therapieën.

Hoofdstuk 5 beschrijft een studie naar de interactie tussen groeifactoren en GAGs, een proces dat essentieel is voor groei. MPS I chondrocyten lieten een verhoging zien in de signalering van fibroblast groeifactor 2 (FGF2) en een verstoring van de extracellulaire distributie van GAGs. Tevens vertoonden botten van MPS I muizen verstoorde groei na incubatie met FGF2 en GAGs, en verstoring van de extracellulaire distributie van GAGs en de groeifactoren FGF2 en Indian hedgehog. Onze resultaten suggereren dat de verstoorde interactie en distributie van GAGs en groeifactoren waarschijnlijk bijdraagt aan het ontstaan van MPS I botziekte. Tevens speculeren wij dat het beïnvloeden van groeifactor regulatie een belangrijke aanvulling op therapie voor MPS I botziekte kan vormen.

In hoofdstuk 6 en 7 worden de effecten van genisteine op gekweekte cellen van MPS I patiënten en in een diermodel (MPS I muis) beschreven. Genisteine, een plantaardige stof die zonder recept verkrijgbaar is, wordt steeds meer gebruikt als (experimentele) behandeling voor MPS II en III en soms ook voor MPS I. In onze studie naar de effecten van genisteine in fibroblasten en chondrocyten van MPS I patiënten observeerden we echter verhoogde aanmaak en stapeling van GAGs. Tevens vertoonden MPS I muizen die behandeld waren met genisteine verminderde botgroei en afwijkingen aan het scrotum (hernia/hydrocèle). Deze studies laten zien dat, zolang bijwerkingen nog niet grondig onderzocht zijn, genisteine gebruik buiten een gecontroleerde medische situatie, zoals een klinische studie, zeer onverstandig kan zijn voor MPS I patiënten.

Tot slot bevat hoofdstuk 8 een algemene discussie over de toekomstperspectieven van neonatale screening en behandeling van MPS I botziekte. Om de resultaten van de neonatale screening voor MPS I optimaal te kunnen benutten, zijn ge valideerde instrumenten (zoals ons algoritme) of biomarkers die het MPS I fenotype kunnen voorspellen, nodig. Tevens
Samenvatting

moeten longitudinale follow-up programma’s voor het volgen van het klinisch beloop van patiënten, met een tot dusver onbekende mutatie, opgestart worden. Voor de behandeling van MPS I botziekte moeten studies naar de zeer vroege start van huidige therapiën, maar ook experimentele therapiën, uitgevoerd worden. Tot slot, omdat botziekte waarschijnlijk al ontstaat voor geboorte, zijn studies naar prenatale diagnostiek en behandeling noodzakelijk.
Appendix
PhD portfolio

Curriculum Vitae

Dankwoord
PhD PORTFOLIO

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### International conferences

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### Other conferences

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### Parameters of Esteem

- Second poster price, MPS symposium, Noordwijkerhout, Netherlands, 2012
- Scholarship WORLD symposium, 2013
- Masterclass Amsterdam Kinder Symposium, 2013
APPENDIX

DANKWOORD

“You can design and create, but it takes people to make the dream a reality” – Walt Disney

Dit resultaat van bijna 4 jaar hard werken was niet mogelijk zonder de steun en hulp van vele mensen. Ik kan niet iedereen persoonlijk bedanken, daar zijn teveel goede en lieve personen voor, maar ik zal de belangrijkste personen noemen.

I have to start with prof. dr. Neu and dr. Lorca of the University of Florida. Dear Joe and Graciela, you taught me my love for science, which I will never lose, and for which I am grateful.

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Andere personen die een speciaal bedankje verdienen voor hun bijdrage aan dit proefschrift en de fijne samenwerking: Tom Wagemans, Lodewijk IJlst, Ronald Wanders, Eveline Langereis, Serwet Demirdas, Ton Bronckers, Rene Leen, en zelfs mijn zus Marie-Louise Kingma en vriendin Ilse Landwehr Johann.

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Tijdens mijn promotieperiode op het laboratorium heb ik nog met 1 teen in de kliniek mogen staan. Bedankt, patiënten en ouders van de HGT-SAN studie, Jessica de Ruijter, Lindsey Welling, Evelien Tump, Klaske Honig en Frits Wijburg, voor de welkome, leerzame, maar ook gezellige afwisseling.


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Mijn lieve familie: Paps, Mams, Tania, Marie-Louise, Dennis en Florian, ik prijs mij gelukkig dat ik uit zo een rijk en liefdevol nest kom. Ik hou van jullie. Papa en Mama, bedankt voor jullie onvoorwaardelijke vertrouwen, steun en liefde, en dat jullie mij mijn werklust en discipline, maar ook liefde en passie voor alles wat ik doe, hebben bijgebracht.

“Love and work are the cornerstones of our humanness” – Sigmund Freud
CURRICULUM VITAE
