Immune responses in patients with Lysosomal Storage Disorders treated with Enzyme Replacement Therapy and Haemopoietic Stem Cell Transplantation

A thesis submitted to the University of Manchester for the degree of Doctor of Medicine in the Faculty of Medical and Human Sciences

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School of Medicine
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Abstract

Immune responses in patients with Lysosomal Storage Disorders treated with Enzyme Replacement Therapy and Haemopoietic Stem Cell Transplantation.

By Muhammad A Saif for the degree of MD, 2012.
The University of Manchester.

Lysosomal storage disorders (LSDs) are caused by defective lysosomal degradation of macromolecules resulting in accumulation of substrates in various tissues. This gradually leads to organ dysfunction and the classical clinical presentation with multisystem involvement. Historically the management of LSDs was confined to symptomatic treatment only. More recently other therapies have become available. Treatment options include cellular therapy in the form of Haemopoietic Stem Cell Transplant (HSCT), Enzyme Replacement Therapy (ERT), Substrate Reduction Therapy (SRT), Chaperone Mediated Therapy (CMT) and gene therapy. Whilst HSCT and ERT are established strategies in clinical practice for some LSDs, others are still in the development phase. The easy accessibility of ERT in the developed world (despite a high cost burden of approximately £144,000 per patient per annum in the UK), fewer risks associated with its administration and good metabolic and clinical outcome, have made ERT the treatment of choice for a number of LSDs. In recent years immune response has been identified as a significant factor in attenuating or nullifying the response to ERT. Despite recognition of this problem, there is a lack of reliable diagnostic tools to test and evaluate the antibody responses in the centres delivering ERT and far too little attention has been focused on development, optimisation and standardization of immune assays.

In this project, IgG ELISA and two different functional enzyme inhibition assays (catalytic inhibition and cellular uptake inhibition) were developed and optimized. The immune response to ERT was then studied in recipients of ERT in MPSI, MPSVI and Pompe disease. Our practice of delivering ERT in recipients of allogeneic HSCT prior to transplant provided us with an opportunity to study the immune response in MPSIH patients during ERT and following HSCT. We demonstrated functionally active antibodies in long term recipients of ERT in MPSI and Pompe disease. Allo-immune response in MPSVI did not inhibit the delivered enzyme therapy. A high titre inhibitory immune response was detected in the majority of MPSIH patients after exposure to ERT. This immune response was abrogated by allogeneic HSCT rendering these patients tolerant to replaced enzyme, confirming HSCT as an effective immune tolerance induction mechanism.
DECLARATION
I, Muhammad A Saif, declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Above all I am thankful to all the patients, their parents and relatives who participated in the study and donated blood.
ABBREVIATIONS

4MU  4-Methylumbelliferyl
ANOVA  Analysis of variance
ARSA  Arylsulphatase A
BBB  Blood brain barrier
BCA  Bicinchoninic acid
BEA_{Ps}  Background enzyme activity in patient serum
BEA_{Ns}  Background enzyme activity in normal serum
CIMPR  Calcium independent mannose-6-phosphate receptor
CMT  Chaperone mediated therapy
CRIM  Cross reactive immunological material
CS  Chondroitin sulphate
CSP  Clinical surveillance programme
CNS  Central nervous system
DS  Dermatan sulphate
DMEM  Dulbecco’s modified eagle medium
DNA  Deoxyribonucleic acid
EA_{Ps}  Enzyme activity in the presence of patient serum
EA_{Ns}  Enzyme activity in the presence of normal serum
ECL  Enhanced chemiluminescence
ELISA  Enzyme linked immunosorbent assay
ER  Endoplasmic reticulum
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<tr>
<th>Acronym</th>
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<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
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<td>ERT</td>
<td>Enzyme replacement therapy</td>
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<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>GA</td>
<td>General anaesthesia</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>GVHD</td>
<td>Graft versus host disease</td>
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<tr>
<td>HSA</td>
<td>Human serum albumin</td>
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<td>HSCT</td>
<td>Haemopoietic stem cell transplant</td>
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<tr>
<td>HPC</td>
<td>Haemopoietic precursor cells</td>
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<tr>
<td>HEK</td>
<td>Human embryonic kidney cells</td>
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<tr>
<td>HLA</td>
<td>Human leucocyte antigens</td>
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<tr>
<td>(h)PGK</td>
<td>Human phosphoglycerate kinase</td>
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<tr>
<td>ICD</td>
<td>International statistical classification of diseases</td>
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<tr>
<td>IDUA</td>
<td>α-L-iduronidase</td>
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<td>IDS</td>
<td>Iduronate-2-sulphatase</td>
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<td>LEBT</td>
<td>Lysosomal enzymes from bovine testis</td>
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<td>LSDs</td>
<td>Lysosomal storage disorder</td>
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<td>LV</td>
<td>Lentivirus</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribosomal nucleic acid</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
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<tr>
<td>MPS</td>
<td>Mucopolysaccharidosis</td>
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<td>M6P</td>
<td>Mannose-6-phosphate</td>
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<td>MPS I H</td>
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<td>MPS I HS</td>
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<td>MPS I S</td>
<td>Scheie syndrome</td>
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<tr>
<td>MLD</td>
<td>Metachromatic leukodystrophy</td>
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<td>NOEV</td>
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<td>SRT</td>
<td>Substrate reduction therapy</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>TRM</td>
<td>Transplant related mortality</td>
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1. Introduction
1.1. Lysosomal storage disorders

Lysosomal storage disorders (LSDs) result from defective lysosomal degradation of macromolecules. This leads to progressive intracellular accumulation of unhydrolysed substrates. Accumulation of these substances results in cellular and tissue dysfunction. These disorders are therefore multisystem, progressive and often cause premature death. However the rate of progression varies amongst various disease groups and individuals within a group. LSDs are individually rare but have a combined prevalence of about 1 in 7000 live births worldwide (Meikle, Hopwood et al. 1999; Poorthuis, Wevers et al. 1999). The majority of these disorders are caused by single gene mutations in one of the lysosomal enzymes but some include trafficking defects to the lysosome. Most of these disorders are autosomal recessive but Mucopolysaccharidosis type II (MPSII), Fabry and Danon disease are X-linked (Wynn, Stubbs et al. 2009). Over 70 disorders have been described in this category (Cox and Cachon-Gonzalez 2012). Depending upon a particular mutation, patients either lack an enzyme or produce an enzyme which does not function optimally. These mutations have specific clinical outcomes which explains the heterogeneous phenotype in these illnesses (Vellodi 2005). Specific mutations have been described for each disease. Whilst there may be a correlation between genotype and phenotype for some of these disorders, this relationship is not straightforward and a number of polymorphisms have been described within the encoding genes which result in different clinical outcomes for the same genetic mutation. The clinical phenotype is also dependent upon the level of enzyme secreted. A critical level of lysosomal enzyme is required to effectively metabolize the substrate within a cell (Gartner, Conzelmann et al. 1983). In an individual with LSD the level of enzyme activity not only determines the severity of the disease but also the onset of symptoms, treatment outcomes and immune response to delivered enzyme as outlined in the later sections.
1.2. Pathophysiology of LSD

1.2.1. Lysosomes and lysosomal enzymes

Lysosomes are membrane bound organelles which are present in all mammalian cells except mature erythrocytes. The enzymes (collectively known as hydrolases) contained in these organelles, hydrolyse a variety of macromolecules (De Duve, Pressman et al. 1955; De Duve and Wattiaux 1966). These biochemical reactions take place in a sequential manner in an acidic environment and result in simpler products of metabolism. About 60 different hydrolases have been identified so far (Eskelinen, Tanaka et al. 2003). Some important hydrolases, their substrates and the clinical illnesses arising as a consequence of their deficiency are described in table 1. The lysosomal enzymes not only break down the products of cellular metabolism and extracellular material (endosomes and phagosomes) by formation of secondary lysosomes (Lloyd 1996; Luzio, Rous et al. 2000) but they are also released from the cells by a process called exocytosis which can then be taken up by another cell in proximity.

Synthesis of lysosomal enzymes in the cells is a complex process which takes place in the rough endoplasmic reticulum. The enzymes then travel to the Golgi body where they acquire a mannose-6-phosphate (M6P) ligand. Two enzymes, a phosphotransferase and a diesterase, accomplish this task. This modification in the structure of the enzyme identifies it as lysosomal. This step is important because the lack of M6P moiety results in a clinical disorder resembling mucopolysaccharidosis e.g. Mucolipidoses II and III (Hickman and Neufeld 1972). This step also forms the basis of cross correction; a phenomenon fundamental to the success of HSCT. During cross correction the enzyme with M6P ligand is exocytosed by the normal cell (donor) and taken up by an enzyme deficient cell (recipient) via a M6P or mannose receptor. Cross correction is

Despite considerable improvements in the understanding of pathophysiology of LSDs, a number of questions remain unanswered. The relationship between the severity of biochemical changes and the mutations that occur in LSDs is still not clearly understood. A number of genetic abnormalities including deletions, insertions, duplications and non-sense mutations have relatively straightforward consequences and lead to a complete lack of protein or dysfunctional protein structure. However in some cases a small amount of protein is correctly translated from functional mRNA which leads to an attenuated disease type (Polten, Fluharty et al. 1991; McInnes, Potier et al. 1992). In contrast, the effects of genetic abnormalities such as mis-sense mutation and small deletions are sometimes more difficult to explain based on our current understanding of the pathophysiology of LSDs. Some of these abnormalities lead to a malfunction of active site of the enzyme protein (Huie, Hirschhorn et al. 1994; Zhang, Bagshaw et al. 2000; Garman and Garboczi 2004). This in turn can lead to a significant compromise in enzyme activity leading to a severe disease phenotype. However, in some instances, small in-frame mutations and mis-sense mutations which do not involve the active site, may lead to a severe disease phenotype (Hermann, Schestag et al. 2000). Some defects lead to misfolding of the protein structure during their synthesis in the endoplasmic reticulum (ER). This leads to formation of a protein that cannot be transported out of the ER. Consequently, these proteins are degraded inside the ER and lead to a severe disease phenotype (Paw, Moskowitz et al. 1990; Schestag, Yaghootfam et al. 2002; Poeppel, Habetha et al. 2005). The residual amount of functionally active enzyme is one of the factors which determine the phenotype of the LSDs. It has been proposed that alongside genetics, several other factors including epigenetics and environmental factors determine the disease phenotype. A D645E missense mutation in the α-glucosidase gene was shown to have an extremely heterogenous disease phenotype in patients of different ethnic origins.
Similarly, monozygotic twins carrying the same gene mutation were reported to have remarkably different disease phenotypes suggesting a role of environmental factors in disease pathology (Lachmann, Grant et al. 2004). A disease phenotype involving one of the most frequent genetic mutations (substitution of asparagine 370 by serine) in Gaucher disease has been studied in great detail. A consequence of this mutation is the development of a dysfunctional protein which leads to a base line enzyme level of approximately 10-20% of normal. A huge variation in the clinical outcome and disease severity was reported in individuals carrying the same genetic abnormality in this cohort (Beutler, Nguyen et al. 1993; Zhao and Grabowski 2002).

1.2.2. Cofactors for lysosomal enzymes

Several cofactors are required for the function of lysosomal enzymes. Deficiency of two important cofactors results in clinically significant disorders. These cofactors are broadly categorized as “saposins” and “cathepsin like proteins” which are described below in more detail.

Saposin
Saposins (sphingolipid activator proteins) metabolize glycosphingolipids. Two genes encode saposins. One gene encodes GM2 gangioliside and the other encodes prosaposin. Prosaposin is a precursor for saposins A, B, C and D. Deficiency of these cofactors also results in LSDs. The clinical disorder resulting from a cofactor deficiency depends upon the enzyme it activates. Unlike most LSDs, where deficiency of an enzyme would result in one particular disease, the cofactor deficiency can lead to a more complex clinical phenotype sometimes resembling more than one clinical entity. Prosaposin, being a precursor molecule for many cofactors results in more severe illness (Conzelmann and

**Cathepsin like proteins**

A protective protein which has cathepsin A activity, protects the lysosomal B galactosidase against proteolytic degradation. Lack of this cofactor causes a combined deficiency of B galactosidase and sialidase (Hoogeveen, Verheijen et al. 1983; Galjart, Morreau et al. 1991).

**1.2.3. Effect of lysosomal enzyme deficiency**

Accumulation of unhydrolysed substances in the cells gives rise to structural and functional changes. In some illnesses the structural changes can be recognized on light microscopy (The foam cells or sea- blue histiocytes in Niemann-Pick A, B and C disease and the typical Gaucher cell in Gaucher disease) but in others ultrastructural analysis is required to visualize the morphological anomalies (Terry and Weiss 1963; Vanier, Wenger et al. 1988). Anatomical and physiological stress causes cellular and systemic dysfunction. This dysfunction can be a consequence of either complex autocrine dysregulation resulting in altered cellular response e.g. macrophages activation (Hollak, Evers et al. 1997; Barak, Acker et al. 1999) or simple mass effect due to the increased size of the cells and tissues (Elleder, Bradova et al. 1990). The pathogenesis of LSDs is complicated and the phenotypic severity of certain diseases is not entirely explained by the current understanding of this group of disorders. The interaction between cytokines/chemokines and macrophages, neuroinflammation (Wu and Proia 2004), mitochondrial dysfunction (Jolly, Brown et al. 2002), and intracellular calcium (Lloyd-Evans, Pelled et al. 2003; Pelled, Lloyd-Evans et al. 2003) play an important role. Some of these factors have been studied in detail but still the pathogenesis of LSDs remains largely enigmatic.
<table>
<thead>
<tr>
<th>Lysosomal protein</th>
<th>Disease</th>
<th>Natural substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactosidase A</td>
<td>Fabry</td>
<td>Globotriasylceramide and blood-group-B substances</td>
</tr>
<tr>
<td>Ceramidase</td>
<td>Farber lipogranulomatosis</td>
<td>Ceramide</td>
</tr>
<tr>
<td>Glucosidase</td>
<td>Gaucher</td>
<td>Glucosylceramide</td>
</tr>
<tr>
<td>Sphingomyelinase</td>
<td>Niemann–Pick A and B</td>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>Sphingolipid activator</td>
<td>Sphingolipid activator deficiency</td>
<td>Glycolipids</td>
</tr>
<tr>
<td>Galactosidase</td>
<td>GM1 gangliosidosis</td>
<td>GM1 ganglioside</td>
</tr>
<tr>
<td>Hexosaminidase A</td>
<td>GM2 gangliosidosis (Tay–Sachs)</td>
<td>GM2 ganglioside and related glycolipids</td>
</tr>
<tr>
<td>GM2-activator protein</td>
<td>GM2 gangliosidosis (GM2-activator deficiency)</td>
<td>GM2 ganglioside and related glycolipids</td>
</tr>
<tr>
<td>Iduronidase</td>
<td>MPS I (Hurler, Scheie, Hurler/Scheie)</td>
<td>Dermatan sulphate and heparan sulphate</td>
</tr>
<tr>
<td>Iduronate-2-sulphatase</td>
<td>MPS II (Hunter)</td>
<td>Dermatan sulphate and heparan sulphate</td>
</tr>
<tr>
<td>Heparan N-sulphatase (sulphamidase)</td>
<td>MPS IIIA (Sanfilippo)</td>
<td>Heparan sulphate</td>
</tr>
<tr>
<td>N-Acetyl-glucosaminidase</td>
<td>MPS IIIB (Sanfilippo)</td>
<td>Heparan sulphate</td>
</tr>
<tr>
<td>Acetyl-CoA-glucosamide</td>
<td>MPS IIIC (Sanfilippo)</td>
<td>Heparan sulphate</td>
</tr>
<tr>
<td>N-Acetylglicosamine-6-sulphatase</td>
<td>MPS IIIID (Sanfilippo)</td>
<td>Heparan sulphate</td>
</tr>
<tr>
<td>N-Acetylgalactosamine-6-sulphate-sulphatase</td>
<td>MPSIV (Morquio-A disease)</td>
<td>Keratan sulphate, chondroitin-6-sulphate</td>
</tr>
<tr>
<td>Galactosidase</td>
<td>MPSIV (Morquio-B disease)</td>
<td>Keratan sulphate</td>
</tr>
<tr>
<td>N-Acetylgalactosamine-4-sulphatase</td>
<td>MPS VI (Maroteaux–Lamy)</td>
<td>Dermatan sulphate</td>
</tr>
<tr>
<td>Beta-Glucuronidase</td>
<td>MPS VII (Sly)</td>
<td>Heparan sulphate, dermatan sulphate, chondroitin-4- and -6-sulphates</td>
</tr>
<tr>
<td>alpha-Glucosidase</td>
<td>Pompe (glycogen-storage-disease type II)</td>
<td>Glycogen</td>
</tr>
<tr>
<td>Cystinosin</td>
<td>Cystinosis</td>
<td>Cystine</td>
</tr>
<tr>
<td>LAMP2</td>
<td>Danon disease</td>
<td>Cytoplasmic debris and glycogen</td>
</tr>
<tr>
<td>Sialin</td>
<td>Infantile sialic-acid-storage disease and Salla disease</td>
<td>Sialic acid</td>
</tr>
<tr>
<td>Mucolipin-1</td>
<td>Mucopolipidosis (ML) IV</td>
<td>Lipids and acid mucopolysaccharides</td>
</tr>
<tr>
<td>NPC1 and 2</td>
<td>Niemann–Pick C (NPC)</td>
<td>Cholesterol and sphingolipids</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Substrate</td>
<td>Disease</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>------------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Cathepsin A</td>
<td>Galactosialidosis</td>
<td>Sialyloligosaccharides</td>
</tr>
<tr>
<td>UDP-N-acetylglucosamine, N-acetylglucosaminyl-1-phosphotransferase</td>
<td>Pseudo-Hurler polydystrophy (ML II and ML III, respectively)</td>
<td>Oligosaccharides, mucopolysaccharides and lipids</td>
</tr>
<tr>
<td>Formylglycine-generating enzyme</td>
<td>Multiple sulphatase deficiency</td>
<td>Sulphatides</td>
</tr>
<tr>
<td>CLN1 (protein palmitoylthioesterase-1)</td>
<td>Neuronal ceroid lipofuscinosis (NCL1) (Batten disease)</td>
<td>Lipidated thioesters</td>
</tr>
<tr>
<td>CLN2 (tripeptidyl amino peptidase-1)</td>
<td>NCL2 (Batten disease)</td>
<td>Subunit c of the mitochondrial ATP synthase</td>
</tr>
<tr>
<td>Arginine transporter</td>
<td>NCL3 (Batten disease)</td>
<td>Subunit c of the mitochondrial ATP synthase</td>
</tr>
</tbody>
</table>

Table 1.1  Selected important hydrolases, their natural substrates and Lysosomal Storage Disorders resulting from their deficiency

Various classifications are used for this diverse group of illnesses. The World Health Organization has given ICD (international statistical classification of diseases) codes to each of the diseases in this category. This system is not particularly useful in a clinical setting as many diseases share symptomology. The most widely used classification is based on the type of substrate accumulated. Five major groups according to this classification include Mucopolysaccharidosis, Lipid storage disorders (Sphingolipidosis, Gangliosidosis and Leukodystrophies), Mucolipidosis, Glycoprotein storage disorders and Glycogen storage disorders. Some important LSDs are described in table 1.1.
1.3. Clinical manifestations

LSDs can present in infancy, childhood and adult age groups. Infantile presentations are generally more severe and more frequently present with neurological symptoms. These are more often rapidly progressive and may be fatal. Presentation in adults is mostly insidious but it can cause significant morbidity and disability over a period of time. Juvenile (childhood) forms are intermediate in severity. These multisystem disorders can involve any organ or system and frequently affect the central nervous system (CNS), musculoskeletal (Kovac 2011), reticuloendothelial, cardiovascular and respiratory systems (van den Berg, de Vries et al.; Mohan, Hay et al. 2002; Grabowski, Andria et al. 2004; Hoffmann and Mayatepek 2005; Pastores and Meere 2005; Eng, Fletcher et al. 2007; Yagci, Salor et al. 2009; Damme, Stroobants et al. 2010; Malek, Chojnowska et al. 2010; Berger, Fagondes et al. 2012; Golda, Jurecka et al. 2012). The neurological symptoms vary from subnormal intelligence to severe brain stem disorders (Jones, Alroy et al. 1997; Ashworth, Biswas et al. 2006; Hamano, Hayashi et al. 2008). LSDs can have attenuated phenotypes which are milder in severity e.g. MPSI has three subtypes known as MPSI H (Hurler syndrome), MPSI HS (Hurler Scheie syndrome) and MPSI S (Scheie syndrome). MPSI H is the most severe form and symptoms include progressive neurodegeneration, visceromegaly, respiratory problems, airway obstruction, and musculoskeletal abnormalities. This form is diagnosed early (within the first year of life) and leads to premature death (Cleary and Wraith 1995). Scheie syndrome (MPSI S) is the mildest form of the disease which is diagnosed much later (on average at 5 years of age) and has a less severe phenotype. The symptoms include joint stiffness, facial changes and mild cardio-respiratory abnormalities. These patients are likely to have a normal life span (Wraith 1995). MPSI HS is intermediate in severity. However, this may result in severe visceral abnormalities leading to reduced life expectancy. The skeletal abnormalities in LSDs are quite variable. These changes are sometimes
referred to as dysostosis multiplex. Short stature and widespread skeletal abnormalities are frequent manifestations. Patients with severe vertebral abnormalities can present with spinal cord compression leading to long term disability. Organomegaly with hepatic dysfunction and recurrent abdominal pain can be a presenting feature in some LSDs. Impaired cardiac function is a cause of serious morbidity in infantile Pompe and can be a limiting factor in delivering potentially cardiotoxic chemotherapy as a conditioning regimen prior to HSCT (Kishnani, Hwu et al. 2006; Wynn, Stubbs et al. 2009). Hydrops fetalis has been reported in some LSDs e.g. Farber’s disease (Kattner, Schafer et al. 1997) and galactosidosis (Haverkamp, Jacobs et al. 1996). More recently, this was reported in other LSDs such as MPS IV, MPS VII and NPC (Whybra, Mengel et al. 2012). Clinical manifestations in patients with LSDs treated with Enzyme replacement therapy (ERT) are summarized in table 1.2.
<table>
<thead>
<tr>
<th>Type of LSD</th>
<th>Clinical manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPS I</td>
<td><strong>Hurler</strong></td>
</tr>
<tr>
<td></td>
<td>Short stature, skeletal abnormalities, hepatosplenomegaly, corneal clouding, cardiac dysfunction, airway obstruction, coarse facial features, severe progressive neurodegeneration, premature death.</td>
</tr>
<tr>
<td></td>
<td><strong>Scheie</strong></td>
</tr>
<tr>
<td></td>
<td>Corneal clouding, cardiac dysfunction, respiratory disease, joint stiffness, normal intelligence or mild intellectual impairment.</td>
</tr>
<tr>
<td></td>
<td><strong>Hurler-Scheie</strong></td>
</tr>
<tr>
<td></td>
<td>Mild cognitive impairment, skeletal abnormalities, hepatosplenomegaly, respiratory disease, cardiac dysfunction, corneal clouding (severity of clinical phenotype is usually intermediate between Hurler and Scheie).</td>
</tr>
<tr>
<td>MPS II (Hunter)</td>
<td>Variable in severity. Skeletal abnormalities (short stature, coarse facial features), hepatosplenomegaly, macroglossia, progressive neurodegeneration (variable severity), respiratory disease, airway obstruction, cardiomyopathy, recurrent infections.</td>
</tr>
<tr>
<td>MPSVI (Maroteaux-Lamy)</td>
<td>Short stature, coarse facial features, skeletal abnormalities, joint stiffness, organomegaly, corneal clouding.</td>
</tr>
</tbody>
</table>
| Pompe            | Infantile form: hypotonia, failure to thrive, respiratory failure, cardiac failure, hepatosplenomegaly, macroglossia  
|                   | Adult form: skeletal muscle abnormalities, respiratory failure (insidious), cardiac dysfunction                                                                                                                          |
| Gaucher          | Hepatosplenomegaly, pancytopenia, liver cirrhosis, osteoporosis, skeletal abnormalities, neurological involvement (convulsion, dementia, muscle apraxia) in type II and III only.                                           |
| Fabry            | Renal failure, cardiac dysfunction, cardiomyopathy, cataract, optic atrophy, Small fibre neuropathy, vasculitis,                                                                                                          |

**Table 1.2 Clinical features of LSDs treated with ERT (Cox and Schofield 1997; Brady and Schiffmann 2000; van der Beek, Hagemans et al. 2006)**
1.4. Treatment strategies

1.4.1. Haemopoietic stem cell transplant (HSCT)

History

A zygote (totipotent stem cell) is the first cell of a new individual. This cell divides to form pluripotent stem cells. Pluripotent stem cells retain the ability to differentiate into any cell derived from the three germinal layers. Multipotent stem cells are more committed cells derived from pluripotent precursors. These can further differentiate into a closely related family of cells. Multipotent haemopoietic stem cells are critical for HSCT as they are capable of reconstituting the entire lympho-haemopoietic system. The first stem cell transplants were done over 50 years ago in patients with leukemia and immunodeficiency. In 1959, Thomas reported the first bone marrow transplant in a patient with end stage leukemia. The source of stem cell was the bone marrow of an identical twin. This patient survived nearly three months (Thomas, Lochte et al. 1959).

The first HSCT for LSDs was reported in a patient with MPSIH in 1981 (Hobbs 1981). The rationale for transplantation in LSDs, is to deliver cellular enzyme replacement therapy from engrafted donor blood cells. Haemopoietic precursor cells (HPC) progeny differentiate into tissue macrophages including Kupfer cells in liver and microglial cells within the brain parenchyma of the recipient. Lysosomal enzymes secreted from these normal donor cells are taken up by the enzyme deficient recipient cells in the vicinity, via the Mannose or M6P receptor. This phenomenon is known as cross-correction (Neufeld 1983) and results in biochemical and phenotypic recovery of recipient cells (Hobbs, Hugh-Jones et al. 1981; Krivit 1983; Krivit, Pierpont et al. 1984) (see figure 1.1).

In 1960, Human leucocyte antigens (HLA) were identified and it became possible to match the HLA types of the donors and recipients. This paved the way for further development of HSCT as a viable treatment option which has evolved considerably since then. Various types of conditioning regimens and
stem cell sources are now in use. HLA matching and immunosuppression is more sophisticated and effective. All these changes have resulted in steady improvement in the outcome of HSCT over the years.

**Principles of HSCT**

**HLA matching**

The process of HSCT involves carefully selecting the donor and source of stem cells. A sibling has a 1/4 chance of being a match for the patient. However, transplantation in genetic disorders, means that 2/3 of the matched siblings are likely to be heterozygote carriers of the disease. This observation is important because the carriers of defective genes may deliver a reduced amount of enzymes to their affected siblings. The patient is typed for HLA class I (A, B and C) and class II (DR and DQ). For unrelated donor transplantation from peripheral blood or bone marrow sources the donor is matched for all 10 loci. It is not always possible to find a 10/10 match. Greater disparity between the donor’s HLA type and the recipient’s can increase transplant complications e.g. Graft versus host disease and transplant related mortality (TRM) (Diaconescu, Flowers et al. 2004; Sorror, Maris et al. 2004; Copelan, Casper et al. 2007).

Umbilical cord blood, on the other hand requires less stringent matching. It is sufficient to match the cord blood at 6 loci instead of 10 (HLA A, B and DR). Many centres use up to a 4/6 match for cord blood transplant in genetic disease.
Innate enzymes are glycosylated (green circles) in the endoplasmic reticulum (ER) of normal cells. Further modification to incorporate the mannose 6-phosphate takes place in the Golgi apparatus (Golgi) where they bind the mannose 6-phosphate receptor (M6PR). The majority (heavy arrows) of the enzymes are then transported to the lysosome (Lys) whilst a minority (fine arrows) are secreted from the cell. The secreted enzyme can bind the cell membrane M6PR or the mannose receptor (ManR), respectively and are internalized. M6PR is universally expressed on all cells, whereas ManR expression is limited to cells of the reticuloendothelial system (Modified from (Sands and Davidson 2006))
Sources of stem cells

The following three sources of stem cells are currently in use for HSCT.

Bone marrow (BM)

Bone marrow is usually taken from the iliac crest by bone marrow needles. This requires general anaesthesia (GA) and multiple needle insertions into the pelvic bone to aspirate the bone marrow. The incidence of graft versus host disease (GVHD) with bone marrow stem cells is lower but the cell engraftment may take longer when compared to peripheral blood as a source of stem cells.

Peripheral blood (PB)

Stem cells are mobilized from the bone marrow into the peripheral blood with the help of granulocyte colony stimulating factor (G-CSF). The mobilized stem cells are then collected by leukapheresis. This does not require GA. A peripheral blood stem cell transplant (PBSCT) tends to engraft quicker than the bone marrow but the incidence of graft versus host disease (GVHD) may be higher (Schrezenmeier, Passweg et al. 2007).

Umbilical cord blood (UCB)

Fetal blood is rich in stem cells. After birth the fetal blood is collected from the placenta. The umbilical cord blood is a suitable source of transplant because it is rapidly available, poses no risk to the donor, contains less risks of disease transmission, and requires less precise matching. The dose of stem cells in cord blood collection is generally lower than peripheral blood and bone marrow, and the engraftment and immune reconstitution can be slower. Recent data has shown that the enzyme delivery, donor chimerism and outcome of HSCT is superior when cord blood is used as a source of stem cells, hence it is has become a preferred method for LSD patients receiving HSCT in many centres (Staba, Escolar et al. 2004; Prasad and Kurtzberg 2008; Prasad, Mendizabal et al. 2008).
Conditioning

Chemotherapy and radiotherapy are used to prepare the recipients for transplantation. This is called conditioning. In children, receiving HSCT for LSD, the conditioning almost always involves chemotherapy without the use of radiotherapy. The purpose of conditioning is to empty the bone marrow niche so that the stem cells from the donor can be engrafted in that space. It also weakens the recipient immune system which facilitates the engraftment of the donor cells. In malignancies, conditioning reduces the disease burden prior to transplant. Various types of conditioning regimens are in use. Broadly the two main types of conditioning are myeloablative and non-myeloablative or reduced intensity. Myeloablative conditioning used for HSCT in LSDs is usually based on busulfan conditioning. Cyclophosphamide and/or Melphlan is used in combination with busulphan. Use of T cell depletion (Campath or Antithymocyte globulin) depends upon the donor type and the risk of GVHD. A number of non-myeloablative conditioning regimens are in use in different transplant centres and may include non-myeloablative doses of chemotherapeutic agents including treosulphan, cyclophosphamide, melphalan etc. Use of chemotherapy inevitably contributes to the risks of HSCT. Major side effects of chemotherapy are organ toxicity, multiorgan failure, myelosuppression, immunosuppression, infertility and risk of secondary malignancies (Shalet, Didi et al. 1995; Michel, Socie et al. 1997; Cohen, Bekassy et al. 2008; Faraci, Bekassy et al. 2008; Friedman, Rovo et al. 2008; Tichelli, Passweg et al. 2008; Tichelli, Rovo et al. 2008).

At the end of conditioning the stem cells are infused to the patient. A period of myelosuppression and immune suppression follows the conditioning chemotherapy. During this phase immunosuppressive therapy is continued to prevent GVHD. Prophylactic antimicrobials are given for a prolonged period of time to prevent opportunistic infections. The short term risks of transplant are mainly due to the toxicity of conditioning, myelosuppression, immunosuppression, acute GVHD and multi organ failure requiring intensive care support. These contribute to the high transplant related mortality (TRM)
immediately after the transplant (Hayes, Lush et al. 1998). HSCT may be associated with long term side effects and significant medium to long term morbidities. These include short stature (Polgreen, Tolar et al. 2008), endocrinopathies (Ho, Lewis et al. 2011), premature cataract, secondary cancers (Niethammer and Mayer 1998) and organ dysfunction due to chronic GVHD. In summary the risks of transplant may be due to the toxicity of chemotherapeutic agents used during conditioning, myelosuppression, immunosuppression and graft versus host disease. In every patient, under consideration for HSCT, the risks and benefits must be weighed carefully.

The outcome of HSCT in lysosomal diseases

The first allogeneic HSCT for LSD in a patient with MPS IH resulted in normal development and intelligence (Boelens, Prasad et al. 2010). Over the last three decades nearly 2000 patients have been transplanted for LSDs in Europe and North America. Early experience of HSCT in MPS I showed that it led to improvements in cardio-respiratory symptoms, ocular changes, organomegaly and psychomotor symptoms (Whitley, Ramsay et al. 1986; Summers, Purple et al. 1989; Braunlin, Hunter et al. 1992; Haddad, Jones et al. 1997; Vellodi, Young et al. 1997). HSCT in MPSVI was shown to improve clinical outcome and neurocognition in the recipient (Krivit, Pierpont et al. 1984). Similarly, encouraging outcomes were reported after HSCT in late onset metachromatic leukodystrophy (MLD) (Imaizumi, Gushi et al. 1994). So far, HSCT has been undertaken in a number of LSDs including MPS II, MPS III, MPS VI, α-Mannosidosis, globoid cell leukodystrophy with variable clinical outcome (Krivit, Pierpont et al. 1984; Vellodi, Young et al. 1992; Coppa, Gabrielli et al. 1995; Wall, Grange et al. 1998; Kapaun, Dittmann et al. 1999; Krivit, Aubourg et al. 1999; Turbeville, Nicely et al. 2011). Current experience of the use of HSCT in LSD is summarized in table 1.3.

In 2008, Moore et al. published data on long term survival of MPS I patients in the UK. The survival of the patients who received HSCT was superior to those who did not receive transplant. The probability of survival after BMT was 68%,
66% and 64% at two, five and ten years (Moore, Connock et al. 2008). Over the last two decades the overall survival (OS) of HSCT in MPS I patients has been reported to be around 40-70% (Peters, Balthazor et al. 1996; Peters, Shapiro et al. 1998; Souillet, Guffon et al. 2003). Data from Eurocord and Duke University showed that the OS in the patients transplanted after 2004 was 75% (and above 80% in registry reported grafts for the most recently transplanted children) and before 1999 it was 53%. In some centres this figure is now approaching 90% (Boelens, Rocha et al. 2009; Wynn, Mercer et al. 2009). The improvement in the overall survival has been attributed to the changes in the clinical practice of HSCT (Vellodi, Young et al. 1997; Grewal, Krivit et al. 2002). A multicentre study looked at the risk factors for graft failure in 2007 and identified three major reasons for improved survival in these patients. Firstly, an effective conditioning regimen was developed which included intravenous busulfan with pharmacokinetic monitoring. Secondly, use of full intensity conditioning regimens which resulted in better engraftment. Thirdly, use of a T cell replete graft which reduced graft rejection resulting in improved outcome (Boelens, Wynn et al. 2007). This group also showed that the metabolic outcome of unrelated donor transplant recipients was superior to those who received ERT alone (Wynn, Wraith et al. 2009). In this study, urinary dermatan sulphate (DS) to chondroitin sulphate (CS) ratio was measured to evaluate metabolic recovery in the patients receiving ERT and this was compared to the recipients of sibling and unrelated donor HSCT. This study showed that unrelated donor transplant most effectively reduced the residual substrate, followed by heterozygous donor transplants (sibling). Substrate reduction was lowest in the recipients of ERT. Even though the enzyme levels and immune response in ERT treated patients were not measured, it is possible that the inferior outcome in this group of patients was due to the reduced level of functional enzyme which was neutralized by inhibitory antibodies. This is supported by a close analysis of the data, in this study, which reveals that some patients showed significantly worse biomarker response when compared to the other ERT recipients (Wynn, Wraith et al. 2009).
For an individual patient undergoing HSCT a number of factors determine the outcome after transplant (Wynn 2011) which include type of disease, genotype/phenotypic characteristics, age at the time of transplant, use of ERT prior to HSCT (Tolar, Grewal et al. 2008), availability and efficacy of alternative treatments, complications of applied therapy and a multidisciplinary approach. Carefully balancing these factors is likely to result in improved outcome of HSCT in LSDs. A number of studies have consistently shown that the HSCT improves the clinical outcome including organomegaly, functional and neurophysiological outcomes, particularly if done earlier in asymptomatic patients (Peters, Balthazor et al. 1996; Vellodi, Young et al. 1997; Guffon, Souillet et al. 1998; Peters, Shapiro et al. 1998; Staba, Escolar et al. 2004).

In neurocognitive disorders, HSCT is superior to ERT due to its ability to correct CNS disease by donor derived mononuclear cells. These cells interact with the adhesion molecules on the surface of the brain capillary endothelium and result in transmigration of cells into the brain parenchyma (Martin-Padura, Lostaglio et al. 1998). This complex process starts with adhesion of leucocytes to endothelial receptors followed by transient opening of endothelial junction to allow migration of the cells through the endothelium (Martin-Padura, Lostaglio et al. 1998). These cells subsequently engraft within the brain tissue and become resident macrophages known as microglial cells, providing deficient enzyme to affected cells by cross correction.
Pharmacological ERT (infused) stays in the circulation and is delivered to the peripheral tissues. However, this ERT cannot penetrate the blood brain barrier and hence fails to correct the CNS disease. In contrast, cellular ERT (allogeneic HSCT) delivers enzyme into the CNS via donor derived microglial cells (Wynn 2011).
### Table 1.3 Indications of HSCT in Lysosomal Storage Disorders

(These classifications were written as a guide to the commissioners of transplant services in the UK)

<table>
<thead>
<tr>
<th>Indication</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard therapy</td>
<td>MPSIH (Hurler)</td>
</tr>
<tr>
<td></td>
<td>MPSVII (Sly)</td>
</tr>
<tr>
<td></td>
<td>alpha-mannosidosis</td>
</tr>
<tr>
<td></td>
<td>Pre-symptomatic Krabbe</td>
</tr>
<tr>
<td></td>
<td>Late onset globoid cell leukodystrophy</td>
</tr>
<tr>
<td></td>
<td>Wolman</td>
</tr>
<tr>
<td>Optional</td>
<td>MPSIH/S</td>
</tr>
<tr>
<td></td>
<td>MPSIS</td>
</tr>
<tr>
<td></td>
<td>MPSVI</td>
</tr>
<tr>
<td></td>
<td>MPSVII</td>
</tr>
<tr>
<td></td>
<td>Late onset Krabbe</td>
</tr>
<tr>
<td></td>
<td>Fucosidosis</td>
</tr>
<tr>
<td></td>
<td>Farber</td>
</tr>
<tr>
<td></td>
<td>Gaucher (Non neuronopathic and norrbottnian)</td>
</tr>
<tr>
<td></td>
<td>Niemann-pick C</td>
</tr>
<tr>
<td>Investigational</td>
<td>MPSIIA</td>
</tr>
<tr>
<td></td>
<td>MPSIIB</td>
</tr>
<tr>
<td></td>
<td>MPSIII A</td>
</tr>
<tr>
<td></td>
<td>MPSIIIB</td>
</tr>
<tr>
<td></td>
<td>MPSIIIC</td>
</tr>
<tr>
<td>Unknown</td>
<td>Gaucher (acute neuronopathic and subacute neuronopathic)</td>
</tr>
<tr>
<td></td>
<td>Tay sachs (early onset)</td>
</tr>
<tr>
<td></td>
<td>MLD (early onset)</td>
</tr>
<tr>
<td></td>
<td>Sandhoff</td>
</tr>
<tr>
<td></td>
<td>Niemann-pick A</td>
</tr>
<tr>
<td></td>
<td>Niemann-pick B</td>
</tr>
</tbody>
</table>
1.4.2. Enzyme replacement therapy (ERT)

The basic concept of enzyme replacement therapy was first described by de Duve in 1964 (Deduve 1964). The initial attempts to treat LSDs by enzyme replacement therapy were made in the 1970s. A number of clinical studies were undertaken to see the effects of intravenous infusion of deficient enzymes in Pompe, Fabry and type I Gaucher disease. Human urine or cord blood was used as a source to extract lysosomal human enzyme. Unfortunately this enzyme treatment failed to achieve therapeutic levels of deficient enzymes and hence was abandoned. Around that time HSCT was introduced as a therapy for LSDs showing promising results in some diseases in this group and resulting in further decline in interest in ERT. In the 1980’s, attempts were made to prepare more purified forms of lysosomal enzyme. Genzyme purified Beta glucosidase from human placenta and manufactured it on an industrial scale. This treatment proved very successful with excellent haematological, biochemical and clinical response in patients and radically changed the management of Gaucher disease (Barton, Furbish et al. 1990; Barton, Brady et al. 1991). The original product (Ceredase) was subsequently replaced by recombinant human enzyme produced in Chinese hamster ovary cells (Cerezyme) (Wraith 2006). Following the commercial success of recombinant human glucocerebrosidase a great deal of interest was generated in this potentially lucrative area. This encouraged the development of new recombinant human enzyme therapies for the treatment of other LSDs. During this treatment patients receive regular intravenous infusions. This is essentially a lifelong therapy to replace the deficient enzyme and may require insertion of long term tunneled intravenous catheters. After infusion into the veins the administered enzyme is taken up by the deficient cells by endocytosis. As explained earlier, delivery of the enzyme to the lysosomes within a cell is dependent upon mannose or M6P receptors. Therefore it is crucial that the delivered recombinant human enzyme is recognized by the appropriate receptor. This is achieved by modification of the glycoprotein structure during the manufacture of recombinant human enzyme (Richards).
Since the development of alglucerase (Cerezyme) for type 1 Gaucher Disease (1991), a number of other LSDs have been treated with ERT. This treatment however cannot correct the CNS disease due to its inability to penetrate through the blood brain barrier (BBB). This limitation is discussed in detail in a later section. Currently available ERTs are shown in the table 1.4.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Defective enzyme</th>
<th>Substrate accumulation</th>
<th>Enzyme replacement therapy</th>
<th>Incidence of IgG antibody (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gaucher Disease</td>
<td>Acid beta glucosidase &lt;br&gt;(glucocerebrosidase)</td>
<td>Glucosylceramide</td>
<td>Alglucerase (Ceredase)</td>
<td>12.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Imiglucerase (Cerezyme)</td>
<td>13.8</td>
</tr>
<tr>
<td>Fabry Disease</td>
<td>Alpha galactosidase A</td>
<td>Globotriaosylceramide</td>
<td>Agalsidase beta &lt;br&gt;(Fabrazyme)</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Agalsidase alfa &lt;br&gt;(Replagal)</td>
<td></td>
</tr>
<tr>
<td>Hurler Syndrome (MPSI)</td>
<td>Alpha L-iduronidase</td>
<td>dermatan and heparan sulfates</td>
<td>Iduronidase &lt;br&gt;(Aldurazyme)</td>
<td>91</td>
</tr>
<tr>
<td>Pompe Disease (GSD II)</td>
<td>Acid alpha glucosidase</td>
<td>Glycogen</td>
<td>Myozyme</td>
<td>89</td>
</tr>
<tr>
<td>Hunter’s (MPSII)</td>
<td>Iduronate-2-sulfatase</td>
<td>Dermatan and heparin sulfates</td>
<td>Elaprase</td>
<td>51</td>
</tr>
<tr>
<td>Niemann-Pick B Disease</td>
<td>Sphingomyelinase &lt;br&gt;NPC1 and 2</td>
<td>Cholesterol and sphingolipids</td>
<td>Recombinant human acid sphingomyelinase &lt;br&gt;(rhASM)</td>
<td>NA</td>
</tr>
<tr>
<td>Maroteaux-Lamy (MPS VI)</td>
<td>N-acetylgalactosamine-4-sulfatase</td>
<td>Dermatan sulphate</td>
<td>Naglazyme arylsulfatase B</td>
<td>97</td>
</tr>
</tbody>
</table>

**Table 1.4**  Currently available Enzyme Replacement Therapies (ERT) and incidence of antibodies in Lysosomal Storage Disorder patients treated with ERT.
The outcome of ERT

An ideal ERT should be able to deliver therapeutic enzyme levels, have minimal side effects associated with its administration, stop the disease progression and correct the existing disease manifestations in patients. The outcome of ERT in Gaucher disease and MPSI has probably been scrutinized more than any other LSD. In 2002, Weinreb et al published 2-5 year follow up data on 128 patients with Gaucher disease. Patients on ERT experienced significant improvement in various parameters evaluated during this study including haematological indices, hepatomegaly (30-40% reduction), splenomegaly (50-60%) and disease related episodes of pain (half of the patients were pain free within two years). Recombinant human acid beta glucosidase is now the standard treatment for patients with Gaucher disease (Weinreb, Charrow et al. 2002). Similar results were reported in a phase I study in MPSI patients who received ERT (recombinant human alpha-L-iduronidase- laronidase). In an initial study, 12 patients with MPSI (age 5-22 years) had clinical radiological and biochemical assessment on week 6, 12, 26 and 56 after commencing the treatment. Radiological investigations included magnetic resonance imaging of the brain and abdomen and echocardiogram of the heart. A significant improvement in clinical outcome was described. Hepatosplenomegaly improved in all patients (liver size in 8 patients was reported to be within normal range by six months). Cardiac dysfunction ameliorated in all patients and ejection fraction improved on echocardiogram (Kakkis, Muenzer et al. 2001). Subsequently a double blind placebo controlled trial compared the clinical outcome of 22 patients (18 MPSI HS and 4 MPS IS) treated with laronidase with that of 23 patients who received placebo. An improvement in visceromegaly, forced vital capacity (FVC) and 6 minute walk test was reported in laronidase group (Wraith, Clarke et al. 2004). This study, which was originally designed for 26 weeks, was extended into an open label clinical trial for a period of 72 weeks and confirmed ongoing clinical response in the patients treated with laronidase (Wraith 2005).
To evaluate the safety and efficacy of recombinant human iduronate-2-sulfatase (idursulfase) in the treatment of MPS II, 96 patients were enrolled in a double-blind, placebo-controlled trial. At the time of evaluation at one year, patients in ERT group showed statistically significant improvements in clinical parameters compared to the placebo group (Muenzer, Gucsavas-Calikoglu et al. 2007). In another randomised control study in MPS II patients (n=12), idursulfase group had lower urinary GAG and showed an improvement in hepatosplenomegaly and exercise tolerance (Muenzer, Wraith et al. 2006). Several other studies subsequently confirmed the efficacy of ERT in MPS II (Giugliani, Federhen et al. 2010; Schulze-Frenking, Jones et al. 2010).

A number of phase I-III trials of arylsulfatase B (recombinant human N-acetylgalactosamine 4-sulfatase) for MPS VI, confirmed the superior clinical and biochemical outcome of patients treated with ERT (Harmatz, Giugliani et al. 2006; Harmatz, Yu et al. 2010). Recently the European clinical surveillance programme (CSP) published an initial report of an intended 15 yearlong study which showed significant ongoing improvement in organomegaly and clinical outcome in patients on ERT (Hendriksz, Giugliani et al. 2011). Several other reports suggested improved outcome in ERT treated MPS VI patients (Decker, Yu et al. 2010; Giugliani, Federhen et al. 2010; Lin, Chen et al. 2010).

Results of two clinical trials were published for Fabry disease in 2001. Both agalsidase alfa (Schiffmann, Kopp et al. 2001) and agalsidase beta (Eng, Banikazemi et al. 2001; Eng, Guffon et al. 2001) showed promising results in phase I-III trials and improved clinical and biochemical parameters. Thurberg et al. also reported reversal of histological changes in Fabry disease patients treated with agalsidase beta (Thurberg, Randolph Byers et al. 2004). The enzyme replacement therapy also improved symptoms in female carriers of the disease who had some disease manifestations (Baehner, Kampmann et al. 2003).

Initial reports of recombinant human alpha-glucosidase as a treatment in Pompe disease patients were encouraging and confirmed its safety (Van den Hout,
Reuser et al. 2001). Winkell et al. reported three wheelchair-bound patients who showed signs of disease stabilization and some recovery in skeletal muscle function (Winkel, Van den Hout et al. 2004). An open-label study of ERT in 44 late-onset Pompe disease patients showed improvement in 6-min walk tests and serum creatinine kinase levels (Strothotte, Strigl-Pill et al. 2010). A UK survey of 20 infantile Pompe disease patients treated from 2000 to 2009 with a median duration of treatment of 31 months showed an overall survival of 65% and ventilator-free survival of 35% (Chakrapani, Vellodi et al. 2010). However, in this study, 35% (7/20) patients were reported to have died at a median age of 10 months which was worse than the original clinical trials on the recombinant enzyme. This was attributed to the late diagnosis and a higher number of infantile Pompe disease patients with a more severe clinical illness. Improvement in the clinical and biochemical parameters in Pompe disease has also been reported in other publications(Van den Hout, Reuser et al. 2001; Van den Hout, Kamphoven et al. 2004; Chakrapani, Vellodi et al. 2010; Strothotte, Strigl-Pill et al. 2010).

1.4.3. Substrate reduction therapy (SRT) and chaperone mediated therapy (CMT)

As discussed earlier, ERT and HSCT work by delivering the enzyme to the enzyme deficient individual. This in turn reduces the substrate accumulation in the body by metabolizing the substrate. In contrast SRT works by slowing down the accumulation of substrate in the cells or driving substrates down alternative degradation pathways. Platt et al. in 1994 described an N-alkylated imino-sugar compound, N-butyl-deoxynojirimycin (Miglustat), to be a potent inhibitor of the ceramide-specific glucosyltransferase. This substance inhibited synthesis of glycosphingolipids and prevented accumulation of glucosylceramide in an invitro model of Gaucher disease. Radin and colleagues in 1996 proposed that it would be feasible to slow the synthesis of glucosylceramide by an inhibitor drug,
1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), to attain clinical and biochemical recovery in Gaucher disease (Radin 1996). In 2000 Cox et al published data from a human trial in Gaucher disease patients receiving oral Miglustat. This study showed modest clinical and haematological improvement (Cox, Lachmann et al. 2000). Miglustat (Zavesca) was approved for use in type I Gaucher disease by European Committee for Proprietary Medicinal Products (CPMP) in July 2002 and subsequently the Food and Drug Administration (FDA) granted approval for its use in USA in July 2003 (Pastores 2006). The results of miglustat phase I and II studies in Niemann-Pick type C (Pineda; Pineda, Wraith et al. 2009) and chronic GM2 gangliosidosis type Sandhoff (Masciullo, Santoro et al.) show modest responses, but this treatment is not curative for any of these LSDs.

In recent years, there has been an increasing interest in a naturally occurring tyrosine kinase inhibitor (genistein) which can cross the BBB. It reduced the liver lysosomal compartment size in MPSIIIB mouse model after eight weeks of treatment (Malinowska, Wilkinson et al. 2009). In a recent study involving 9 months of continuous treatment with genistein in the same mouse model, significant improvement in lysosomal storage, substrate reduction and neuroinflammation were noted. These changes translated into correction of behaviour in the mice (Malinowska, Wilkinson et al. 2010).

Chaperones are small molecular weight ligands which bind to mutated lysosomal proteins and stabilize them. This results in functional activation of the defected proteins. Chaperone mediated therapies (CMT) e.g. imino acids (N-butyldeoxynojirimycin) and N-octyl-4-epi-beta-valienamine (NOEV) have been tested in glycosphingolipidoses (Priestman, Platt et al. 2000; Butters, Dwek et al. 2005) and beta-galactosidosis (Matsuda, Suzuki et al. 2003) respectively. In a recent study, 1-deoxynojirimycin derivatives were shown to be more potent inhibitors of D-galactosidases. This has resulted in renewed interest in studying their efficacy in the treatment of GM1-gangliosidosis (Frohlich, Furneaux et al. 2011).
These therapies are inexpensive, easy to administer, generally well tolerated and have a promising side effect profile. However, limited clinical efficacy remains the main limitation of these treatments. Although neither SRT nor CMT is curative as a single agent therapy, their utility as combination or adjuvant therapies in milder disease phenotypes is yet to be assessed.

1.4.4. Gene therapy

Gene therapy aims to replace a mutated gene with functional copies of a normal gene to correct the phenotype of the disease (Ponder and Haskins 2007). Once incorporated into the cellular DNA, the gene is transcribed and translated to provide a permanent source of enzyme which can correct the disease phenotype (Langford-Smith, Wilkinson et al. 2012). Gene therapy can be delivered by several techniques which mostly utilize a delivery mechanism known as a vector. However, it is possible to deliver the gene directly and achieve a limited local response (Sikes, O'Malley et al. 1994; Yoo, Soker et al. 1999). A major disadvantage of this approach is rapid degradation of gene product in vivo. A number of vectors have been used to deliver stable copies of gene into the cells. Vectors can be broadly classified as viral vectors and non-viral vectors. Adeno-Associated viral vector (Koeberl, Alexander et al. 1997; Alba, Bosch et al. 2005; Haisma and Bellu 2011), retroviral vectors (Dani 1999) and lentiviral vectors (Zufferey, Dull et al. 1998) have been used in gene therapy approaches. Non-viral vectors involve delivery of gene via chemical or physical carrier (Niidome and Huang 2002). HSCT can be used to deliver gene therapy (Emery, Nishino et al. 2002). Due to the improvement in outcome of HSCT, gene delivery by HSC using a viral vector such as lentivirus (LV-HSCT) has become feasible. This approach has been used in animal models of several neurodegenerative LSDs. LV-HSCT has shown promising results in metachromatic leukodystrophy (Biffi, Capotondo et al. 2006), MPSI (Visigalli, Delai et al. 2010) and MPS IIIA (Langford-Smith, Wilkinson et al. 2012). Even
though gene therapy is still a research tool, based on the recent data, a clinical trial is underway to evaluate the efficacy of gene therapy in MLD.

1.5. Limitations of treatments

The limitations of the two main treatments used in clinical practice i.e. HSCT and ERT are discussed in detail in following sections.

1.5.1. Limitations of HSCT

As mentioned earlier, HSCT is now a standard treatment for some LSDs e.g. MPSIH. However, even in this disorder the clinical outcome is inconsistent in different tissues. It is less effective in prevention and reversal of some disease manifestations e.g. musculoskeletal and orthopaedic problems (Masterson, Murphy et al. 1996; Weisstein, Delgado et al. 2004). Early reports of HSCT in several LSDs including Hunter syndrome (Shapiro, Lockman et al. 1995; McKinnis, Sulzbacher et al. 1996) and Gaucher (Rappeport and Ginns 1984; Tsai, Lipton et al. 1992) showed some efficacy but relatively high morbidity and mortality. HSCT is ineffective in other LSDs where the results of transplant are not encouraging e.g. the initial reports of HSCT in patients with MPSIII (Sivakumur and Wraith 1999) showed that the HSCT did not prevent neurological deterioration even if the transplant was performed early in life. More recently a better outcome was reported in 19 patients with MPSIII in a single centre study. 12 patients survived the transplant and 9 of them were reported to have some degree of disease stabilization. In this cohort most of the patients were transplanted late in life and only two patients received HSCT before the age of 2 years. Even in the patients who were transplanted late during their life (3-5 years), some improvement was reported in cognitive skills. In this cohort the clinical outcome was assessed by various clinical and neurophysiological parameters and was found to be better than the non-transplanted patients. However in comparison to the normal controls a generalized developmental delay was reported. Despite these initial
encouraging results, it is too early to conclude about the long term outcome of transplant in these patients (Prasad, Mendizabal et al. 2008). The reason for poor outcome in some LSDs is not entirely clear. In animal models of MPSIII, the transplant reduced the heparan sulphate derived disaccharides by 27% in brain parenchyma but the secondary cholesterol and GM3 ganglioside storage was not significantly altered and hence did not translate into clinical improvement (Lau, Hannouche et al. 2010). This transmigration does not deliver therapeutic enzyme levels in the brain and fails to achieve neurological recovery by cross correction. It has been proposed that the delivery of enzyme into the tissue could be improved by either increasing the enzyme available to the tissue or improving the tissue specific transport coefficient (Wynn et al 2009). Graft manipulation to achieve higher gene expression could deliver supraphysiological levels of enzyme to the brain tissue. This might offer an effective cellular therapy in these patients in future.

In summary HSCT is a promising treatment for some LSDs but it has some limitations. It is not effective in all LSDs. Even though the outcome of HSCT has been steadily improving, it still carries significant morbidity and mortality. Apart from short term risks of relatively high transplant related mortality (TRM), the later complications of transplant, including risk of secondary malignancies and infertility, need to be considered prior to contemplating an HSCT. Also, this therapy does not improve the clinical phenotype completely in some patients, particularly if offered late in life. It does not alter the clinical course of illness in some organs e.g. musculoskeletal system. The maximum effect of treatment is achieved if given early (with in the first two years) which is not always possible due to late presentation in some LSDs or delay in diagnosis due to a lack of awareness of these rare disorders amongst the wider medical community.
1.5.2. Limitations of ERT

Even though ERT improved outcome and reversed some clinical symptoms, it failed to show universal benefits in halting and reversing the disease progression and clinical course of disease (Mercimek-Mahmutoglu, Reilly et al. 2009). There is increasing evidence from animal studies and human trials to suggest that the ERT is less effective in reversing disease pathology in some organs and clinical recovery in other systems can take a long time. Bone disease prior to ERT is not corrected during the treatment and some bone pathologies e.g. osteopenia and osteoporosis may take over 5 years to resolve. ERT is less effective in slowing and reversing the disease pathology in bone, cartilage, musculoskeletal system, heart valves and brain (Schiffmann, Kopp et al. 2001; Wraith 2006; Cox-Brinkman, van den Bergh Weerman et al. 2010). In a follow up study of MPSI patients which lasted 6 years, clinical improvement was slow and took several years in some patients, despite relatively quick recovery in urinary GAG and enzyme levels (Sifuentes, Doroshow et al. 2007).

A major problem with the use of ERT is its inability to correct the CNS disease. This is because the blood brain barrier (BBB) prevents the delivered therapy from penetrating into the brain tissue (Begley, Pontikis et al. 2008; Enns and Huhn 2008). BBB is meant to protect the brain tissue from potentially harmful substances and microorganisms present within the circulation. This barrier is formed by tight endothelial junctions and astrocyte projections (glia limitans) surrounding the brain capillaries. This mechanical and electrical barrier prevents most solutes including ERT in peripheral circulation from entering the brain parenchyma freely (Begley and Brightman 2003). In order to effectively treat neurological involvement in LSD the treatment should be able to cross the BBB either by disruption of the barrier or by direct infusion of the ERT into the brain. Recently ERT was successfully delivered into cerebrospinal fluid via cerebellomedullary cistern in animal models of MPSIIIB (Jolly, Marshall et al. 2010). The success of this approach has led to the initiation of a clinical trial in MPSIIIA patients via the intrathecal route.
As discussed earlier, the cost of treatment is an important consideration in a climate where resources are becoming increasingly scarce. ERT carries a relatively high cost burden estimated at $150,000-300,000 per patient per annum in MPS I (Connock, Juarez-Garcia et al. 2006) and for all ERT treatable LSDs, averages £144,000 per patient in the UK. This makes it almost impossible to administer this treatment in patients in the developing world. Even in the industrialized nations, current fiscal challenges may make it impossible to continue unrestricted delivery of this treatment.

Another problem that was predicted at the time of development of ERT was immunological response to recombinant human proteins. This was initially identified in animals and subsequently recognized in patients receiving the ERT.

1.5.3. Human immune responses to recombinant proteins

A number of recombinant human proteins are in clinical use. A detailed review on these products revealed that even though the immune response could potentially reduce the efficacy of the products, in the majority of cases it was not clinically significant (Porter 2001). Some of the factors which influence the immunogenicity of recombinant proteins include denaturing of the molecules during production, impurities, aggregation of particles, dose of administration, route of administration, frequency of treatment and structural homology to microorganism and non-human antigens (Richards ; Porter 2001). In the majority of cases, IgG and IgM antibodies are produced in response to immune stimulation and only occasionally IgE antibodies are implicated. Whilst IgG and IgM antibodies are known to neutralize the effects of the humanized proteins, IgE antibodies are mostly associated with allergic reactions including anaphylaxis. One example of a humanized protein which is associated with clinically significant immune response is that of recombinant human coagulation factors used in Haemophilia patients. Antibodies against recombinant factor VIII and factor IX (Haemophilia inhibitors) pose serious clinical problems which are
associated with significant morbidity and mortality. In a 3 year longitudinal study on Haemophilia A patients, about a quarter of the treated patients developed neutralizing antibodies. The risk of development of inhibitors in this cohort was estimated to be 12%, 27% and 49% at days 8, 10 and 25 of protein exposure respectively (Bray, Gomperts et al. 1994). Antibodies from both IgG and IgM class are implicated in inhibition of coagulation factors in vivo and in vitro (Yoshioka, Fukutake et al. 2003). The functional assays performed on patients with high titer antibodies demonstrate a considerable inhibition of replacement therapy reducing the factor activity to less than 1% in some cases. In Haemophilia patients with refractory immune responses, this poses a major challenge in clinical management. Insulin is another example of replacement therapy where immune responses can cause significant clinical impact. Immune responses to both human and animal insulin can reduce the clinical efficacy of the product. However, recombinant human insulin seems to generate milder immune responses with less inhibition of administered insulin compared to the porcine insulin (Spijker, Poortman et al. 1982; Fineberg, Galloway et al. 1983; Fineberg, Galloway et al. 1983; Di Mario, Arduini et al. 1986). Studies on various recombinant human proteins e.g. growth hormone (Gunnarsson and Wilton 1987; Buzi, Buchanan et al. 1989; Takano, Shizume et al. 1989; Zeisel, Lutz et al. 1992), granulocyte colony stimulating factor (G-CSF) (Laricchia-Robbio, Moscato et al. 1997), erythropoietin (Nielsen and Thaysen 1989; Urra, de la Torre et al. 1997) and interferon (Spiegel, Spicehandler et al. 1986) show similar results. The immunogenicity of all these products is variable, influenced by multiple factors (mentioned earlier) and sometimes results in reduced clinical efficacy of the therapy delivered. The adverse clinical effects including treatment failure are mediated by the ability of these antibodies to neutralize the delivered protein and hence functional assay is a better predictor of the clinical outcome.

In haemophilia A and B the risk factors for development of neutralizing antibodies (inhibitors) have been studied in great detail. It is estimated that complete or near complete gene deletions result in inhibitor development in 20-
80% of the patients who are exposed to recombinant human factor VIII. Their risk is significantly higher than those with smaller gene deletions resulting in inhibitor development in less than 5% (Oldenburg and Pavlova 2006). This difference in incidence is due to the presence of a small amount of residual factor VIII produced by the patients who have minor gene deletions. This constant exposure of the immune system to extremely low levels of functional/non-functional factor VIII is sufficient to induce immune tolerance. This residual protein is termed as cross reactive immunological material (CRIM). However exceptions to this rule exist e.g. severe Haemophilia A patients with intron 22 inversion only show modest inhibitor antibody response in the absence of CRIM.

1.5.4. Immune response to ERT

As a part of pharmacovigilance, the pharmaceutical companies developing ERT are required to monitor the immune response to their product. The generation of immune antibodies was reported in phase I and II studies during development of ERT.

In a randomized double blind placebo controlled study undertaken by Genzyme, all patients exposed to Aldurazyme developed IgG immune response. The median time to seroconversion was 25.8 days. The antibodies reported were mostly of IgG class. Rarely, IgM and IgE antibodies were also reported. IgE antibodies were mostly associated with severe systemic allergic reaction (Lipinski, Lipinski et al. 2009). The patients with mild immune response showed a robust decrease in urinary GAG whereas more severe immune responses were associated with variable reduction in disease biomarker (Wraith, Clarke et al. 2004). In a double blind placebo controlled study to evaluate the efficacy of ERT in MPSI, 91% patients developed IgG response to laronidase during treatment. The mean time to seroconversion was 52 days (range, 20 to 106). Close to the end of the follow up period the majority of patients were spontaneously tolerized. There were no significant differences between tolerized
and non-tolerized groups in terms of substrate reduction, clinical severity, radiological and cardiac imaging. The authors suggested that seroconversion did not significantly alter the tolerability and clinical efficacy of laronidase (Wraith, Clarke et al. 2004). In another longitudinal study of 10 MPSI patients receiving ERT (recombinant human -L-iduronidase) the antibody titers were studied to evaluate the immune response to therapy and compared with normal controls receiving the same treatment. At the start of treatment there was no difference in antibody titers between the patient and the control group. Five of the patients subsequently developed high titre immune response. By week 104, all patients had low antibody titer or no antibodies. In this study there was no significant difference in clinical outcome in the two treated groups regardless of their immune response. The authors argued against the use of immune tolerance induction in these patients due to potential morbidity associated with conventional immune tolerance induction treatments (Kakavanos, Turner et al. 2003).

Various studies describing the effects of ERT in other LSDs e.g. MPSII (Hunters Syndrome), MPSVI, Gaucher, Pompe and Fabry disease also showed development of immune response in ERT treated patients occasionally associated with adverse reactions necessitating cessation of treatment (Richards, Olson et al. 1993; Rosenberg, Kingma et al. 1999; Amalfitano, Bengur et al. 2001; Hunley, Corzo et al. 2004; Muenzer, Wraith et al. 2006; Muenzer, Gucsavas-Calikoglu et al. 2007; Vedder, Linthorst et al. 2007; White, Argento Martell et al. 2008; Tesmoingt, Lidove et al. 2009).
1.5.5. Functional and clinical impact of immune response

The antibody bound to the ERT can reduce its efficacy by various other mechanisms including altered enzyme distribution, altered intracellular enzyme trafficking and increased enzyme turnover (Brooks 1999). Whilst it is possible that the *in vitro* enzyme inhibition does not reflect *in vivo* ERT failure subsequent studies showed a correlation between the high antibody titre, *in vitro* enzyme inhibition (catalytic activity and cellular uptake), reduced metabolic clearance of GAG and adverse clinical outcome in some ERT treated LSDs. In a recent publication Cox-Brinkman et al. described the effect of ERT and HSCT on dermal fibroblast morphology in 12 patients with MPSI. The skin biopsies were taken on regular intervals before and during 2 years of ERT as well as before and 6 months after HSCT. This study showed that both ERT and HSCT improved the tissue morphology of skin fibroblasts based on vacuolation score of histological sections. However, three of the 12 patients in this study did not show any improvement in their dermal fibroblast morphology. All of them had IgG antibodies. Due to the small size of the study it was not possible to show statistical correlation between treatment failure and incidence of these antibodies. The follow up of the patients who received HSCT showed rapidly declining levels of antibodies in these patients. These data are interesting as they provide evidence of immune tolerance induction by HSCT and show that the donor lymphocytes despite being educated in recipient reticuloendothelial system, retain the donor immune response (Cox-Brinkman, van den Bergh Weerman et al. 2010). In a recent study urinary GAG and HCII-T levels in 9 MPS I and 11 MPS II were evaluated and correlated with antibody titres. There was a clear correlation between the antibody levels and HCII-T. These levels improved at the onset of treatment but deteriorated after generation of immune response often to values above the baseline. In contrast urinary GAG levels were less responsive to antibody titres. In severe MPS I patients serum HCII-T levels never corrected to normal in all patients treated with ERT (Clarke, Hemmelgarn et al. 2012).
1.5.6. Effect of severity of immune response

Dickson et al. evaluated the effects of anti IDUA antibodies in animal models of MPSI (dogs) treated with ERT and demonstrated that the presence of high titres of anti-IDUA antibodies in serum reduced the uptake of ERT to less than 10% compared to the serum from canines with low or negative titres. There was a correlation between the high titre antibodies and urinary GAG excretion in this study. Dose escalation and immune tolerance both reduced urinary GAG excretion. Tissue histology revealed a remarkable improvement in the appearance of kidneys and synovia but less response was observed in lymph nodes and heart valves. Improvement in renal histology correlated with urinary GAG excretion. The animals which had no antibodies or very low levels had greater substrate reduction compared to those with high titre immune response. Published data from other animal studies supports the findings in the above study and shows that even though high titre immune responses reduce the clinical efficacy of ERT, some clinical benefit can still be achieved when compared to untreated animals (Dickson, Peinovich et al. 2008; Ponder 2008). The response to ERT in the presence of antibodies is even more disappointing in other tissues e.g. cartilage and heart valves (Shull, Kakkis et al. 1994). In a study involving MPSI patients (70% of patients with iduronidase-null mutations), all patients developed anti Iduronidase antibodies. Antibody titres greater than 1:10,000 were associated with a lower reduction in urinary GAG levels when compared to the patients who had low or undetectable titres (Wraith, Beck et al. 2007).

CRIM negative infantile Pompe disease is another example of LSD where the immune response to ERT appears to make the treatment ineffective. In a longitudinal study the outcome of ERT (recombinant human acid alpha glucosidase) was evaluated in 21 CRIM positive and 11 CRIM negative infantile Pompe patients. Clinical evaluation of these patients included monitoring motor development and assessment of cardiac function. Metabolic outcome and
immune response was monitored by substrate clearance and IgG antibody ELISA assays. At 52 week follow up, nearly half of the CRIM negative and 5% of CRIM positive children had died. After 27 months although none of the CRIM negative patients survived, 80% of CRIM positive patients survived. In comparison to the CRIM positive patient, those who were CRIM negative generated the immune response quicker with higher titres which stayed at higher levels during the follow up period. This study gave direct evidence of the poor outcome of the disease in presence of high titre antibodies to ERT and propensity of CRIM negative patients to develop a more severe and sustained immune response. These data were in contrast to the initial data from MPSI patients who were reported to develop immune tolerance spontaneously with continuing infusions of ERT (Wang, Lozier et al. 2008; Kishnani, Goldenberg et al. 2010). More recently, a study evaluating the impact of allo-immune response in Pompe disease revealed a worse clinical response in CRIM positive patients with high titer antibodies when compared to CRIM positive patients with low titer immune response (Banugaria, Prater et al. 2011). In this study the overall survival of CRIM negative patients was similar to CRIM positive patients with high titer antibodies which underlines the significance of high titer immune response. In a recent publication De Vries et al. described the immune response and the effects of antibodies in a patient of Pompe disease. This 39 year old patient experienced adverse reaction associated with infusion of ERT. Further studies on antibody revealed significant inhibition of cellular uptake (on average 25% lower in the presence of patient serum compared to normal serum). These antibodies failed to show quantifiable catalytic inhibition of enzyme in vitro but there was some indirect evidence of enzyme activity inhibition by western blot analysis and measurement of enzyme activity. This patient was reported to have a worse clinical outcome compared to the reference subjects who did not have significant immune response. The clinical assessment was done by monitoring forced vital capacity, 6 minute walk, manual muscle testing and other isometric muscle contractions. Based on the antigen antibody binding data, the authors suggested that approximately 37.5% of the infused enzyme at recommended
dosage was captured by the antibodies at the time of maximum immune response in this particular patient. (de Vries, van der Beek et al.).

Neutralizing antibodies have also been reported in other LSDs including MPS II, Gaucher and Fabry disease which were associated with deterioration in clinical response in some cases (Ponce, Moskovitz et al. 1997; Rubio, Kim et al. 2003; Zhao, Bailey et al. 2003; Linthorst, Hollak et al. 2004; White, Argento Martell et al. 2008).

1.5.7. Assays for detection and quantification of immune response

In order to understand the antibody response, its functional impact and limitations in the existing practice of antibody testing, it is important to comprehend the basic principles of antibody detection tests and functional assays. A simple description of the principles of these assays is as follows.

1) Assays for detection of the immune response

The recommendations for the development of immunological assays to detect antibodies against any therapeutic protein also apply to ERT. These have been published previously (Mire-Sluis, Barrett et al. 2004; Shankar, Shores et al. 2006). The assays should include the use of a highly sensitive technique e.g. ELISA. A positive result then needs to be verified to ensure that the positive response is due to the antibodies and is specific to the antigen (ERT in this case). This can be achieved by performing specific assays e.g. immunoprecipitation assays or Western blot analysis. Briefly, during ELISA the antigen (recombinant enzyme) is fixed on a solid support i.e. ELISA plate either specifically via capture by a specific antibody in a sandwich ELISA, or non-specifically by direct binding of the antigen to the surface. Patient serum is then added to the fixed antigen to capture antibodies. The antibodies in human serum bind to the antigen. A detection antibody which is usually an antihuman antibody is exposed to the plates which form a covalent bond to the antigen
antibody complex. After exposure to a specific substrate the complex produces a visible signal which is read by light absorption or transmission. IgG, IgM and IgE antihuman antibodies can be used to detect serum antibodies of respective classes. This assay needs optimisation and validation which involves confirmation of specificity of the ELISA assay by immunoprecipitation tests or Western blot analysis. The presence of antibody in patient serum is not an evidence of enzyme inhibition. This requires functional enzyme assays.

2) Catalytic enzyme inhibition assay
This assay determines neutralization of catalytic activity of enzyme by the antibodies in the patient serum. This can be performed by various different techniques and there is no consensus on the most appropriate method. The demonstration of enzyme activity inhibition would inevitably require mixing studies. This can be achieved by mixing a patient’s serum or antibodies isolated from serum with a validated potency enzyme assay. The inhibition of enzyme activity of the solution after mixing the patient’s serum gives a quantifiable measure of direct catalytic enzyme inhibition. A standard should be established and validated for the comparison and quantification of enzyme activity inhibition. Enzymes from various sources (recombinant human and innate) can be used for inhibition studies.

3) Cellular uptake inhibition assay
This assay differs from enzyme activity inhibition as it evaluates a different effect of antibody i.e. its ability to inhibit the uptake of enzyme by enzyme deficient cells. This is determined by developing a standard cell line for each disease group (usually non FcR expressing fibroblast). These cells are then incubated with enzyme solution in the presence and absence of patient serum (with antibodies). The uptake of enzyme by the cells can be measured by various methods. Broadly, it can be measured either directly by flow cytometric analysis of the cells to detect the labeled enzyme present in the fibroblasts, or indirectly by measuring the enzyme activity of cell lysate. The comparison of enzyme
uptake by the cells in the presence or absence of antibodies gives a measure of cellular uptake inhibition.

1.5.8. Limitations of existing assays

Currently the assays for antibody detection, quantification and assessment of functional impact are being carried out by the pharmaceutical companies. There is no consensus on methodology, standardisation or reporting of these assays. A number of different ELISA techniques have been used for detection of antibodies in patient serum which are not comparable and lack a robust external quality assessment mechanism. Recently, efforts have been made to optimize and standardize these assays (Sellos-Moura, Barzegar et al. 2011). It is vital that the catalytic inhibition assays actually measure the drop in catalytic activity of the enzyme after the mixing of enzyme solution with the antibodies from patient serum. In some instances binding and precipitation of the enzyme by the antibodies has been implied as catalytic inhibition. Whilst antigen antibody binding will inevitably be required to inhibit the catalytic activity, it may not do so in all cases due to the polyclonal nature of IgG antibodies. The substrates used to detect catalytic inhibition are artificial and might not reflect true in vivo enzyme inhibition. Similarly there is no standard cell line in use, to study the cellular uptake inhibition for various diseases and hence it is difficult to compare the results from various centres. This problem is compounded by a lack of efficient and reliable biomarkers which predict the treatment failure instantaneously.

1.6. Diagnosis, monitoring and disease biomarkers

LSDs are diagnosed by direct enzyme assays. This is available for most of the disorders but in a small minority the diagnosis is made on clinical findings coupled with radiological investigations, electroretinogram, tissue biopsy and mutation analysis. Enzyme levels can be tested in peripheral blood (serum, leucocytes), tissue fibroblasts and urine (Meikle, Hopwood et al. 1999; Wilcox
In most disorders it is possible to make a prenatal diagnosis by chorionic villus sampling or amniocentesis. Carriers should only be identified by mutation analysis as they might have normal or near normal levels of enzyme which sometimes are difficult to differentiate from normal individuals.

Urinary GAG assay has been in use for some time as a biomarker of the disease. In the clinical settings enzyme levels and urinary GAG are used to confirm the diagnosis in new patients. More recently urinary dermatan sulphate (DS) to chondroitin sulphate (CS) ratio was found to be a superior biomarker in MPSI compared to urinary GAG (Church, Tylee et al. 2007). The development of heparin cofactor II-thrombin (HCII-T) as a reliable biomarker in MPSI, II, III, VI and VII (serum and dried blood spot) has opened new avenues for using this biomarker as a cheap neonatal screening tool and a biomarker to study the disease progression in these illnesses (Langford-Smith, Arasaradnam et al. 2010; Langford-Smith, Mercer et al. 2010).

1.7. Immune tolerance induction

Recently, immunogenicity was recognized as one of the most important “modifiable factors” compromising the long term efficacy of ERT (Lacana, Yao et al. 2012). This has resulted in increased emphasis now placed on development of strategies to induce immune tolerance in recipients of ERT. Development of immune tolerance is a complex process. Most of the immune responses generated by adaptive immune system rely on B and T lymphocytes. After sensitization and stimulation by helper T cells, B cells evolve to form antibody secreting cells (plasma cells). However, antibodies generated by the human immune system in response to some infused protein are independent of T cell interaction. B1B cells, which are not regulated by helper T cells are capable of producing antibodies without interaction with helper T cells. As most of the IgG antibodies produced in response to recombinant human protein belong to IgG1 and IgG4 classes, it is widely accepted that the helper T cell dependent mechanisms are also involved in generating antibody responses (Herzog, Fields et al. 2002; Withers, Fiorini et al. 2007). This makes both B and T lymphocytes a
target for immune tolerance induction in these patients. Various immune
tolerance induction regimens have been tried to improve the metabolic outcome
of ERT. Conventional tolerance regimens used in severe Haemophilia patients
with inhibitors do not show promising results in ERT treated LSD patients with
neutralizing antibodies. Serious side effects e.g. systemic allergic reactions and
nephrotic syndrome have been reported in patients who were treated with
escalating doses of ERT to induce immune tolerance (Hunley, Corzo et al.
2004).

Immune tolerance induction regimens used in clinical practice can be
categorized into two groups. The first involves immune suppression or
myelosuppression which impedes the adaptive immune response by either
cellular inhibition or switching off the humoral component of the immune system.
The second includes repeated exposure of the immune system to the deficient
protein to achieve tolerance. The potential mechanism by which the immune
system becomes tolerized using the former technique is not clear but it probably
involves steric hindrance, inhibition of degradation of infused protein or
immunomodulation (Astermark 2011). In LSDs, this hypo-sensitization can
either be achieved by regular infusion of the exogenous recombinant enzyme or
delivery of innate enzyme in the form of cellular therapy (HSCT) which delivers
enzyme produced by the donor cells after engraftment. The two strategies can
be used concurrently to enhance and expedite immune tolerance induction. In
the past, gene therapy was incorporated into this strategy to achieve immune
tolerance induction. In animal models of Haemophilia successful immune
tolerance using HSCT based gene transfer has been described (Bigger, Siapati
et al. 2006; Pichard, Bellodi-Privato et al. 2006). More recently HSCT based
gene therapy was evaluated in the murine model of Pompe disease. Lentivirus
mediated gene transfer was able to partially correct the phenotype of the
disease. Expression of deficient protein in haemopoietic precursor cells did not
produce immune response in immunocompetent mice but instead tolerized them
to the delivered ERT (Douillard-Guilloux, Richard et al. 2009). A similar strategy has also been employed in MPSIIIA (Langford-Smith, Wilkinson et al. 2012).

Whilst most LSDs are slowly progressive disorders where a gradual induction regimen is a suitable management strategy, in other disorders e.g. infantile Pompe this is not acceptable. In infantile Pompe the development of high titre antibody to ERT is invariably fatal and the development of a reliable, successful and quick immune tolerance induction regimen is crucial. In order to achieve this, a number of conventional immune tolerance induction treatments were studied in a knock-out mouse model of Pompe disease. In this study mycophenolate mofetil, methotrexate and cyclosporine with azathioprine were evaluated as immune tolerance induction regimens. Empirical treatment with low dose methotrexate (3 weekly) started within 24 hours of administration of the first dose of recombinant human acid alpha glucosidase was the only successful regimens. This treatment prevented the development of high titre antibody response to the ERT. The exact mechanism of action of methotrexate in this context is not clear. Further experiments on these mice revealed B1 B cell dysregulation as demonstrated by the consistent expansion of paritoneal B1B cell clones. However it is not yet clear how this cellular expansion leads to the development of immune tolerance induction to ERT (Joseph, Munroe et al. 2008). In another study on a canine model of MPSI a different induction therapy was found to be beneficial. In this study a regimen consisting of cyclosporine A and azathioprine combined with low dose weekly infusions of recombinant human alpha-L-iduronidase (strategy 1 and 2 combined) was evaluated. The induction regimen was given over a period of 60 days which successfully prevented a high titre immune response to therapeutic doses of enzyme (once weekly infusion) for a period of 6 months. During this study ciclosporine levels were also monitored and sub-therapeutic cyclosporine was shown to have an inferior outcome (Kakkis, Lester et al. 2004). In the past, immune tolerance was induced in a patient with Gaucher disease by using a combination of plasma
exchange, cyclophosphamide, intravenous immunoglobulins and large doses of Ceredase (Brady, Murray et al. 1997).

Whilst more complex regimens and combinations of immune suppression might be promising, they increase the risks associated with therapy. In Haemophilia, immune tolerance induction success rates vary between 40-80 per cent depending upon the therapeutic regimens. These regimens include low dose chemotherapy, administration of high doses of FVIII twice daily (Bonn protocol), low FVIII doses three times weekly (van Creveld protocol), immune adsorption followed by immune suppression (Malmö protocol) and modified Malmo-Bonn regimen (acquired Haemophilia). However the relapse rates are high. More complex regimens using combination treatments and immune suppression are associated with significant morbidity. Most of the side effects are due to immunosuppression but more serious adverse reactions including anaphylaxis have been reported during the use of escalating doses of replacement therapy in an effort to achieve immune tolerance (Berntorp, Astermark et al. 2000; Carlborg, Astermark et al. 2000; Kreuz, Ettingshausen et al. 2003; Wight, Paisley et al. 2003; Curry, Misbah et al. 2007). In a study on a knock-out mouse model of Pompe disease, various immune tolerance regimens including mycophenolate mofetil, methotrexate and cyclosporine with azathioprine were evaluated. Empirical treatment with low dose methotrexate successfully induced immune tolerance (Joseph, Munroe et al. 2008). In another study on a canine model of MPS I, cyclosporine A (within therapeutic levels) and azathioprine combined with low dose weekly infusions of laronidase (strategy 1 and 2 combined) successfully prevented development of high titer antibody response (Kakkis, Lester et al. 2004). A successful regimen involving combination of plasma exchange, cyclophosphamide, intravenous immunoglobulins and large doses of Ceredase (Brady, Murray et al. 1997) is described in Gaucher disease patients.

Rapid and effective immune tolerance induction is critical in some infantile LSDs where development of high titer antibodies leads to catastrophic outcome very rapidly. Intensification of the immune tolerance induction regimens by using
combination chemotherapies could achieve rapid immune tolerance, but there is a risk of increased morbidity associated with these approaches. Use of novel treatment approaches e.g. monoclonal antibodies (anti-CD20 antibody) have been successfully used to induce immune tolerance in some congenital disorders (Franchini, Mengoli et al. 2008; Barnes, Davis et al. 2010) but there is very little data on the use of these treatment options in ERT treated LSD patients (Mendelsohn, Messinger et al. 2009). Recently Messinger et al reported successful use of a combination of Rituximab (anti-CD 20 antibody), methotrexate and immunoglobulins to induce immune tolerance in two CRIM negative Pompe disease patients. In addition, two patients (both CRIM negative) were given prophylactic Rituximab and Methotrexate and remain tolerant to recombinant human alpha glucosidase at 18 and 35 months of age (Messinger, Mendelsohn et al. 2012). Addition of bortezomib to conventional immunomodulatory immune tolerance strategy showed promising results in Pompe disease patients (Banugaria, Prater et al. 2012).

HSCT has previously been used to eradicate the immune response in a number of auto-immune disorders such as systemic lupus erythematosus (Jayne, Passweg et al. 2004; Lisukov, Sizikova et al. 2004; Burt, Traynor et al. 2006) and rheumatoid arthritis (Snowden, Passweg et al. 2004; Teng, Verburg et al. 2005). In 2010, Uprichard et.al. published a case report of a 22 year old patient with severe Haemophilia A with refractory high titre inhibitor to factor VIII replacement therapy, who received allogeneic HSCT to induce immune tolerance (Uprichard, Dazzi et al. 2010). Unfortunately, this patient died of Klebsiella pneumonia septicaemia on day 46 post transplantation. However, during the very brief follow up period, the inhibitor to infused factor VIII re-emerged; suggesting a persistence of immune response mediated by memory T cells which were not completely ablated by reduced intensity conditioning. The authors suggested that a subsequent donor lymphocyte infusion could eradicate this immune response completely as is shown in animal models (Van Wijmeersch, Sprangers et al. 2007). Currently there is no such data available on the role of allogeneic HSCT in LSDs. Allogeneic HSCT could induce immune
tolerance in LSD patients with refractory immune response and bring both replacement of the defective enzyme (through hematopoietic stem cells and their differentiated progeny) and eradication of inhibitory immune response. With the elimination of the recipient immune system as part of conditioning for HSCT, enzyme-reactive B and T cells are removed. Following the acute period of immune reconstitution after the transplant, in which the risk of GvHD is managed by immune suppression, newly developing donor T and B cells would display tolerance to specific donor and host proteins.

Unlike many other allo and auto immune disorders where novel treatment approaches e.g. monoclonal antibodies (Rituximab, Campath) have been tested in large patient series, there is very little data on the use of these novel treatment options in ERT treated LSD patients.

1.8. Project aims

1.8.1. Development of quantitative and functional immune assays for LSDs

The first aim of this research project was to develop quantitative and functional immune assays in MPSI, MPSVI and Pompe disease patients who were exposed to ERT. In view of high sensitivity and specificity, ELISA was selected as the basis for the detection assay. Two different types of functional assays were to be developed in order to evaluate inhibition of catalytic enzyme activity and cellular uptake inhibition.
1.8.2. To study the incidence and impact of immune response in MPSI-H patients treated with ERT and HSCT

The current practice of delivering ERT to MPS I patients around the time of allogeneic HSCT, afforded us the opportunity to study the kinetics of the allo-immune response in Aldurazyme (recombinant human IDUA) treated patients with MPS I. The aim was to study the incidence of allo-immune response with IgG ELISA and assess the functional impact of allo-antibodies in recipients of ERT and HSCT. We also wanted to explore HSCT as an immune tolerance induction strategy.

1.8.3. To evaluate the nature and incidence of immune response in other LSDs

Based on immune assays developed in aim 1, all patient samples collected over the period of research and archive samples, collected prior to the start of study, were to be evaluated to explore the incidence of functional nature of immune response in MPSI HS, MPS VI and Pompe disease patients. This would include patients on long term ERT.
2. Materials and methods
2.1. Patients and sample preparation

Blood samples from LSD patients who were treated and followed up in Royal Manchester Children’s Hospital over a period of four years were collected with informed consent under ethical permission REC 08H101063. Patient blood samples were processed within 6 hours of collection. The serum samples (with no anti-coagulant) were centrifuged at 3000 G at room temperature and then serum was separated and stored at -80°C. Samples were grouped into longitudinal series (with two or more time points) and cross sectional series (single time point) for each LSD.

2.2. Principles of Immune assays

2.2.1. ELISA

During this assay, an antigen (enzyme) is fixed on a surface (EIA plate) overnight. The plate is then washed with washing buffer and blocked by a blocking solution containing human serum albumin. This ensures that the non-specific binding sites, on the surface, are blocked and reduces the background interference. After thoroughly washing the plates, a primary antibody (patient serum) is applied to the antigen. The plates are washed between every step to minimize contamination. A secondary antibody (anti-human IgG antibody) is then applied to the EIA plates. These antibodies are conjugated with a substance which changes colour on exposure to a specific substrate. This reaction can be quantified accurately by measurement of light absorption as shown in the figure 2.1. Due to the unavailability of commercially available standardized human IgG antibodies to respective enzymes, a standard curve could not be created in the assays. Therefore, a negative control was defined
for each dilution. For each disorder, a different set of experiments were undertaken to define a negative cut off and optimize enzyme concentrations used for the ELISA. This is elaborated in respective sections in chapter 3.

Figure 2.1 IgG Enzyme linked immunosorbent assay

Antigen (enzyme) is applied to a surface (EIA plate). Primary antibodies (patient serum) bind to the antigen. These antibodies are detected by a secondary antibody (HRP conjugated, anti-human IgG antibodies) which changes the colour of the reaction when exposed to the substrate (OPD).

2.2.2. Catalytic inhibition

For all disorders studied in this project, functional enzyme assays were optimized in 96 well plates. These assays were then modified to develop mixing assays. The inhibitory antibodies in patient serum may block the catalytic site of the enzyme. Mixing of patient serum with an enzyme solution of known enzyme
concentration, will then inhibit the enzyme activity of the solution. This inhibition can be quantified by comparing enzyme activity of the solution in the absence of serum and presence of normal serum and patient serum as shown in figure 2.2. An extensive series of experiments had to be undertaken, in order to define optimum conditions for each disorder.

Figure 2.2 Catalytic inhibition assay

Catalytic inhibition assay. Antibodies in patient serum bind to the enzyme and reduce the ability of enzyme to breakdown artificial substrate resulting in reduced release of 4MU. This is then compared to a standard (normal human serum) to quantify the inhibition of enzyme activity.
2.2.3. Cellular uptake inhibition

Cellular uptake inhibition assays are based on functional enzyme activity assay and hence they determine the ability of the antibodies to block the cellular uptake of functionally active enzyme. For each disorder, human fibroblasts from patients with severe disease phenotype (deficient in enzyme) were acquired and maintained in culture media. These cells were seeded into 6 well culture plates. Under optimum conditions, these fibroblasts were exposed to the deficient enzyme in the presence and absence of antibodies (in patient sera). The cells were subsequently lysed by freezing and sonication. The amount of enzyme within the cells was accurately measured by enzyme assays. Cellular uptake inhibition was quantified by comparing the amount of enzyme delivered into the cell in the presence and absence of antibodies (see figure 2.3). For various disorders, the experiment conditions such as exposure time and enzyme concentrations had to be changed considerably to elicit cellular uptake inhibition as described in detail in chapter 3.
Cellular uptake inhibition assay. Antibodies in patient serum bind to the enzyme and reduce its uptake by enzyme deficient patient fibroblasts. The cells are lysed and the enzyme activity is measured accurately. The comparison of enzyme activity in fibroblasts in the presence and absence of antibodies gives an accurate measure of inhibition of enzyme uptake by antibodies.
2.3. MPSI

2.3.1. IgG ELISA

In this assay, the antigen (recombinant human IDUA, Aldurazyme), is fixed on the enzyme immunoassay (EIA) plate. Antibodies present in patient serum, which are specific to the enzyme (Aldurazyme), bind to this antigen. These antibodies are then detected by secondary anti-human IgG antibodies conjugated with a substrate.

In our assay, 96 well EIA plates were coated with enzyme-carbonate solution (10µg/ml in 0.1M NaHCO₃, pH8.5) overnight and blocked with blocking agent containing 1% HSA. Patient serum (50µl) was added (two fold serial dilutions, see figure 2.4) to each well in duplicate and incubated at room temperature for 1 hour.

![Figure 2.4 Preparation of serial dilution of specimens](image)

One hundred microlitres of IgG goat anti human Horseradish peroxidase antibody (1:5000 dilutions) was added to each well and incubated at room temperature (RT) for 1 hour. Following this period, 100µl o-Phenylenediamine dihydrochloride (OPD) solution was added to each well and left for 10 minutes in a dark place. The reaction was stopped by 2.5M H₂SO₄ (50µl into each well) and the light absorbance was read at 492nm immediately. The normal range was determined for each dilution by testing 13 normal sera (Normal volunteers). A positive cut off value was defined as two SD above the absorbance value for the normal sera at that concentration. To quantify ELISA, the lowest titre with
absorbance value above the cut off was reported as positive serum dilution for that patient sample. Positive responses were confirmed by western blot to show the specificity of the IgG antibody to Aldurazyme.

2.3.2. Enzyme activity assay

Our catalytic inhibition assays were based on functional enzyme activity assay measured by using 4-Methylumbelliferyl (4MU) α-L-iduronide substrate (Glycosynth, Warrington, UK) as previously described (Stirling, Robinson et al. 1978; Kakkis, Matynia et al. 1994). The α-L-iduronidase cleaves α-L-iduronic acid from the substrate to yield the fluorescent product 4-methylumbelliferone (4-MU). The liberated 4-methylumbelliferone is measured using a luminescence spectrophotometer against a standard of known concentration.

\[ \text{α-L-iduronidase} \]

\[
\text{4-methylumbelliferyl-α-L-iduronide} \rightarrow \text{4-methylumbelliferone} + \text{iduronic acid}
\]

The assay was performed in duplicate. Samples were prepared in a 96 well plate. Patient sample (20 µl) and 20µl artificial substrate (4-methylumbelliferyl-α-L-iduronide; Glycosynth) in substrate buffer (0.4M formate buffer, pH 3.5, 0.9%NaCl) were added into each well. The plate was wrapped in cling film and covered with aluminum foil to prevent light interference. The plate was incubated in a shaking incubator at 37°C for 1 hour and reaction was stopped by adding 160 µl of stop buffer (0.2M sodium carbonate, 0.1M sodium hydrogen carbonate). The fluorescence generated was read at excitation 360nm (wavelength absorbed by the substrate) and emission of 450nm (wavelength emitted). This fluorescence was then compared to a standard by developing a standard curve using a fluorescent standard as shown in table 2.1.
Table 2.1  Fluorescent standard

2.3.3. Mixing assay (quantification of catalytic inhibition)

The above protocol was modified to develop and optimize mixing assays which determine inhibition of enzyme activity by patient sera. This assay determines the ability of a patient serum (with anti IDUA IgG antibodies) to inhibit the enzyme activity of Aldurazyme solution with a known enzyme concentration. This is then compared to the normal serum and baseline enzyme activity (no serum) to quantify the percentage enzyme inhibition. The enzyme activity is measured by functional enzyme activity assays using the artificial substrate 4-methylumbelliferyl-α-L-iduronide as previously described (see figure 2.2).

The assay was performed in duplicate on the highest titre patient sample in the longitudinal series. A series of two fold dilutions (starting from 30ng/ml to optimize the catalytic inhibition) of recombinant human α-L-iduronidase (Aldurazyme, Genzyme, MA) in dilution buffer (PBS, 0.05% Tween, 0.01% HSA) were used to assess inhibition of enzyme activity. Ten microlitres of each enzyme solution was then mixed with the same volume of patient sera and normal sera in 96 well black plates to minimize the auto fluorescence and background light scatter. The sera were added at fixed dilutions (1:4) and
incubated at room temperature in a shaking incubator for two hours. 4MU substrate (20 µl) in substrate buffer (0.4M formate buffer, pH 3.5, 0.9%NaCl) was added to each well and incubated for an hour at room temperature in the dark. The enzyme activity in the absence and presence of sera (for patient serum and normal serum) was measured. We observed some inhibition of enzyme activity with normal serum and hence for each experiment normal serum was used as a standard to compare and quantify the inhibition caused by the antibodies in patient sera. The enzyme activity in the presence of patient serum was also compared with enzyme activity of the plain enzyme solution for each dilution. We also used IDUA from human embryonic kidney cells (HEK) and mouse liver cells to assess the inhibition of innate enzyme by patient sera. Enzyme activity in normal serum and patient serum was also measured. This was then subtracted from the observed enzyme activity of the solution to remove the background enzyme activity which ensured that the observed activity in Aldurazyme solution was due to the recombinant enzyme only.

The % inhibition of the catalytic activity for each enzyme dilution was measured as follows,

\[
\text{% Inhibition} = 100 - 100 \times \frac{E_{A_{Ps}} - B_{E_{A_{Ps}}}}{E_{A_{Ns}} - B_{E_{A_{Ns}}}}
\]

\(E_{A_{Ps}}\): Enzyme activity in the presence of patient serum
\(E_{A_{Ns}}\): Enzyme activity in the presence of normal serum
\(B_{E_{A_{Ps}}}\): Background enzyme activity in patient serum
\(B_{E_{A_{Ns}}}\): Background enzyme activity in normal serum


**2.3.4. Cellular uptake inhibition**

IgG antibodies in patient serum can bind to the infused enzyme at 6MP site and inhibit the uptake of enzyme by the patient cells. This assay determines the inhibition of enzyme uptake by non-FCR expressing, IDUA deficient fibroblasts in the presence of antibodies. Enzyme activity in cells is measured by using 4-Methylumbelliferyl \( \alpha \)-L-iduronide as described earlier (see figure 2.3).

MPSI fibroblasts (MPSI-A171) from a Hurler patient were established and maintained in DMEM, 10% FBS, 1% glutamine at 37\(^\circ\)C and 5% CO\(_2\). The assays were performed in duplicates. Six well culture plates were seeded with 1.5x10\(^5\) fibroblasts into each of the six wells. The cellular uptake inhibition was performed once the wells were 90-95% confluent, 24-48 hours later. The enzyme was diluted in culture medium (DMEM with 1% glutamine) to below ½ Km for Aldurazyme (100 ng/ml). Patient serum or normal serum was added in a volume of 10 µl (1 in 100 dilutions) to the diluted enzyme solution (1000 µl volume) and incubated for two hours at room temperature. The culture medium in seeded wells was replaced with the enzyme mixed medium incubated with either normal or patient serum. The cells were incubated for another one hour in 5%CO\(_2\) at 37\(^\circ\)C. The cells were then washed with PBS and harvested by trypsinization. The cells were resuspended and washed twice with PBS and final suspension made in homogenization buffer (0.5M NaCl/0.02M Tris pH7-7.5). Following a cycle of freeze-thaw, the cells were sonicated, centrifuged at 2045G for 10 minutes. The supernatant was tested for enzyme activity using 4MU assay (described earlier). The protein concentration was determined by Bicinchoninic acid (BCA) assay as described previously (Smith, Krohn et al. 1985). No uptake inhibition was observed with normal sera and hence the percent inhibition was calculated against plain enzyme solution as follows:

\[
\text{% Inhibition} = 100 - \frac{100 \times \text{Enzyme activity of lysate incubated in the presence of patient serum}}{\text{Enzyme activity of lysate incubated with enzyme}}
\]
2.4. Pompe disease

2.4.1. ELISA

96 well flat bottomed EIA plates were coated with 50µl/well enzyme carbonate solution (1µg/ml myozyme in 0.1M NaHCO₃, pH8.5) and stored at 4°C overnight. Excess enzyme-carbonate solution was carefully aspirated next day and the plate was washed gently 5x with wash buffer (PBS, 0.1% Tween, pH7.4). To each well, 200µl of blocking Agent (1% w/v HSA in 0.02M Tris /HCl, 0.25M NaCl buffer pH7.0) was added and the plate stored at RT for 1 hr. After carefully aspirating the excess blocking agent, plates were washed again with wash buffer x 5. Using dilution buffer (PBS, 0.05% Tween, 0.01% HAS), 2 fold dilutions (16 dilutions in total) of specimens to be tested, positive control and negative control were prepared.

To each well, 50µl of standard, control or sample were added and plates covered with an adhesive cover slip and incubated at RT for 1 hr. After aspirating the tested samples the plates were washed 5x with wash buffer. IgG goat anti human HRP antibody was added (100µl) at a 1:5000 dilution to each well and incubated at RT for 1 hr. At the end of incubation, the plates were washed 5x and 100µl of OPD was added to each well. The plates were kept for 10 minutes at RT in a dark place. The reaction was stopped by adding 50µl 2.5M H₂SO₄ and the absorbance of the plate was read at 492nm immediately.

2.4.2. Enzyme assay in 96 well plate

The activity of the lysosomal enzyme α-glucosidase was measured using an artificial substrate 4-methylumbelliferyl-α-D-glucopyranoside. The assay was based on the previously described method (Cooper, Hatton et al. 1988) which was modified and optimized for a 96 well plate. Enzyme α-glucosidase cleaves
α-D-glucose from the substrate to yield the fluorescent product 4-methylumbelliferone. The liberated 4-methylumbelliferone is then measured using a luminescence spectrophotometer against a standard of known concentration.

\[
\text{α-glucosidase} \\
4\text{-methylumbelliferyl-α-D-glucopyranoside} \quad \rightarrow \quad 4\text{-methylumbelliferone} + \text{glucose}
\]

The enzyme α-glucosidase is a glycosidase which hydrolyses terminal α1-4 and α1-6 glucose residues in the degradation of the glycogen within lysosomes. The assay was performed in duplicate. Samples were prepared in a 96 well plate. Specimens to be tested and artificial substrate (4-methylumbelliferyl-α-D-glucopyranoside), prepared in substrate buffer (0.1M citrate/0.2M phosphate buffer at pH 4.0), were added to each well of the plate in equal volumes (20µl). The plate was wrapped in cling film and covered with aluminum foil to prevent light interference. The plate was incubated in a shaking incubator at 37°C for 30 minutes and reaction was stopped by adding 160 µl of stop buffer (0.2M sodium carbonate, 0.1M sodium hydrogen carbonate). The fluorescence generated was read at excitation 360nm and emission of 450nm. This fluorescence was then compared to a standard by developing a standard curve using a fluorescent standard as described previously.

**2.4.3. Catalytic inhibition assay**

Enzyme assays were modified to develop mixing assays in order to determine the inhibition of enzyme activity by patient serum. The assay was performed in duplicate on the highest titre patient sample. A series of two fold dilutions (starting from 12.5µg/ml) of recombinant human alglucosidase α (Myozyme) in dilution buffer (PBS, 0.05% Tween, 0.01% HSA) were used to assess inhibition.
of enzyme activity. Ten microlitres of each enzyme solution was then mixed with the same volume of patient sera and normal sera in 96 well black plates. The sera were added at fixed dilutions (1:4) and incubated at 4C for 24 hours. At the end of first incubation, 4MU substrate (20 µl) in substrate buffer (0.4M formate buffer, pH 3.5, 0.9%NaCl), was added to each well and incubated for 30 minutes at room temperature in the dark. The enzyme activity in the absence and presence of sera (for patient serum and normal serum) was measured. The enzyme activity in the presence of patient serum was also compared with enzyme activity of the plain enzyme solution for each dilution. Background enzyme activity was measured in normal and patient serum.

The % inhibition of the catalytic activity for each enzyme dilution was measured as follows,

\[
\% \text{ Inhibition} = 100 - 100 \times \frac{EA_{Ps} - BEA_{Ps}}{EA_{Ns} - BEA_{Ns}}
\]

\(EA_{Ps}\): Enzyme activity in the presence of patient serum
\(EA_{Ns}\): Enzyme activity in the presence of normal serum
\(BEA_{Ps}\): Background enzyme activity in patient serum
\(BEA_{Ns}\): Background enzyme activity in normal serum

### 2.4.4. Cellular uptake inhibition

This assay determines the inhibition of enzyme uptake by, acid alpha glucosidase deficient fibroblasts in the presence of antibodies. Fibroblasts from a Pompe disease patient were established and maintained in DMEM, 10% FBS, 1% glutamine at 37°C and 5% CO₂. The assays were performed in duplicates. Six well culture plates were seeded with \(1.5 \times 10^5\) fibroblasts into each of the six wells. The cellular uptake inhibition was performed once the wells were 90-95% confluent, 24-48 hours later. The enzyme (Myozyme) was diluted in culture medium (DMEM with 1% glutamine) at concentrations of 12.5µg/ml and 6.25µg/ml to ensure penetration of enzyme into the enzyme deficient cells (Yang, Kikuchi et al. 1998). Patient serum or normal serum was added in a
volume of 10 µl (1 in 100 dilutions) to the diluted enzyme solution (1000 µl volume) and incubated for one hour at room temperature. The culture medium in seeded wells was replaced with the enzyme mixed medium incubated with either normal or patient serum. The cells were incubated for another six hours in 5%CO₂ at 37°C. The cells were then washed with PBS and harvested by trypsinization. The cells were resuspended and washed twice with PBS and final suspension made in homogenization buffer (0.5M NaCl/0.02M Tris pH7-7.5). Following a cycle of freeze-thaw, the cells were sonicated, centrifuged at 2045G for 10 minutes. The supernatant was tested for enzyme activity using 4MU assay (described earlier). The protein concentration was determined by Bicinchoninic acid (BCA) assay (Smith, Krohn et al. 1985). The percent inhibition was calculated against plain enzyme solution as follows:

\[
\% \text{ Inhibition} = 100 - \left( \frac{\text{Enzyme activity of lysate incubated with enzyme}}{\text{Enzyme activity of lysate incubated in the presence of patient serum}} \right) 
\]

2.5. MPS VI

2.5.1. ELISA

96 well flat bottomed EIA plates were coated with 50µl/well enzyme carbonate solution (10µg/ml of Naglazyme in 0.1M NaHCO₃, pH8.5) and stored at 4°C overnight. After aspirating the excess enzyme next day the plate was washed gently 5x with wash buffer (PBS, 0.1% Tween, pH7.4). 200ul of blocking Agent (1% w/v HSA in 0.02M Tris /HCl, 0.25M NaCl buffer pH7.0) was added to each well and plate stored at RT for 1 hr. The plates were then washed again with wash buffer x 5. Using dilution buffer (PBS, 0.05% Tween, 0.01% HAS), two fold dilutions (16 dilutions in total) of all specimens to be tested, were prepared. To each well, 50µl of standard, control or sample were added and plates were covered with an adhesive cover slip and incubated at RT for 1 hr. After aspirating the tested samples the plates were washed 5x with wash buffer. IgG goat anti human HRP antibody was added (100µl) at a 1:5000 dilution to each
well and incubated at RT for 1 hr. At the end of incubation, the plates were washed 5x and 100µl of OPD was added to each well. The plates were kept for 10 minutes at RT in a dark place. The reaction was stopped by adding 50µl 2.5M H₂SO₄ and the absorbance of the plate was read at 492nm immediately.

2.5.2. Enzyme assay in 96 well plates

The activity of the lysosomal enzyme arylsulphatase B (N-acetylgalactosamine-4-sulphatase) was measured using the artificial substrate 4-methylumbelliferyl-sulphate. Previously described assays (Fluharty, Stevens et al. 1974) were modified to measure enzyme activity using 96 well plates. The arylsulphatase B cleaves sulphate from the substrate to yield the fluorescent product 4-methylumbelliferone. The liberated product is then measured using a luminescence spectrophotometer against a standard of known concentration.

\[
\text{Arylsulphatase B} \\
4\text{-methylumbelliferyl-sulphate} \rightarrow 4\text{-methylumbelliferone} + \text{sulphate}
\]

This assay measures the collective activity of arylsulphatase B and its isoenzymes including arylsulphatase A and arylsulphatase C, hence measurement of Arylsulphatase B requires separation of arylsulphatase B by chromatography. However, this step in not necessary during the mixing assays where purified recombinant human Arylsuphatase B (Naglazyme) is used and there is no contamination of other isoenzymes at any stage.

The enzyme activity assay was performed in duplicate. Specimens to be tested and artificial substrate (4-methylumbelliferyl-sulphate), prepared in substrate buffer (0.1M acetate buffer pH 5.4) at a concentration of 5mg/ml, were added to each well of the plate in equal volumes (20µl). The plate was wrapped in cling film and covered with aluminum foil to prevent light interference. The plate was incubated in a shaking incubator at 37°C for 2 hours and reaction was stopped
by adding 160 µl of stop buffer (0.2M sodium carbonate, 0.1M sodium hydrogen carbonate). The fluorescence generated was read at excitation 360nm and emission of 450nm. This fluorescence is then compared to a standard by developing a standard curve using a fluorescent standard as described previously.

2.5.3. Catalytic inhibition assay

Enzyme assays were modified to develop mixing assays in order to determine the inhibition of enzyme activity by patient serum. The assay was performed in duplicate on the highest titre patient sample in a longitudinal series. A series of two fold dilutions (starting from 1.25µg/ml) of recombinant human Arylsulphatase B (Naglazyme) in dilution buffer (PBS, 0.05% Tween, 0.01% HSA) were used to assess inhibition of enzyme activity. Ten microlitres of each enzyme solution was then mixed with the same volume of patient sera and normal sera in 96 well black plates to minimize the auto fluorescence and background light scatter. The sera were added at fixed dilutions (1:4) and incubated at room temperature for 1 hour. 4MU substrate (20 µl) in substrate buffer (0.1M acetate buffer pH 5.4) was added to each well and incubated for 2 hours at room temperature in the dark. The enzyme activity in the absence and presence of sera (for patient serum and normal serum) was measured. The enzyme activity in the presence of patient serum was also compared with enzyme activity of the plain enzyme solution for each dilution. Background enzyme activity in patient serum and normal serum was measured.
The % inhibition of the catalytic activity for each enzyme dilution was measured as follows,

\[
\text{% Inhibition} = 100 - 100 \times \frac{\text{EA}_{\text{Ps}} - \text{BEA}_{\text{Ps}}}{\text{EA}_{\text{Ns}} - \text{BEA}_{\text{Ns}}}
\]

\text{EA}_{\text{Ps}}: \text{Enzyme activity in the presence of patient serum}
\text{EA}_{\text{Ns}}: \text{Enzyme activity in the presence of normal serum}
\text{BEA}_{\text{Ps}}: \text{Background enzyme activity in patient serum}
\text{BEA}_{\text{Ns}}: \text{Background enzyme activity in normal serum}

2.5.4. Cellular uptake inhibition

This assay determines the inhibition of enzyme uptake by N-acetylgalactosamine-4-sulphatase deficient fibroblasts in the presence of antibodies. Fibroblasts from a MPS VI patient (MPS VI 94/097) were established and maintained in DMEM, 10% FBS, 1% glutamine at 37°C and 5% CO₂. The assays were performed in duplicate. Six well culture plates were seeded with 2.0x10⁵ fibroblasts into each of the six wells. The cellular uptake inhibition was performed once the wells were over 95% confluent, 24-48 hours later. The enzyme (Naglazyme) was diluted in culture medium (DMEM with 1% glutamine) at concentrations of 1.25µg/ml and 160ng/ml to ensure delivery of enzyme into the cells. Patient serum or normal serum was added in a volume of 10 µl (1 in 100 dilutions) to the diluted enzyme solution (1000 µl volume) and incubated for two hours at room temperature. The culture medium in the wells was replaced with the enzyme mixed medium incubated with either normal or patient serum. The cells were incubated for another two hours in 5% CO₂ at 37°C. The cells were then washed with PBS and harvested by trypsinization. The cells were resuspended, washed twice with PBS, and sonicated after a cycle of freeze-thaw. After centrifugation at 2045G for 10 minutes, the supernatant was tested for enzyme activity using 4MU assay (described earlier). During the assay, the baseline arylsulphatase A and C activity of fibroblasts was measured (prior to enzyme exposure) and subtracted from the final enzyme activity.
activity (post Naglazyme exposure) to reveal the arylsulphatase B enzyme delivered into the cells.

Figure 2.5 Cellular uptake inhibition in MPSVI

The protein concentration was determined by Bicinchoninic acid (BCA) assay (Smith, Krohn et al. 1985). Arylsulfatase B activity was measured as follows,

Fibroblast arylsulphatase B activity = (Enzyme activity post Naglazyme exposure) – (baseline Arylsulphatase A & C activity)

The per cent inhibition was calculated against plain enzyme solution as follows:

% Inhibition= 100- 
\[
\frac{\text{Enzyme activity of lysate incubated in the presence of patient serum}}{\text{Enzyme activity of lysate incubated with enzyme}} \times 100\%
\]
2.6. MPS II

2.6.1. ELISA

96 well flat bottomed EIA plates were coated with 50µl/well enzyme carbonate solution (10µg/ml of elaprase in 0.1M NaHCO₃, pH8.5) and stored at 4°C overnight. After aspirating the excess enzyme next day the plate was washed gently 5x with wash buffer (PBS, 0.1% Tween, pH7.4). 200ul of blocking Agent (1% w/v HSA in 0.02M Tris /HCl, 0.25M NaCl buffer pH7.0) was added to each well and plate stored at RT for 1 hr. The plates were then washed again with wash buffer x5. Using dilution buffer (PBS, 0.05% Tween, 0.01% HAS), 2 fold dilutions (16 dilutions in total) of specimens to be tested, a positive control and negative control were prepared. To each well, 50µl of standard, control or sample was added and plates were covered with an adhesive cover slip and incubated at RT for 1 hr. After aspirating the tested samples the plates were washed 5x with wash buffer. IgG goat anti human HRP antibody was added (100µl) at a 1:5000 dilution to each well and incubated at RT for 1 hr. At the end of incubation, the plates were washed 5x and 100µl of OPD was added to each well. The plates were kept for 10 minutes at RT in a dark place. The reaction was stopped by adding 50µl 2.5M H₂SO₄ and the absorbance of the plate was read at 492nm immediately.

2.6.2. Enzyme assay in 96 well plates

Hunter syndrome (MPS II) results from a defect in the activity of the enzyme iduronate-2-sulphatase (IDS). This fluorimetric assay for IDS uses 4-methylumbelliferyl-α-iduronate 2-sulphate (MU-αldoA-2S) as a substrate. To exclude a dialysis step, lead acetate is added to the reaction mixture of crude homogenate to precipitate phosphate and sulphate ions. Liberation of methyl umbelliferone (MU) from MU-αldoA-2S requires the sequential action of two enzymes, desulphation by IDS followed by hydrolysis of MU-α-iduronide by α-
iduronidase. To avoid the second step being rate limiting, a second incubation in the presence of additional α-iduronidase is undertaken. This exogenous enzyme source of α-iduronidase also contains IDS activity but this is completely inhibited by the high concentration of phosphate used in the second incubation.

\[
\text{Iduronate-2-sulphatase} \quad \xrightarrow{\alpha\text{-iduronidase}} \quad \text{4-MU-\alpha\text{-IdoA-2S}} \rightarrow \text{4-MU-\alpha\text{-Ido}} \rightarrow \text{4-MU}
\]

The liberated 4-methylumbelliferone is measured using a luminescence spectrophotometer against a standard of known concentration. The enzyme activity assay was performed in duplicate. Specimens to be tested and artificial substrate (MU-\alpha\text{-IdoA-2S}) prepared in substrate buffer (0.1M sodium acetate/acetic acid buffer pH 5.0 containing 10mM lead acetate) were added to each well of the plate in equal volumes (20μl). The plate was sealed with adhesive film and incubated in a shaking incubator at 37°C for 4 hours. Phosphate citrate buffer (double concentrated McIlvains phosphate / citrate buffer) was added (20 μl) to all samples and 10 μl purified lysosomal enzymes from bovine testis (LEBT, available in the kit) was added and mixed in a shaking incubator at 37°C for 24 hours. At the end of the incubation, 140 μl of stop buffer (0.5 M NaHCO₃/0.5 M Na₂CO₃ buffer pH 10.7 + 0.025% Triton X-100) was added and the fluorescence emission generated was measured using the microplate reader. A 4-MU standard curve was prepared as described previously. The fluorescence generated was read at excitation 360nm and emission of 450nm. This fluorescence is then compared to a standard by developing a standard curve using a florescent standard as described previously.
2.6.3. Catalytic inhibition assay

The above enzyme activity assay was modified to develop catalytic inhibition assay. The assay was performed in duplicate on the highest titre patient sample in a longitudinal series. A series of two fold dilutions (starting from 5µg/ml) of recombinant human iduronate-2-sulphatase (Elaprase) in dilution buffer (PBS, 0.05% Tween, 0.01% HSA) were used to assess inhibition of enzyme activity. Ten microlitres of each enzyme solution was then mixed with the same volume of patient sera and normal sera in 96 well black plates to minimize the auto fluorescence and background light scatter. The sera were added at fixed dilutions (1:4) and incubated at 37°C for 1 hour in a shaking incubator. The enzyme activity of the mixture was then measured using 4MU substrate as described earlier. This involved two additional incubation steps. The enzyme activity in the absence and presence of sera (for patient serum and normal serum) was measured. The enzyme activity in the presence of patient serum was also compared with enzyme activity of the plain enzyme solution for each dilution. The % inhibition of the catalytic activity for each enzyme dilution was measured as follows,

\[ \text{% Inhibition} = 100 - 100 \times \frac{E_{Aps} - B_{Eps}}{E_{Ans} - B_{EAn}} \]

\(E_{Aps}\): Enzyme activity in the presence of patient serum
\(E_{Ans}\): Enzyme activity in the presence of normal serum
\(B_{Eps}\): Background enzyme activity in patient serum
\(B_{EAn}\): Background enzyme activity in normal serum
2.6.4. **Cellular uptake inhibition assay**

Due to a failure in developing a reliable mixing assay (catalytic inhibition) and unavailability of a specific artificial substrate, development of cellular uptake inhibition assay was not undertaken. This is discussed in more detail in chapter 3.

2.7. **Western blot**

The specificity of the IgG ELISA was confirmed by western blot analysis. Proteins were separated using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). This allowed various proteins to be separated based on their molecular weight. Ten wells of SDS PAGE gel (10%) were prepared in duplicate. Samples with protein concentration of 0.6μg per well were loaded in the loading buffer. After loading enzymes and marker, an electric field was applied (100 volt) until a ladder (marker) became apparent as proteins separated (resolved) in the gel. At this point the electric current was increased to 150 volts. The proteins were transferred to nitrocellulose membrane using the semi dry transfer system (Constant 150 volt for 45 minutes). The membrane was removed from the transfer system and blocking solution (5% milk in PBS-Tween) was applied for 1 hour. The membrane was washed 3x in PBS (5 minutes per wash). The primary antibody (patient serum) was applied in a dilution of 1:1000 and kept overnight at 4°C. Membrane was washed again 3x in PBS-Tween (10 minutes per wash). The secondary antibody (anti human IgG antibody) was applied at a dilution of 1:2500 for 1 hour at RT. The membrane was washed again 3X in PBS-Tween (20 minutes per wash). Enhanced chemiluminescence (ECL) solution was applied and photographic film was prepared to image the antibody binding to the protein.
Figure 2.6 Western blot analysis
The figure describes the principle of western blot analysis. Proteins are separated, based on their molecular weight, by applying an electric current in a gel. These proteins are then transferred onto a membrane. Antibodies against a specific protein are identified by a ladder which marks the position of the protein on the nitrocellulose membrane.

2.8. Biomarkers and chimerism analysis
The ratio of DS to chondroitin sulphate (CS), a glycosaminoglycan (GAG) which is not stored in cells in MPSI, is a better marker of disease progression than urinary GAG (Church, Tylee et al. 2007; Langford-Smith, Arasaradnam et al. 2010; Langford-Smith, Mercer et al. 2010). We therefore, retrospectively collected data on DS/CS ratio in our longitudinal patient series. Urinary GAG and DS/CS ratio were performed in our clinical laboratory using two dimensional electrophoresis (originally described by Whiteman (Whiteman 1973)) and LabWorks software (Anachem, Luton, UK) (Church, Tylee et al. 2007). Analysis of donor engraftment was performed by variable number tandem repeats
(VNTR) analysis using UVP gel photography system and LabWorks software (Anachem Luton UK).

2.9. Statistics

Statistical analysis to compare multiple means of the same parameter was performed by one-way analysis of variance (ANOVA) with the post-hoc Tukey’s test. Analysis of data with more than two parameters was done by two-way ANOVA via the standard least square method and post-hoc Tukey’s multiple comparison. JMP software (SAS institute inc.) was used for these analyses. Error bars represent standard deviation of mean and were calculated using Microsoft Excel software.
3. Development of quantitative and functional immune assays for Lysosomal Storage Disorders
3.1. Introduction

To date, the majority of data on allo-immune responses to ERT have emerged from the clinical trials conducted by the pharmaceutical companies developing these therapies. These immune assays were developed, in order to comply with the pharmaco vigilance guidelines, mandatory for the pharmaceutical industry. These assays were based on highly sensitive immune antibody quantification and detection technique i.e. ELISA (Mire-Sluis, Barrett et al. 2004; Shankar, Shores et al. 2006). However, no clear strategy was developed by the pharmaceutical industry, in order to study the functional effects of these antibodies on delivered therapy. The multiplicity of antibody–enzyme interactions means that a number of functional assays are required to thoroughly investigate the effects of antibodies. Recombinant enzymes, in clinical use, have a three dimensional structure with loci for interaction with substrate, co-factors, cell surface receptors as well as other loci that are redundant (Rempel, Clarke et al. 2005). Binding of antibody may block enzyme activity (directly or indirectly), inhibit cellular enzyme uptake, change the cellular compartment reached, enhance enzyme catabolism or may not have any measurable impact at all.

Despite recent efforts to optimize and standardize these assays (Sellos-Moura, Barzegar et al. 2011), these are not widely available and no clinically accredited laboratories are currently offering these assays as a service. Development and optimisation of these assays requires careful consideration of a number of aspects of interaction between the antibodies and enzymes. It is important to distinguish the binding and/or precipitation of the enzyme by the antibodies from true catalytic or cellular uptake inhibition. Defining assay conditions is a major challenge, and under suboptimum assay conditions, a strong inhibitory specimen may not reveal true enzyme inhibition. In order to address this issue, a series of experiments were undertaken to determine the optimum conditions
to elicit the functional nature of the antibodies. This required a number of experiments performed under different conditions. A sensitive ELISA technique was optimized to detect and quantify immune responses to ERT. Two different types of functional assays were developed for MPSI, Pompe disease and MPSVI. These included assays to evaluate inhibition of enzyme activity in vitro by antibodies in patient serum (catalytic enzyme inhibition), and assays to demonstrate the inhibition of enzyme uptake by patient fibroblasts which were deficient in respective enzymes (cellular uptake inhibition).

3.2. MPSI

Aldurazyme is available as an ERT for patients with MPSI. The commercially available product (Aldurazyme) was used in the experiments to develop quantitative and functional assays.

3.2.1. ELISA

The first step in developing a sensitive IgG ELISA for Aldurazyme, is to define the optimum concentration of enzyme to coat the EIA plates. During development of ELISA, a negative cut off was defined for each dilution of serum. Specificity of the assay was determined by simultaneously testing a serum with IgG anti-IDUA antibodies and another serum sample with IgG anti-aglucosidase alpha antibodies. Serum specimens from longitudinal patient series were tested to assess sensitivity of the immune assay. The assay was repeated using EIA plates prepared approximately 2 weeks prior to testing, to assess the stability of plates.

A range of Aldurazyme concentrations were used to coat the plates and the absorbance was measured after incubation with serum from patients with MPSI treated with Aldurazyme as described in chapter 2. There was very little absorbance detected on Aldurazyme concentrations of less than 1µg/ml. This absorbance increased on higher enzyme concentrations and seemed to plateau at 10 µg/ml and hence this concentration was selected as optimum Aldurazyme
concentration to coat the EIA plates as shown in figure 3.1. Serum from thirteen normal individuals were used to define the mean negative control and a negative cut off was defined as two standard deviations above the mean, in order to include the 95% confidence interval for the value at a specific concentration (figure 3.2). The ELISA was repeated using a positive control (MPSI patient with IgG antibodies) and another patient with Pompe disease who had IgG anti aglucosidase alpha antibodies in serum. The ELISA was specific to IDUA antibodies and the Pompe disease patient serum showed an absorbance below the negative cut off consistent with a negative result (figure 3.3). Serum samples from an MPSIH patient over a period of time (longitudinal series) were tested which showed the escalating immune response after exposure to Aldurazyme peaking at a level of over 1:250000 dilutions which was the limit of serum dilutions used for these assays (figure 3.4). ELISA plates showed the same results when repeated 2 weeks or longer after preparation of plates (figure 3.5).

![Effect of differing concentrations of Aldurazyme (µg/ml) EIA plate coating](image)

**Figure 3.1 Optimisation of EIA plate coating**
The figure shows absorbance of light as detected by microplate reader at a wavelength of 492nm. Serum dilutions ranged from 1:8 to 1:1024. Aldurazyme concentration ranging from 0.8 to 20µg/ml was used. The data shows that absorbance continues to increase till 10 µg/ml and then seems to plateau above that level.
Figures 3.2-3.5  Optimisation of ELISA
Figure 3.2 shows positive cut off (red line) for ELISA based on average absorption values from 13 different normal human sera (blue line). Please see text for details. Figure 3.3 shows negative ELISA using serum of a patient with IgG antibodies to aglucosidase alpha (green line) whilst the serum with anti IDUA antibodies is strongly positive (blue line). Figure 3.4 shows longitudinal ELISA data in a MPSI patient. Note positive ELISA at a dilution of 1:250000 on day 600 post ERT, confirming the highly sensitive nature of the assay. Figure 3.5 shows high absorbance of ELISA when tested in plates prepared two weeks earlier confirming the stability of coated plates over a period of time.
The specificity of ELISA was also demonstrated using a western blot analysis as shown in figure. This figure shows that in the absence of antibodies in the pre ERT sample, there is no antibody binding to Aldurazyme (recombinant IDUA) or Myozyme (recombinant human alglucosidase alfa, Genzyme, Framingham, USA). After exposure to ERT, the IgG antibodies in patient serum only bind to Aldurazyme and not to Myozyme (figure 3.6).

**Figure 3.6 Western blot analysis for MPSI**

Western blot for two patients depicting the specificity of ELISA for IDUA (Aldurazyme). In the absence of antibodies in the pre ERT sample (left panel), there is no antibody binding to IDUA or Myozyme (MZM). After exposure to ERT, the IgG antibodies in patient serum only bind to IDUA and not to MZM (right panel). Lower two panels are controls showing position of IDUA and MZM. Mkr (Marker) region shows the position of various bands based on their molecular weight (kDa) according to the ladder.

### 3.2.2. Catalytic enzyme assay

In clinical practice, enzyme activity assays are mostly performed in test tubes which require large volume of substrates and patient samples. Due to availability of only a limited volume of patient specimens, Aldurazyme enzyme activity assays were optimized in 96 well plates. These assays were then modified to develop catalytic inhibition assays. An inhibition of enzyme activity was seen when normal serum was mixed with enzyme solution with known enzyme activity. This response was seen with both normal serum and patient serum. A number of experiments were undertaken to define the conditions
which would reveal the inhibition caused by antibodies in patient serum. This included studying the effect of change in serum dilution, enzyme concentration, pH, incubation times, using different dilution buffers and splitting the incubation period.

The highest inhibition of enzyme activity was seen by using undiluted (neat) serum. However, due to the availability of a limited amount of serum a dilution of 1:4 was used for the final analysis of patient specimens. The same inhibition pattern was seen across the range of enzyme concentrations (15, 30 and 60ng/ml) as shown in figure 3.7. During this experiment an inhibition of over 25% was observed by both patient and normal serum at all enzyme concentrations but there was no statistically significant difference between normal serum and patient serum.
Figure 3.7  Effect of serum dilutions on catalytic inhibition

The figure shows inhibition of enzyme activity by neat serum and at three different dilutions (2, 4 and 8). Maximum inhibition was seen by neat specimens of patient and normal serum. This trend was observed in all three enzyme concentrations (15, 30 and 60ng/ml) used to assess the effect of serum dilutions. Data was analysed by 2-way ANOVA using post-hoc Tukey's test. NS indicates no significant difference. Error bars refer to standard deviation of the mean.
The IDUA enzyme assays for MPSI require an optimum acidic pH. In the previous experiment, the pH of the buffer used for serum dilution was 7. Despite the very small volume of dilution buffer (10μl), it could be argued that the use of a dilution buffer at a higher pH could have caused some drop in enzyme activity. In order to see the effect of change in pH, a dilution buffer with low pH (3.5) was used which showed persistent inhibition by serum (figure 3.8) and there was no statistically significant difference between dilution buffers at pH 7.0 when compared to the dilution buffer at pH 3.5. This confirmed that the inhibition seen by the sera is due to the inhibitory effect of sera and not due to the altered pH.

![Effect of change in PH](image)

**Figure 3.8 Effect of change in pH**

The figure shows the effect of change in pH. No significant difference in enzyme inhibition was observed at two different pH. Data was analysed by 2-way ANOVA using post-hoc Tukey’s test. NS indicates no significant difference. Error bars refer to standard deviation of the mean.

Early ELISA experiments had shown that the dilution buffer (buffer 2) used in ELISA did not compromise the binding of antibodies to the enzyme coated plates. During early catalytic inhibition assay, homogenizing buffer (buffer 1) was used. Failure to demonstrate additional inhibition by antibodies in catalytic inhibition could be due to denaturing of the antibodies during the process of
serum dilution. Therefore, inhibition assays were repeated using buffer 1 and buffer 2 (figure 3.9). Dilution buffer 2 was the same buffer which was used in ELISA experiments. Although these experiments showed significant inhibition of enzyme activity, no significant difference was seen between the normal serum and patient serum. Even though the difference between patient serum and normal serum was not statistically significant, inhibition of enzyme activity by patient serum was slightly higher than normal serum when buffer 2 was used and hence buffer 2 was selected for subsequent experiments.

**Figure 3.9  Effect of change in dilution buffers**

The figure shows the effect of change in dilution buffers. Significantly higher enzyme inhibition was seen using buffer 2. However the difference between normal serum and patient serum was not statistically different. Data was analysed by 2-way ANOVA using post-hoc Tukey's test. * indicates a P value ≤ 0.05, ** indicates P value ≤ 0.01, *** represents P value ≤ 0.001. NS indicates no significant difference. Error bars refer to standard deviation of the mean.
Some antibodies require a longer incubation period to bind to the enzymes to neutralize their effect. In order to evaluate the effect of incubation time, two different incubation periods were compared to assess the enzyme inhibition. These experiments showed that there was a statistically significant difference in enzyme activities between each group. However, within each group the only significant difference was between no serum to serum values (both patient and normal serum). Again despite higher inhibition by patient serum, there was no statistically significant difference between patient serum and normal serum. (see figure 3.10)

Figure 3.10  Effect of change in incubation time

The figure shows the effect of change in incubation time. Reducing the incubation time to 30 minutes (figure b) results in loss of inhibition (compared to standard). Data was analysed by 2-way ANOVA using post-hoc Tukey’s test. ** indicates P value ≤ 0.01, *** represents P value ≤ 0.001. NS indicates no significant difference. Error bars refer to standard deviation of the mean.
At this stage the incubation period was split into two incubations. The first incubation period involved mixing of patient serum with enzyme solution and the second incubation started after the substrate was added to the serum and enzyme mixture. This experiment showed a statistically significant difference in enzyme activity between baseline enzyme activity (no serum) and sera (patient and normal serum). Also, there was statistically significant difference between the patient serum and normal serum (figure 3.11).

Figure 3.11  Inhibition using two separate incubations

The figure shows the effect of splitting the incubation period. There is significantly more inhibition by patient serum when compared to normal serum and no serum. Data was analysed by one-way ANOVA using post-hoc Tukey's test. ** indicates P value ≤ 0.01, *** represents P value ≤ 0.001. Error bars refer to standard deviation of the mean.
Splitting the incubation period, increasing the incubation time for first incubation and using low enzyme concentration increased the difference between the inhibition by patient serum and normal serum. As there was some inhibition seen by normal serum, the enzyme activity in the presence of normal serum was used as a standard to describe the inhibition caused by patient serum. There was significant difference in enzyme activity across the enzyme concentrations (p<0.01). See figure 3.12.

![Figure 3.12](image_url)

**Figure 3.12  Effect of change in enzyme concentrations**

After optimizing the experiment conditions, the assay was performed across the Aldurazyme concentration (60ng/ml to 0.45 ng/ml). The same inhibition pattern was observed across the enzyme concentrations. This effect becomes more marked at low enzyme concentration. Data was analysed by 2-way ANOVA using post-hoc Tukey’s test. ** indicates P value ≤ 0.01. Error bars refer to standard deviation of the mean.
The experiment was repeated using human innate enzyme from HEK cells. A similar pattern of inhibition was seen. Figure 3.13 shows that the enzyme activity in the presence of serum drops to nearly 60% of the standard at a low concentration of enzyme lysate (6.25 μg/20μl). In subsequent experiments, serum with no antibodies showed no significant inhibition of enzyme activity confirming the specificity of this response (figure 4.5).

![Two incubations across enzyme concentrations (innate enzyme)](image)

**Figure 3.13  Inhibition of innate enzyme (human embryonic kidney cells lysate)**

The experiment was repeated using human innate enzyme from human embryonic kidney (HEK) cells. Inhibition of innate enzyme becomes more marked when using lower concentrations of enzyme (6.25 and 12.5 μg/20μl of HEK cells protein).
In order to demonstrate that this inhibition was caused by antibodies in patient serum, the experiment was repeated using a patient serum before HSCT (with high titre of IgG antibodies) and the same patient serum after HSCT (with no detectable IgG antibodies). The same assay was repeated using innate IDUA mouse liver IDUA at different concentrations. An inhibition of above 20% was demonstrated, which suggested cross reactivity of human antibodies with innate enzymes from both humans and other species (figure 3.14).

![Innate enzyme (mouse IDUA) inhibition](image)

**Figure 3.14** Inhibition of innate enzyme (mouse IDUA enzyme)

*Figure shows that mouse enzyme is inhibited by patient serum suggesting cross reactivity of antibodies to mouse IDUA.*

Based on optimisation experiments, buffer 2 with two hour split incubation was selected for final assays. A range of enzyme concentrations was chosen to evaluate the catalytic inhibition (see chapter 2).
3.2.3. Cellular uptake inhibition

A suitable enzyme concentration for cellular uptake was determined by exposing enzyme deficient fibroblasts to a range of enzyme concentrations. Inhibition assay was then performed on selected concentrations to optimise the uptake inhibition. Fibroblasts were seeded in 6 well plates as described in chapter 2 and exposed to increasing concentrations of Aldurazyme for a fixed period of time (one hour) to ensure delivery of enzyme into the cells. Excellent levels of enzyme were delivered into the cells using a wide range of enzyme concentrations (see figure 3.15).

![Figure 3.15 Optimizing Aldurazyme concentration for uptake inhibition assay](image)

Fibroblasts from MPS1H patient were seeded in 6 well plates and exposed to a range of enzyme concentration (6000ng/ml to 93.75 ng/ml). Enzyme delivery into the cells was achieved at all concentrations.

Concentrations at 1/2 Km (Km: maximum enzyme delivered into the cells) and below were selected for further experiments. This was to ensure that the
antibodies are not overcome by excessive enzyme in the incubation medium. Enzyme activity in fibroblast lysate was measured at 93, 187, 375 and 750ng/ml in the presence and absence of serum from the patient with high titre IgG antibodies. This experiment was repeated using the serum from a normal individual. The enzyme activity in the presence and absence of serum was compared to quantify percentage inhibition. Unlike catalytic inhibition, no inhibition of cellular uptake of enzyme was seen at any enzyme concentration by normal serum. The difference between enzyme activity without serum and patient serum increased with reducing enzyme concentrations. Statistically significant difference (p<0.05) between the enzyme activities was seen between no serum and patient serum groups at all enzyme concentrations (figure 3.16). The highest enzyme inhibition was seen at enzyme concentration of 93ng/ml which was selected as the optimum concentration for analysis of serum specimens for final analysis (figure 3.17).

![Optimization of enzyme uptake inhibition](image)

3.16 Cellular uptake inhibition at different enzyme concentrations

Enzyme activity without serum and patient serum in MPSIH fibroblast lysate was assessed at four different Aldurazyme concentrations. Data was analysed by 2-way ANOVA using post-hoc Tukey's test. * indicates a P value ≤ 0.05, ** indicates P value ≤ 0.01, *** represents P value ≤ 0.001. Error bars refer to standard deviation of the mean.
3.17 Percentage cellular uptake inhibition at different enzyme concentrations

Inhibition of enzyme uptake by MPSIH fibroblasts was assessed at four different Aldurazyme concentrations. Maximum inhibition of enzyme uptake was observed at 93ng/ml. Data was analysed by 2-way ANOVA using post-hoc Tukey’s test. * indicates a P value ≤ 0.05, ** indicates P value ≤ 0.01, *** represents P value ≤ 0.001. NS indicates no significant difference. Error bars refer to standard deviation of the mean.
3.3. Pompe disease

Myozyme is licensed for use in Pompe disease patients. Myozyme was purchased and used for developing the immune assays.

3.3.1. ELISA

Similar to MPSI, different enzyme concentrations were evaluated for optimisation of ELISA for Pompe disease. A negative cut off was defined by testing normal sera. Sensitivity and specificity of the assay were evaluated by high titre antibody Pompe disease patient serum and serum from a patient with anti-IDUA antibodies. Three different Myozyme concentrations were used (0.1, 0.5 and 1μg/ml) to coat EIA plates. A concentration of 1μg/ml was chosen as the optimum concentration for coating EIA plates based on the absorbance values of patient serum and normal control (figure 3.18).

![Optimizing Myozyme concentration ELISA](image)

Figre 3.18 Optimizing Myozyme concentration for coating EIA plates

Three different Myozyme concentrations were used to assess the absorbance during development of IgG ELISA. The highest absorbance was seen at 1μg/ml Myozyme concentration as shown in the figures. Error bars refer to standard deviation of the mean.
ELISA for MPSI, a normal cut off was defined as a value, 2 standard deviations above the mean absorbance for that dilution (figure 3.19). Positive results at a serum dilution of 1:250000 demonstrate highly sensitive nature of the assay developed (figure 3.20). The assay was performed using a Pompe disease patient serum and a serum known to have IgG antibodies to Aldurazyme. The results confirmed the specificity of assay (figure 3.21a). Specificity of assays was also confirmed by western blot analysis as shown in (figure 3.21b)

![Cut off and Sensitivity of ELISA graphs](image)

**Figure 3.19**  **Figure 3.20**

**Figure 3.19 and 3.20** Negative cut off and Sensitivity of ELISA

Negative cut off was defined as explained in the text. Figure 3.20 shows very high level of absorbance (positive at all 16 dilutions) in patient sample 2. Error bars refer to standard deviation of the mean.
Figure 3.21 Specificity of ELISA assay

High absorbance seen by Pompe disease patient. However a patient sample with IgG anti IDUA antibodies remains negative at all dilutions tested. Error bars refer to standard deviation of the mean. Western blot analysis (3.21b) shows binding of antibodies in patient serum to Naglazyme only. There is no antibody binding with Myozyme.
3.3.2. Catalytic inhibition

Enzyme activity assay for Myozyme was optimized in a 96 well plate (see figure 3.22) as described in chapter 2. The enzyme activity plateaued at levels above 25μg/ml depicting the highest limit of detection of enzyme activity. From this data, suitable enzyme concentrations were selected to develop mixing assay to study the catalytic enzyme inhibition. A number of parameters including incubation time, enzyme concentration and serum dilutions were evaluated to optimise the assay.

![Optimizing enzyme assay in 96 well plate](image)

**Figure 3.22** Optimizing Myozyme activity assay in 96 well plate  
*Error bars refer to standard deviation of the mean*

Based on understanding of catalytic inhibition, developed during optimisation experiments in MPSI patients, only split incubations were used to develop the enzyme inhibition assays in Pompe disease. An inhibition of enzyme activity was seen both with normal serum and patient serum and the difference was statistically significant as shown in figure 3.23. However, unlike MPSI no significant difference was seen between normal serum and patient serum
despite using experimental conditions similar to those of MPSI. In previously described experiments, de Vries et al. used a much longer incubation period (de Vries et al. 2010) and hence the effect of varying incubation times was studied in subsequent experiments. Incubation time was increased to 4 and 24 hours. A significant inhibition compared to normal serum was only seen after 24 hour incubation. The enzyme activity with patient serum was significantly lower than no serum across the range of concentrations used in this experiment (p<0.05) see figure 3.23.

The effect of varying serum dilutions on a range of enzyme concentrations was tested as shown in the figure 3.24. More inhibition (direct and compared to a standard) was seen by concentrated (1:4 dilution) serum and the difference between patient serum and normal serum was statistically significant at this dilution. However, on dilution of patient serum to 1:8 and 1:16, this inhibition is lost. Due to the availability of a limited amount of patient samples and demonstration of quantifiable inhibition at 1:4 serum dilution, neat serum was not used for the assay.
The figure shows catalytic enzyme inhibition at three different incubations at 2, 4 and 12 hours across a range of enzyme concentrations (12.5 to 0.8 μg/ml). Optimum inhibition (compared to normal serum) is seen at 24 hour incubation. Data was analysed by 2-way ANOVA using post-hoc Tukey's test. * indicates a P value ≤ 0.05, ** indicates P value ≤ 0.01, *** represents P value ≤ 0.001. Error bars refer to standard deviation of the mean.
### Figure 3.24  Effect of serum dilutions on catalytic enzyme inhibition

The figure shows catalytic enzyme inhibition using serum dilutions of 1:4, 1:8 and 1:16. Maximum inhibition (compared to standard) is seen at a serum dilution of 1:4 across the range of enzyme concentrations (12.5 to 0.78 μg/ml). Data was analysed by 2-way ANOVA using post-hoc Tukey’s test. * indicates a P value ≤ 0.05, ** indicates P value ≤ 0.01, *** represents P value ≤ 0.001. Error bars refer to standard deviation of the mean.

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The figure shows catalytic enzyme inhibition using serum dilutions of 1:4, 1:8 and 1:16. Maximum inhibition (compared to standard) is seen at a serum dilution of 1:4 across the range of enzyme concentrations (12.5 to 0.78 μg/ml). Data was analysed by 2-way ANOVA using post-hoc Tukey’s test. * indicates a P value ≤ 0.05, ** indicates P value ≤ 0.01, *** represents P value ≤ 0.001. Error bars refer to standard deviation of the mean.
These experiments were performed at very high enzyme concentrations. However, pharmacokinetic data on Myozyme suggests that the maximum concentration of enzyme achieved after infusion is approximately 200μg/ml. Myozyme has a half-life of about 2.75 hours [Myozyme product literature, scientific discussion EMA 2006]. This suggests that the levels of Myozyme in serum would drop to below 1μg/ml within 24 hours of infusion. This necessitates assessment of catalytic inhibition at lower enzyme concentrations and hence a wide range of enzyme concentrations (12.5μg/ml - 0.1μg/ml) was chosen to study the catalytic inhibition in final assay protocol. During optimising experiments, a different patient serum (with higher antibody titre) inhibited the enzyme activity completely at a concentration of 0.1μg/ml whilst there was detectable enzyme activity at this concentration with normal serum (100% inhibition). See figure 3.25. Based on these experiments, for the final assay protocol, prolonged incubation period (24 hours) at a dilution of 1:4 was selected. The inhibition was tested across a wide range of enzyme concentrations as described in chapter 2.
Figure 3.25 Catalytic inhibition at low enzyme concentrations

Catalytic enzyme inhibition was assessed by lowering the Myozyme concentration. The optimum inhibition was seen at 0.1μg/ml Myozyme. Figure (a) shows the actual enzyme activity and activity in presence of patient and normal serum at four different dilutions. Figure (b) shows the percentage enzyme inhibition. Data was analysed by 2-way ANOVA (a) and one way ANOVA (b) using post-hoc Tukey’s test. * indicates a P value ≤ 0.05, ** indicates P value ≤ 0.01, *** represents P value ≤ 0.001. NS indicates no significant difference. Error bars refer to standard deviation of the mean.
3.3.3. Cellular uptake inhibition

Initial experiment to deliver Myozyme into the fibroblasts from the Pompe disease patient showed no delivery of enzyme into the cells across the range of enzyme concentrations. However, in subsequent experiments higher doses were used and incubation times were increased. These experiments showed that higher Myozyme concentrations were required to deliver the enzyme into the cells compared to Aldurazyme in MPSI experiments. Also, the incubation time had to be increased to achieve quantifiable levels of enzyme in the Pompe disease patient fibroblasts (figure 3.26). Incubation times of both 6 and 24 hours delivered satisfactory levels of enzyme into the fibroblasts and hence a 6 hour incubation period was used in subsequent experiments.

![Enzyme uptake by Pompe fibroblasts](image)

**Figure 3.26 Optimizing enzyme concentrations and incubation time for cellular uptake inhibition assay**

The figure shows enzyme activity of Pompe disease fibroblasts after exposure to a range of Myozyme concentrations (1.56-200μg/ml). No uptake was seen at any concentration when cells were incubated for one hour. Increasing the incubation time resulted in successful delivery of enzyme into the cells.
No significant enzyme uptake inhibition was seen by normal serum when tested at two different enzyme concentrations (see figure 3.27). However, there was significantly low enzyme activity in the presence of patient serum when compared to normal serum and no serum (p<0.05), both at 12.5μg/ml and 6.25 μg/ml. For final analysis, the percentage inhibition was calculated by comparing the enzyme activity in the presence and absence of patient serum.

**Figure 3.27** Cellular uptake inhibition at two different enzyme concentrations

Enzyme activity in the presence of patient serum is reduced to approximately 50% when compared to no serum and normal serum. No inhibition was seen by normal serum and hence percentage inhibition was measured by comparing enzyme activity in the presence of patient serum with that of the enzyme activity in the absence of serum. Data was analysed by one-way ANOVA using post-hoc Tukey’s test. * indicates a P value ≤ 0.05, ** indicates P value ≤ 0.01, *** represents P value ≤ 0.001. Error bars refer to standard deviation of the mean.
Figure 3.28 shows the enzyme activity in fibroblast lysate in the presence and absence of patient serum (with antibodies). Enzyme inhibition was seen only by Pompe disease patient serum with IgG whilst no significant inhibition was seen in the absence of an immune response.

![Specificity of assay (Pompe disease)](image)

**Figure 3.28  Specificity of Cellular uptake assay**

Data was analysed by one-way ANOVA using post-hoc Tukey’s test. * indicates a P value ≤ 0.05. Error bars refer to standard deviation of the mean.
3.4. MPS VI

Commercially available product Naglazyme was used to develop immune assays for MPSVI patients.

3.4.1. ELISA

Two different Naglazyme concentrations were used to optimize enzyme concentration for coating EIA plates. Enzyme concentration of 10μg/ml showed excellent absorbance values and hence this concentration was selected for coating the plates (figure 3.29).

![Optimizing Naglazyme concentration for EIA plates](image)

3.29 Optimizing Naglazyme concentration for EIA plates

Error bars refer to standard deviation of the mean.
A cut off was defined similarly to ELISA for MPSI and Pompe disease i.e. 2 standard deviation above the mean value of absorbance at a given concentration. Specificity of assay was determined by quantifying immune response in serum of a patient with MPS VI and serum with IgG antibodies to Aldurazyme (figure 3.30).

![Figure 3.30 Specificity of ELISA](image)

The figure shows positive ELISA when using serum from an MPSVI patient treated with Naglazyme. Absorbance value of serum with IgG anti IDUA antibodies remained below the threshold (negative result). Figure also shows a negative cut off for the MPS VI ELISA. Error bars refer to standard deviation of the mean. A western blot analysis shows binding of antibodies to Naglazyme only.
ELISA assay on a longitudinal series of samples from a patient with MPS VI showed a positive result at the limit of dilution (1:250000) showing high sensitivity of the assay (figure 3.31).

**Figure 3.31 Sensitivity of ELISA**

The figure shows results of a longitudinal sample series of an MPSVI patient. The highest response suggests a positive ELISA at a serum dilution of (1:250000). Error bars refer to standard deviation of the mean.
3.4.2. Catalytic inhibition

In order to develop mixing assays, Nagalzyme activity assay was optimized in 96 well black plates. To evaluate the sensitivity of substrate, two different concentrations of artificial substrate (10mg/ml and 5 mg/ml) were used. Both substrate concentrations resulted in highly sensitive assays. In view of difficulty in dissolving 10mg of artificial substrate per millilitre of substrate buffer, only 5mg/ml was selected as the optimum substrate concentration for subsequent assays (figure 3.32).

![Optimization of Arylsulphatase enzyme assay in 96 well plate](image)

**Figure 3.32 Optimisation of Arylsulphatase enzyme assay in 96 well plate using to different substrate concentrations.**

No significant difference was seen between the two concentrations. 4MU depicting the enzyme activity tended to plateau at 40μg/ml suggesting the upper limit of detection of enzyme activity. Error bars refer to standard deviation of the mean.

However, unlike MPSI and Pompe disease, no inhibition of enzyme activity was seen on mixing patient serum with enzyme concentrate (see figure 3.33). There was no statistically significant difference between no serum (Enzyme activity), patient serum and normal serum groups at any enzyme concentration tested during this experiment.
Figure 3.33 Catalytic inhibition assay using a range of Naglazyme concentrations (0.01 to 1.25 μg/ml)

Data was analysed by 2-way ANOVA using post-hoc Tukey’s test. No significant difference was seen between the three groups (p>0.05) at all concentrations tested suggesting no enzyme inhibition either by patient serum or normal serum. There was an overall statistically significant concentration effect (p<0.01). Error bars refer to standard deviation of the mean.

The experiment was repeated across a range of enzyme concentrations using different serum dilutions and incubation times (2, 4 and 24 hours) but no underlying enzyme inhibition was demonstrated either by patient serum or normal serum (see figures 3.34 and 3.35).
Figure 3.34 Effect of change in incubation time

The figure shows catalytic enzyme inhibition at three different incubation periods (2, 4 and 24 hours). No statistically significant difference was seen between patient serum, normal serum and no serum groups at any concentration using different incubation periods. There is however, an overall statistically significant concentration effect (P<0.01) between all concentrations. Data was analysed by 2-way ANOVA using post-hoc Tukey's test. Error bars refer to standard deviation of the mean.
No significant difference was seen in enzyme activity using different serum dilutions. Data was analysed by 2-way ANOVA using post-hoc Tukey's test. Error bars refer to standard deviation of the mean.
3.4.3. Cellular uptake inhibition

A high level of enzyme was delivered into the MPS VI patient fibroblasts when they were exposed to Naglazyme. Increasing the exposure time (incubation) to six hours significantly enhanced the intracellular enzyme delivered into the cells (figure 3.36). However, due to an adequate level of enzyme delivery into the cells at one hour, this incubation period was selected to study the enzyme uptake inhibition.

![Enzyme uptake by MPS VI fibroblasts](chart.png)

**Figure 3.36 Optimizing Naglazyme concentration for cellular uptake assay**

Excellent enzyme delivery was seen into the MPSVI fibroblasts at one hour incubation. Increasing the incubation time, significantly increased the uptake. Data was analysed by 2-way ANOVA using post-hoc Tukey's test. * indicates a P value ≤ 0.05, ** indicates P value ≤ 0.01, *** represents P value ≤ 0.001. Error bars refer to standard deviation of the mean.
Enzyme inhibition was tested at two different enzyme concentrations (1.25μg/ml and 160ng/ml). However, no significant difference was seen between the enzyme activity in the absence of serum and in the presence of patient serum (with high titre antibodies), see figure 3.37. Increasing the concentration of patient serum to 1:50 dilution also did not change the pattern of enzyme uptake and no enzyme uptake inhibition was seen by either patient or normal serum (figure 3.38).

![Effect of enzyme concentration](image)

**Figure 3.37**

![Effect of serum dilution](image)

**Figure 3.38**

**Figure 3.37 and 3.38** Cellular uptake inhibition assay at different enzyme concentrations and serum dilutions

Data was analysed by 2-way ANOVA using post-hoc Tukey’s test. No significant difference was seen between normal serum and patient serum at the two concentrations suggesting no inhibition. Also, no significant difference was seen using more concentrated serum (dilution 1:50). Error bars refer to standard deviation of the mean.
3.5. MPSII

Recombinant enzyme therapy (Elaprase) is available for clinical use in MPSII patients. This product was purchased and used to develop immune assays for MPSII patients treated with Elaprase.

3.5.1. ELISA

Two different Elaprase concentrations were used to optimize EIA coating and 10μg/ml Elaprase was selected as the optimum coating concentration (figure 3.39). A cut off was defined as two standard deviation above the mean value of absorbance for a concentration. ELISA specificity was demonstrated by testing MPS II patient serum and serum of MPSI patient with IgG antibodies to IDUA (see figure 3.40).

![Optimizing Elaprase concentration for EIA plates](image)

Figure 3.39 Optimzing Elaprase concentration for coating EIA plates
Figure 3.40  Specificity of ELISA and negative cut off

Specificity of ELISA is demonstrated by a negative result when using a serum with IgG anti IDUA antibodies. A negative cut off is shown in the figure.

3.5.2. Catalytic inhibition

As described previously, the enzyme activity assay for Elaprase is a two step process which involves sequential action of two enzymes. Desulphation of artificial substrate by iduronate-2-sulphatase is followed by action of $\alpha$-iduronidase which releases 4MU. This enzyme activity assay was successfully optimized in a 96 well plate (see figure 3.41), however, mixing assay required addition of a third incubation period. The initial assays demonstrated no significant inhibition of enzyme activity (figure 3.42) but repeat experiments on highly complex assays (with three incubation periods) showed extremely high inter assay variability as shown in figure 3.43 and 3.44.
Figure 3.41 Optimizing iduronate-2-sulphatase enzyme activity assay in 96 well plate

Figure 3.42 Catalytic inhibition assay

The figure shows no inhibition of enzyme activity in the presence of normal serum and patient serum. A high enzyme activity is seen when patient serum or normal serum is mixed with enzyme solution at some dilutions. Data was analysed by 2-way ANOVA using post-hoc Tukey’s test. * indicates a P value ≤ 0.05, ** indicates P value ≤ 0.01, *** represents P value ≤ 0.001. Error bars refer to standard deviation of the mean.
**Figures 3.43**

**Figures 3.44 and 3.45**  Catalytic inhibition at two enzyme concentrations

Variable enzyme activity was seen after inhibition assay as shown in the figures. Data was analysed by 2-way ANOVA using post-hoc Tukey’s test. Error bars refer to standard deviation of the mean.
3.6. Discussion

Even though the immune assays for different LSDs were based on the same principle, the conditions of each experiment had to be changed considerably. This highlights the different characteristics and variable nature of antibodies produced in response to different ERTs. ELISAs for all LSDs were highly sensitive and antibodies were still detected at extremely dilute serum samples of over 1: 200,000 dilution. In contrast, catalytic inhibition assays were more difficult to perform and required a combination of specific sets of conditions to elicit the underlying inhibition by antibodies in patient serum. It is interesting to note that in MPSI and Pompe disease, both the normal serum and patient serum (with high titre antibodies) inhibit the enzyme activity. This could be due to the inhibition by non-specific immunoglobulins and complement proteins in normal serum. Therefore the enzyme activity of the enzyme-patient serum mixture was compared with enzyme-normal serum mixture (standard) to reveal the inhibition caused by antibodies in patient serum. Initial experiments for catalytic inhibition assay, showed that there was no statistically significant difference in enzyme activity when tested under different enzyme concentrations, dilutions, incubation time, dilution buffers and pH. Increasing the incubation time to 2 hours increased the inhibition by patient serum compared to normal standard but this difference was not statistically significant. Likewise, catalytic inhibition using buffer 2 as a dilution buffer, was higher than buffer 1 (p<0.01) and hence buffer 2 was adopted for subsequent assays. In the final assay protocol, the incubation time was increased and two incubations were used. The first incubation involved mixing the patient serum/normal serum with enzyme at room temperature. At the end of this incubation, the substrate was added to the mixture, followed by a second incubation. A wider range of enzyme concentrations was used for final analysis of samples as outlined in the assay protocol described in chapter 2. At low enzyme concentrations, the percentage inhibition by patient serum in comparison to normal serum and no serum appears high. Assessment of catalytic inhibition at low concentration is important because the serum enzyme concentrations, unlike leucocyte enzyme
concentration, drop very rapidly after ERT infusion due to the short half-life and rapid clearance of enzyme from the circulation. This is discussed in more detail in chapter 4. The assays were repeated in the tolerized (no antibodies) and non-tolerized (with high titre antibodies) serum, from the same patients, to confirm that the inhibition against the standard (normal serum) was due to the antibodies in the serum. In order to minimize background interference, baseline enzyme activity in sera was measured during each assay and subtracted from the final enzyme activity for patient and normal sera. A similar pattern of inhibition was seen when innate human and mouse IDUA was used. This phenomenon of cross reactivity of antibodies with innate enzymes of animal origin has been described previously (Brooks, King et al. 1997). In contrast, cellular uptake inhibition assays showed higher levels of inhibition at high enzyme concentrations. It can be argued that the cellular uptake inhibition assays are more meaningful because they incorporate two aspects of enzyme inhibition. Firstly, they look at the ability of antibodies to block the enzyme from getting into the cells which is a critical step required for the action of infused exogenous enzyme. Secondly, due to the measurement of enzyme uptake using a functional assay, one can only determine the catalytically active enzyme taken up the cells. Hence these assays actually measure the uptake of catalytically active enzyme, incorporating both aspects of functional assays. Catalytic inhibition assays, in Pompe disease, required a much longer incubation period of 24 hours. This necessitated carrying out a first incubation at 4°C. A different set of enzyme concentrations had to be used based on optimisation experiments, in 96 well plates. Cellular uptake inhibition assay required considerably higher enzyme concentration in 6 well plates seeded with Pompe disease patient fibroblasts. However, increasing the enzyme concentration was not enough to deliver the enzyme into the cells which is a prerequisite to the measurement of cellular enzyme uptake inhibition. The exposure time had to be increased to six hours. This finding is consistent with the pharmacokinetic data on Myozyme (Amalfitano, Bengur et al. 2001)(Myozyme product literature). In clinical practice, the recommended dose
of Myozyme for Pompe disease patients is significantly higher than the dose of Aldurazyme for MPSI patients. This poses an important question of whether the current dosage strategies deliver adequate levels of enzyme into the enzyme deficient cells of Pompe disease patients who develop high titre immune response. Based on the data, it can be argued, that in the presence of high titre immune response, either the dose of infusion or frequency of infusion need to be increased to ensure delivery of therapeutic levels of Myozyme into the patient fibroblasts.

MPS VI patients in contrast to MPSI and Pompe disease do not show evidence of in vitro enzyme inhibition. This could be due to three reasons. It is possible that, despite our experiments, we were unable to define the optimum conditions to demonstrate enzyme inhibition. This will be supported by the fact that previous experiments in animals and humans (White, Argento Martell et al. 2008) showed some evidence of in vitro enzyme inhibition. However, these techniques did not involve the use of functional enzyme assays and did not look at the true uptake of enzyme by the patient fibroblasts. A second possibility is that the antibodies produced in MPSVI are not inhibitory as is shown in our experiments. This is supported by the fact that there is no credible data showing a correlation between the antibody titres and biomarkers of disease progression or clinical deterioration. A third reason for a failure to show an inhibition could be the relatively small number of patient samples in this group. We only tested three patients and it is possible that none of them had inhibitory antibodies.

MPSII enzyme activity assay is a more complex assay which requires two incubations. First step is desulphation, which is followed by IDUA exposure to free 4MU from the artificial substrate. These steps are highly sensitive to time, pH and temperature (Fluharty, Stevens et al. 1974; Mercelis, Van Elsen et al. 1979; Voznyi, Keulemans et al. 2001). This makes it considerably more difficult to alter the experiment conditions to develop and optimize a catalytic inhibition assay for MPSII patients using the currently available 4MU artificial substrate. This was confirmed by initial experiments which gave inconsistent results during the development of catalytic inhibition assay. Development of reproducible,
consistent and reliable functional assays for MPSII will require a different substrate which is more specific, robust and does not require multiple incubation steps.
4. Haemopoietic stem cell transplantation improves the high incidence of neutralizing allo-antibodies observed in MPSI-Hurler after pharmacological enzyme replacement therapy
4.1. Introduction

Mucopolysaccharidosis type I (MPS I) is caused by deficiency of α-L-iduronidase (IDUA). This results in intracellular accumulation of dermatan sulphate (DS) and heparan sulphate (HS) and a progressive, multisystem clinical disorder. Recombinant human ERT for LSDs first became available in the 1990s (Barton, Brady et al. 1991) and is currently in clinical use for six LSDs at considerable financial cost (Connock, Juarez-Garcia et al. 2006). ERT is delivered into the enzyme deficient cells via M6P receptors. In order to effectively clear the accumulated substrate inside the cells, the ERT has to be metabolically active and penetrate the cells to reach the lysosomal compartment.

An allo-immune response is widely reported to ERT and is summarized in table 1.4 (Ponder 2008). This phenomenon is not unique to ERT and antibodies are reported in response to a number of infused recombinant human proteins. (Porter 2001; Clarke, Wraith et al. 2009). Early data did not support the functional nature and significant clinical impact of antibodies in MPSI (Kakkis, Muenzer et al. 2001) but subsequently a prospective, open-labelled, multinational study revealed a suboptimal biomarker response in the presence of high antibody titres (> 1:10,000) when compared to patients with no allo-immune response (Wraith, Beck et al. 2007). More recent studies in canine models of MPSI show a high level of enzyme uptake inhibition by anti IDUA antibodies (Dickson, Peinovich et al. 2008). There is increasing evidence, mostly in animal models of the LSDs, of an inverse correlation between an observed antibody response and metabolic and clinical outcome. Even though the immune response currently reported in patients with MPSI is 91% (Brooks, Kakavanos et al. 2003), the true incidence of functionally active (neutralizing) antibodies is unknown.

The consequences of persistent, functional allo-immune response can be serious and range from reduced efficacy of ERT to catastrophic, rapid progression of disease and high mortality (Shull, Kakkis et al. 1994; Wraith, Beck et al. 2007; de Vries, van der Beek et al. 2010; Kishnani, Goldenberg et al.
A number of immune tolerance induction regiments have been reported in illnesses such as Haemophilia and MPSI (Kakkis, Lester et al. 2004). Allogeneic HSCT can replace the enzyme naïve immune system with that of the donor, thereby tolerizing the individual to the replaced enzyme. The donor immune system is naturally tolerized to this protein the donor possesses normal levels of this enzyme.

In children with severe MPSI (MPSI H, Hurler Syndrome) the standard of care is allogeneic HSCT which is superior to ERT due to its ability to deliver enzyme into the CNS. Pharmacological ERT is ineffective in neurocognitive disorders and does not prevent neurological disease. In our centre, we deliver ERT to patients with MPSI H prior to HSCT to improve the somatic (including cardiac) manifestations of the disease and optimize the clinical status before potentially toxic transplant conditioning (Wynn, Mercer et al. 2009). This enabled us to collect blood samples from the recipients of MPSIH before and during the ERT and HSCT.

We studied the incidence, pattern and impact of immune response in MPSIH patients prior to receiving any treatment and following ERT and HSCT. We also determined the relationship between these antibody responses and metabolic biomarkers of the disease.

### 4.2. Patient demographics

The patients were categorized into two groups. One group included patients who were followed longitudinally and comprised patients who received ERT prior to HSCT (n=8). The second was a cross sectional group (n=20) of patients all of whom were at least one year post HSCT. All patients in these two groups had severe disease phenotype (MPSI H, Hurler Syndrome). One patient (patient 5) rejected the donor cells following HSCT and had autologous cells returned to restore hematological function. This patient subsequently received a successful second allogeneic HSCT. Longitudinal data for this patient during both transplants are included in the results. Patient demographics, enzyme levels,
details of HSCT and immunosuppression for all patients in longitudinal series and cross sectional series are described in table 4.1 and table 4.2 respectively.

<table>
<thead>
<tr>
<th>No</th>
<th>Gender</th>
<th>Genotype</th>
<th>Age at HSCT (months)</th>
<th>Age at ERT (months)</th>
<th>Duration of ERT (days)</th>
<th>Type of HSCT</th>
<th>Source of stem cells</th>
<th>Conditioning</th>
<th>GVHD Prophylaxis</th>
<th>Enzyme level at diagnosis</th>
<th>Latest Enzyme post HSCT</th>
<th>Donor engraftment (VNTR)</th>
<th>Complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>W402X/ W402X</td>
<td>6.9</td>
<td>4.2</td>
<td>83</td>
<td>MUD</td>
<td>CB</td>
<td>Bu, Flu, ATG</td>
<td>CSA, Pred</td>
<td>0.22</td>
<td>55.5</td>
<td>100%</td>
<td>Autoimmune haemolysis; Rhinovirus pneumonia</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>W402X/ W402X</td>
<td>11.5</td>
<td>9.2</td>
<td>64</td>
<td>MUD</td>
<td>CB</td>
<td>Bu, Cy, ATG</td>
<td>CSA, Pred</td>
<td>0.22</td>
<td>27.6</td>
<td>100%</td>
<td>VOD (late); skin GVHD; renal tubular acidosis secondary to CSA</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>W402X/ W402X</td>
<td>12.2</td>
<td>8.5</td>
<td>96</td>
<td>MUD</td>
<td>PBSC</td>
<td>Bu, Cy, Campath</td>
<td>CSA, MTX</td>
<td>0.14</td>
<td>49.9</td>
<td>100%</td>
<td>RSV pneumonitis; Pneumothoraces</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>Q70X/ W402X</td>
<td>8.3</td>
<td>4.5</td>
<td>114</td>
<td>MUD</td>
<td>BM</td>
<td>Bu, Cy, Campath</td>
<td>CSA</td>
<td>0.31</td>
<td>15.7</td>
<td>98%</td>
<td>Cy-induced acute cardiomyopathy</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>Q70X/ Q70X</td>
<td>7.5</td>
<td>5.3</td>
<td>87</td>
<td>MUD</td>
<td>CB</td>
<td>Bu, Cy, ATG</td>
<td>CSA, Pred</td>
<td>0.2</td>
<td>38.3</td>
<td>99%</td>
<td>Adenovirus; Gastrointestinal &amp; skin GVHD; Pneumonia; Graft failure</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>R628X/ R628X</td>
<td>11.5</td>
<td>8.1</td>
<td>112</td>
<td>Sibling</td>
<td>BM</td>
<td>Bu, Cy, Campath</td>
<td>CSA</td>
<td>0.05</td>
<td>33.8</td>
<td>100%</td>
<td>VOD, CMV reactivation</td>
</tr>
<tr>
<td>7</td>
<td>Female</td>
<td>W402X/Un-known</td>
<td>13.2</td>
<td>10.2</td>
<td>113</td>
<td>MUD</td>
<td>CB</td>
<td>Bu, Cy, ATG</td>
<td>CSA, Pred</td>
<td>0.09</td>
<td>17.1</td>
<td>100%</td>
<td>Norovirus gastroenteritis</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>R628X/ R628X</td>
<td>5.0</td>
<td>0.9</td>
<td>124</td>
<td>Sibling</td>
<td>BM</td>
<td>Bu, Flu, Campath</td>
<td>CSA, MTX</td>
<td>0.06</td>
<td>20.1</td>
<td>70%</td>
<td>VOD, adenovirus infection</td>
</tr>
</tbody>
</table>

Table 4.1 Patient demographics (longitudinal patient series)

MUD; Matched unrelated donor, CB; Cord blood, BM; Bone marrow, PBSC; Peripheral blood stem cell, CSA; Ciclosporine A, Pred; Prednisolone, VNTR; Variable number tandem repeats, VOD; Veno occlusive disease, GVHD; Graft versus host disease, Bu; Busulfan, Cy; Cyclophosphamide, ATG; Antithymocyte globulin, RSV; Respiratory syncytial virus, Flu; Fludarabine, MTX; Methotrexate.
<table>
<thead>
<tr>
<th>No.</th>
<th>Gender</th>
<th>Type of HSCT</th>
<th>Age at transplant (months)</th>
<th>Source of stem cells</th>
<th>Conditioning</th>
<th>Immunosuppression</th>
<th>Duration of immune suppression (days)</th>
<th>Duration of ERT before HSCT</th>
<th>IgG Antibody</th>
<th>Donor engraftment (VNTR)</th>
<th>Complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>MUD</td>
<td>14.4</td>
<td>PBSC</td>
<td>Bu, Cy, Campath</td>
<td>CSA, Pred</td>
<td>154</td>
<td>120</td>
<td>Negative</td>
<td>100%</td>
<td>GVHD (grade I), idiopathic pneumonitis, CMV reactivation</td>
</tr>
<tr>
<td>2</td>
<td>Female</td>
<td>MUD</td>
<td>11.0</td>
<td>CB</td>
<td>Bu, Cy, ATG</td>
<td>CSA, Pred</td>
<td>126</td>
<td>90</td>
<td>Negative</td>
<td>100%</td>
<td>GVHD (grade I)</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>MUD</td>
<td>10.7</td>
<td>PBSC</td>
<td>Bu, Cy, Campath</td>
<td>CSA, MMF</td>
<td>122</td>
<td>72</td>
<td>Negative</td>
<td>100%</td>
<td>Chronic skin GVHD (grade I)</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>MUD</td>
<td>15.4</td>
<td>CB</td>
<td>Bu, Cy, ATG</td>
<td>CSA, Pred</td>
<td>270</td>
<td>110</td>
<td>Negative</td>
<td>100%</td>
<td>EBV reactivation</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>Sibling</td>
<td>11.0</td>
<td>BM</td>
<td>Treo,flu</td>
<td>CSA, MTX</td>
<td>90</td>
<td>150</td>
<td>Negative</td>
<td>100%</td>
<td>No significant problems</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>Sibling</td>
<td>17.7</td>
<td>BM</td>
<td>Bu, Cy</td>
<td>CSA, MTX</td>
<td>105</td>
<td>90</td>
<td>Positive</td>
<td>100%</td>
<td>No significant problems</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>MUD</td>
<td>21.5</td>
<td>CB</td>
<td>Bu, Fly, ATG</td>
<td>CSA</td>
<td>122</td>
<td>95</td>
<td>Negative</td>
<td>NA</td>
<td>No significant problems</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>MUD</td>
<td>25.4</td>
<td>CB</td>
<td>Bu, Cy, ATG</td>
<td>CSA</td>
<td>229</td>
<td>240</td>
<td>Negative</td>
<td>100%</td>
<td>EBV reactivation, Neutropenic sepsis, Cardiomyopathy</td>
</tr>
<tr>
<td>9</td>
<td>Female</td>
<td>MUD</td>
<td>15.3</td>
<td>BM</td>
<td>Cy,Bu,Campath</td>
<td>CSA, Pred</td>
<td>150</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>100%</td>
<td>GVHD, Sepsis, cardiac arrest</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>Sibling</td>
<td>7.5</td>
<td>BM</td>
<td>C,Bu</td>
<td>CSA</td>
<td>210</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>100%</td>
<td>No significant problems</td>
</tr>
<tr>
<td>11</td>
<td>Male</td>
<td>Sibling</td>
<td>31.3</td>
<td>BM</td>
<td>Bu, Cy</td>
<td>CSA, MTX</td>
<td>115</td>
<td>No prior ERT</td>
<td>Weak positive (1:128)</td>
<td>100%</td>
<td>No significant problems</td>
</tr>
<tr>
<td>12</td>
<td>Male</td>
<td>Sibling</td>
<td>10.8</td>
<td>BM</td>
<td>Bu, Cy, ATG</td>
<td>CSA, Pred</td>
<td>140</td>
<td>No prior ERT</td>
<td>Weak positive (1:64)</td>
<td>90%</td>
<td>Neutropenic sepsis</td>
</tr>
<tr>
<td>13</td>
<td>Male</td>
<td>Sibling</td>
<td>15.2</td>
<td>BM</td>
<td>Bu, Cy, ATG</td>
<td>CSA</td>
<td>188</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>90%</td>
<td>GVHD skin (grade I), Neutropenic sepsis</td>
</tr>
<tr>
<td>14</td>
<td>Male</td>
<td>Unrelated</td>
<td>16.6</td>
<td>CB</td>
<td>Cy, Melphalan, ATG</td>
<td>CSA</td>
<td>94</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>45%</td>
<td>PTLPD, neutropenic sepsis</td>
</tr>
<tr>
<td>15</td>
<td>Male</td>
<td>MUD</td>
<td>18.8</td>
<td>BM</td>
<td>Cy, Melphalan, ATG</td>
<td>CSA</td>
<td>85</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>100%</td>
<td>CMV reactivation, Neutropenic sepsis, EBV reactivation , PTLPD</td>
</tr>
<tr>
<td>16</td>
<td>Male</td>
<td>Sibling</td>
<td>11.8</td>
<td>BM</td>
<td>Bu, Cy, ATG</td>
<td>CSA</td>
<td>154</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>70%</td>
<td>Neutropenic sepsis, Gram negative sepsis</td>
</tr>
<tr>
<td>17</td>
<td>Female</td>
<td>MUD</td>
<td>22.7</td>
<td>BM</td>
<td>Cy,Bu,ATG,Flu</td>
<td>CSA</td>
<td>186</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>100%</td>
<td>No significant problems</td>
</tr>
<tr>
<td>18</td>
<td>Female</td>
<td>Sibling</td>
<td>13.4</td>
<td>BM</td>
<td>Cy,Bu,ATG,Flu</td>
<td>CSA, Pred</td>
<td>330</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>100%</td>
<td>EBV reactivation, neutropenic sepsis, chronic limited skin GVHD</td>
</tr>
<tr>
<td>19</td>
<td>Male</td>
<td>MUD</td>
<td>15.0</td>
<td>BM</td>
<td>Bu, Cy, Campath</td>
<td>CSA</td>
<td>NA</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>NA</td>
<td>No significant problems</td>
</tr>
<tr>
<td>20</td>
<td>Male</td>
<td>MUD</td>
<td>14.9</td>
<td>PBSC</td>
<td>Bu, Cy, Campath</td>
<td>CSA</td>
<td>243</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>100%</td>
<td>GVHD (grade I), Gram negative sepsis, EBV reactivation</td>
</tr>
</tbody>
</table>

Table 4.2: Patient demographics (Cross sectional patient series)
MUD; Matched unrelated donor, CB; Cord blood, BM; Bone marrow, PBSC; Peripheral blood stem cell, CSA; Cyclosporine A, Pred; Prednisolone, VNTR; Variable number tandem repeats, VOD; Veno occlusive disease, GVHD; Graft versus host disease, EBV; Ebstein-barr virus, CMV; Cytomegalovirus, Bu; Busulphan, PTLPD; Post transplant lymphoproliferative disorder, Cy; Cyclophosphamide, ATG; Antithymocyte globulin, Flu; Fludarabine, MTX; Methotrexate.
4.3. Pattern of immune response

4.3.1. ELISA

In the longitudinal patient series, high titres of IDUA reactive antibodies were seen in 87.5% (n=7) patients during IgG ELISA. Figure 4.1 shows the immune response after first exposure to ERT up until HSCT in these patients. One patient (patient 8) demonstrated detectable IgG antibodies only at low titre (1:256). These patients were followed up for a median period of 216 days (range 155-685). The median time from first ERT exposure to a first positive IgG antibody result was 38.5 days and the peak response 55.5 days (range 17-129). Antibody titres in seven patients (immediate post-transplant data not available for patient 6 in this group) who received HSCT had dropped to less than 1:10,000 by a median time of 101 days (range 26-137) despite having a normal level of innate IDUA. Summary of immune response in all these patients is shown in table 4.3.

![Figure 4.1 Immune response (Pre-HSCT) in ERT treated MPSI patients](image)

**Figure 4.1** Immune response (Pre-HSCT) in ERT treated MPSI patients

Longitudinal data describing the immune response in 8 patients (1-8) in longitudinal series from start of ERT to just before HSCT is shown baselined to the time of first starting ERT. All patients raise antibody responses to enzyme.
Median time to first positive ELISA test | 38.5 days (range 14-129)
---|---
Median time to highest immune response | 55.5 days (range 17-129)
Median time to immune tolerance | 101 days (range 26-137)
Incidence of high titre antibodies | 87.5% (7/8)
Incidence of catalytic inhibition | 62.5 (5/8)
Incidence of cellular uptake inhibition | 75% (6/8)
Immune tolerance 1 Year post HSCT | 100% (28/28)

**Table 4.3. Summary of immune response in longitudinal patient series.**
ELISA results from all patients over the entire follow up period are shown in figure 4.2 base lined to the first HSCT. None of the patients had detectable antibodies on the most recent ELISAs performed a year or more after the HSCT. It is interesting to note that Patient 5 in this series had a primary graft rejection with autologous hematological recovery after infusion of stored autologous cells. This resulted in escalation of immune response following subsequent exposure to IDUA which was later eradicated by a second unrelated donor transplant.

Analysis of patient sera in cross sectional group (n=20) showed that 85% (n=17) patients, tested one year or more after HSCT had no detectable antibodies despite normal IDUA levels. ERT was given to 8 patients prior to HSCT. Three patients in this group had weakly positive IgG antibody response to IDUA (<1:250). Chimerism data was not available for two patients in this group. The majority of patients (n=14, 78%) had achieved full donor engraftment whilst 22% (n=4) were chimeric with donor leukocyte engraftment of 45%, 70%, 90% and 90% respectively. Median duration of immune suppression for Graft versus host disease (GVHD) prophylaxis after HSCT in cross sectional and longitudinal patient series was 150 days (range 90-330) and 169 days (range 110-250) respectively (see table 5 and 6).
Figure 4.2 Immune response (Pre & post HSCT) in ERT treated MPS I patients

Longitudinal data describing the immune response in 8 patients (1-8) in longitudinal series is shown baselined to the time of first HSCT. Antibody titre is presented on primary vertical axis in logarithmic scale. Note a rapid decline in antibody titre after HSCT (following approximately 3 months of ERT). Patient 5 rejected his first graft which was followed by an escalation of antibody titres. This immune response resolved after second transplant on day 328.
4.4. Enzyme neutralization by antibodies

Catalytic inhibition

Figures 4.3 describes the catalytic inhibition in all patients in longitudinal series (direct enzyme inhibition and inhibition compared to a standard). In this series 62.5% of patients demonstrated catalytic inhibition. The inhibition was more pronounced at lower enzyme concentrations. Three patients (patient 4, 5 and 8) did not show enzyme inhibition at any concentration whereas another 2 patients (patients 7 and 3) showed over 75% inhibition of enzyme activity at concentrations of less than 1ng/ml. Direct inhibition of enzyme at higher concentrations (30ng/ml or higher) ranged from 10-35% but inhibition in comparison to a standard (Normal serum) was lower which is shown in figure 4.4. To confirm that the inhibition in patient serum was due to the antibodies, we assessed the enzyme inhibition in the same patient before and after HSCT i.e. specimen with the highest titre antibodies and no detectable antibodies on ELISA. The data confirmed that there was no catalytic inhibition in the absence of antibodies (figure 4.5).
Figure 4.3  Catalytic enzyme inhibition in MPSIH patients

Figure 4.4  Catalytic enzyme inhibition (Aldurazyme) in eight patients (1-8) and total inhibition in comparison to a standard (normal serum) across various enzyme concentrations.
Figure 4.5 Percentage inhibition in tolerized and non-tolerized serum

Inhibition due to tolerized and non-tolerized serum. Non-tolerized serum taken from a patient (patient 3) on ERT before HSCT with high titre antibodies is compared to tolerized serum taken from the same patient a year after HSCT (with no antibodies) which shows no significant inhibition.
4.4.1. Inhibition of cellular uptake of catalytically active enzyme

Cellular uptake inhibition was also assessed in the samples with the highest antibody titre for each patient in the longitudinal series. Inhibition of enzyme uptake by MPSI fibroblasts was seen in 75% (n=6) patients in this series. Figure 4.6 shows the uptake inhibition assay on these 8 patients. Two patients (patient 6 and 8) did not show any inhibition. It is interesting to note that one of these patients (patient 6) had shown some enzyme inhibition on catalytic inhibition assays. In contrast the two patients (patients 4 and 5) who did not show any evidence of catalytic inhibition showed 25% and 76% inhibition of enzyme uptake respectively. Patients 2 and 7 who showed over 75% inhibition of enzyme activity also demonstrated a high level of cellular uptake inhibition (over 70%). There was no correlation between the catalytic inhibition and cellular uptake inhibition in the rest of the patients suggesting a polyclonal nature of antibodies. The cellular uptake inhibition was repeated in non-tolerized (patient on ERT pre transplant and high titre immune response) and tolerized (post HSCT with no antibodies) specimens from the same patient to confirm that the inhibition was due to the antibodies in the serum (figure 4.7).
Figure 4.6  Cellular uptake inhibition in MPSIH patients

Cellular uptake inhibition in all patients (1-8) in longitudinal series. Patients 6 and 8 show no cellular uptake inhibition.

Figure 4.7  Cellular uptake inhibition in tolerized and non-tolerized MPSIH patients

Tolerized serum (post-transplant with no antibodies) shows no inhibition compared to significant inhibition by non-tolerized serum (prior to transplant) in the same patient.
4.5. Immune response and disease biomarkers

Longitudinal biomarker data (DS/CS ratio) were available for six patients in this series. Figure 7 describes the relationship between antibody titres and DS/CS ratio over the follow up period. Patient 2 showed an immediate improvement in the DS/CS ratio after starting ERT. This improvement in biomarkers was halted by a high titre immune response. This pattern continued even after the HSCT was performed and further improvement in DS/CS ratio resumed only after the immune response was completely abrogated. DS/CS ratio in patient 3 stayed at a high level despite HSCT, with a continuing high titre immune response. The slight improvement in DS/CS ratio seen in this patient was suboptimal and could be considered as a therapeutic failure at this stage. DS/CS ratio after resolution of immune response is not yet available for this patient. The improvement in biomarker levels in patient 4 was significant after ERT was commenced; however, this progress was halted and partially reversed after a high titre immune response was generated. The subsequent improvement in DS/CS ratio followed the resolution of immune response. Patient 5 rejected his first HSCT and had a second successful transplant. His ERT was interrupted during this period. The initial improvement in biomarker levels plateaued during the high titre immune response despite restarting ERT. After a second HSCT, biomarker levels showed some deterioration despite ERT and HSCT which improved after the immune response was completely resolved. Patient 6 showed a suboptimal biomarker response to ERT during the high titre immune response. The DS/CS ratio in patient 7 dropped significantly after ERT but further follow up data was not available during the immune response and following the HSCT.
Figures 4.8-4.13  Correlation between biomarkers (DS/CS ratio) and the antibody titres in six patients in longitudinal series (patients 2-7).
The shaded areas depict the period of ERT and black arrows point to the time of Stem Cell Transplant. 4.8) Patient 2 shows an immediate improvement in the DS/CS ratio after starting ERT which is halted by high titre anti IDUA antibodies. The biomarker levels
improve after the immune response is abrogated. 4.9) Patient 3 shows minimal improvement in biomarkers despite HSCT and ERT during high titre immune response. 4.10) The improvement in biomarker levels in patient 4 is significant after ERT is commenced; however, this progress is arrested and reversed after the high titre immune response is generated. The biomarkers improve after resolution of immune response. 4.11) Patient 5 rejected his first HSCT and had a second successful transplant. The initial improvement in biomarker levels plateau during the high titre immune response despite ERT (note a brief period when ERT was interrupted after first failed HSCT) which improves only after the immune response is completely resolved after second HSCT. 4.12) Patient 6 shows suboptimal response to ERT during the high titre immune response. 4.13) DS/CS ratio in patient 7 drops to a significant level but further follow up data is not available during the immune response and following the HSCT. Biomarker data is not available for patients 1 and 8.
4.6. Discussion

The immune response to replacement recombinant human proteins is widely reported (Porter 2001). However the clinical impact of antibodies ranges from no significant effect (Spijker, Poortman et al. 1982) to almost complete failure of therapy (Yoshioka, Fukutake et al. 2003). In clinical disorders, such as Hemophilia A and B, the immune response (inhibitory antibodies) can make the replacement therapy (recombinant human factor VIII and IX) ineffective in some cases. However availability of robust functional assays (Bethesda assays) makes it easier to monitor the immune response and its impact in these patients. In contrast, monitoring immune response in LSDs, is considerably more challenging due to a lack of sensitive and standardised immune assays. This is further complicated by the marked phenotypic heterogeneity that exists in these disorders. It has been proposed that the allo-antibodies can neutralize the infused enzyme by a number of mechanisms including altered enzyme distribution, altered intracellular enzyme trafficking, increased enzyme turnover and altered pharmacokinetic profile of infused enzyme (Brooks 1999). The polyclonal nature of the IgG antibodies makes it imperative to develop multiple functional assays to encompass the full range of effects due to antibodies (Mire-Sluis, Barrett et al. 2004). Despite recent recognition of significance of antibodies, no clinically accredited laboratories are performing functional immune assays and hence the incidence and impact of these allo-antibodies remains unknown.

Due to the difficulty in delivering ERT in some LSD patients with allergic immune response and recognition of functional nature of antibodies, (de Vries, van der Beek et al. 2010; Kishnani, Goldenberg et al. 2010) a number of immune tolerance induction strategies were evaluated. These strategies had variable outcomes and serious side effects were reported in some cases (Brady, Murray et al. 1997; Hunley, Corzo et al. 2004; Kakkis, Lester et al. 2004; Joseph, Munroe et al. 2008). Intensification of immunomodulatory therapies by using a combination of chemotherapeutic agents and
immunosuppressants could enhance the efficacy of these regimens but this may result in a significantly increased morbidity as was seen in Haemophiliac disorders treated with complex induction regimens (Berntorp, Astermark et al. 2000; Kreuz, Ettingshausen et al. 2003). HSCT has been used as a mean to develop immune tolerance and control the symptoms in severe autoimmune diseases. However, this modality has almost exclusively been used in autoimmune disorders (Sullivan, Muraro et al. 2010). Manipulated graft, using reduced toxicity conditioning can achieve immune tolerance induction in some recipients of solid organ transplant (Kawai, Cosimi et al. 2008; Scandling, Busque et al. 2008). However, despite all these developments and reduction in transplant related mortality, HSCT as an immune tolerance induction mechanism has not been used in allo-immune disorders.

This cohort included only MPSIH patients which is a severe phenotype. These patients are expected to develop a higher titre immune response when compared to more attenuated forms including MPSIHS and MPSIS. Seven patients were homozygous for null mutation which confers a high risk of developing allo-immune responses. Our results revealed a high incidence of antibodies in this subgroup consistent with previous reports. All transplant recipients, in the longitudinal series, cleared their antibodies to clinically insignificant levels within a median period of 101 days despite normal enzyme levels. None of the 20 patients in the cross sectional group had a high titre immune response, one year or more after HSCT and cessation of immunosuppression. Full donor engraftment was not required for immune tolerance as some patients were tolerized despite achieving only mixed chimerism. These data confirm allogeneic HSCT as an effective and quick immune tolerance induction mechanism.

The majority of patients (62.5%) demonstrated evidence of some catalytic inhibition by antibodies. Despite weak catalytic inhibition at high enzyme concentration, this may reflect partial neutralization of infused enzyme due to a
very short half-life of Aldurazyme (3 hours in the absence of antibodies) and a mean maximum plasma concentration ($C_{\text{max}}$) of only 1.2-1.7μg/ml post infusion [ALID-014-02: A phase II Open-Label Clinical Trial of Aldurazyme]. In previous experiments, cross reactivity of these allo-antibodies was seen between exogenous recombinant ERT and innate IDUA. These data show that the antibodies generated in response to infused ERT can potentially neutralize the innate enzyme produced by cellular ERT (allogeneic HSCT). This signifies a need for HSCT induced immune tolerance.

In contrast to catalytic enzyme inhibition, cellular uptake inhibition experiments were undertaken at a much higher enzyme concentration. Enzyme concentration of less than $\frac{1}{2}$ Km (100 ng/ml) and serum dilution of 1:100 were selected during the optimisation experiments. Corrected for dilution, it will equate to just over 6 times the $C_{\text{max}}$ (maximum plasma concentration post infusion) of Aldurazyme. This shows that the treatment becomes virtually ineffective in some patients with high titre immune response. The cellular uptake inhibition at a higher enzyme concentration compared to catalytic inhibition may reflect a stronger inhibition of the M6P binding sites (and enzyme uptake) than the catalytic site of Aldurazyme by anti IDUA antibodies.

Analysis of biomarkers data and antibody titres show that the recovery is slowed or in some cases reversed, in the presence of a high titre immune response. Generally there appears to be a close relationship between the two, although DS/CS ratio and biomarker responses lagged behind antibody responses usually by several days, confirming the in vivo neutralization of ERT by antibodies.

These data show a high incidence of high titre functional immune response in MPSIH patients. A close correlation between the biomarkers of disease and antibody titres, confirm the in vivo effect of functionally active allo-antibodies.
These data also confirm allogeneic HSCT as an effective and quick immune tolerance induction strategy.
5. The incidence and functional nature of allo-antibodies in MPSI patients on long term ERT
5.1. Introduction

Even though a high incidence of allo-immune response was reported in early clinical trials in some LSDs, it was proposed that this response was transient in nature due to seroconversion in some patients. A large multi-centre trial on MPSI patients showed that despite generation of immune response in 90% of patients on ERT, the majority of patients had cleared the immune response within 6 months of treatment (Wraith, Clarke et al. 2004). A lack of sensitive and widely available functional immune assays and an absence of clear correlation between the presence of antibodies and clinical outcome increased the uncertainty surrounding the significance of antibodies, particularly in the long term recipients of ERT. However, persistent allergic reactions including anaphylaxis were reported in patients receiving ERT on a long term basis (Lipinski, Lipinski et al. 2009) suggesting that at least in some patients, refractory immune response to delivered ERT persisted beyond 6 months and remained problematic. Allergic reactions are mostly associated with IgE antibodies whilst functional antibodies usually belong to IgG and IgM classes. Nonetheless, generation and persistence of IgE antibodies, over a prolonged period of time is a reflection of a dysregulated immune system and strongly points towards a propensity in these individuals to develop persistent immune response involving other antibody classes. It is important to remember that unlike IgE mediated immune response which manifests with obvious clinical features, allo-immune response involving other antibodies may remain completely asymptomatic. Demonstration of the functionally active nature of these antibodies means that the immune response can lead to neutralization of delivered therapy even in relatively asymptomatic patients resulting in clinical deterioration over a period of time (Banugaria, Patel et al. 2012). Neutralizing allo-antibodies in long term recipients of ERT may have several implications and hence it is critical to assess the immune response in patients receiving long term ERT.
To address the question of whether functional allo-immune response persists in long term recipients of ERT in MPSI, quantitative and functional immune assays were performed in patients receiving ERT.

5.2. Patient demographics

Blood samples were collected from seventeen patients on long term Aldurazyme therapy. Longitudinal data was available for 5 patients and 12 had a single blood sample, taken at least 2 years after the start of treatment. The median age at the start of ERT in this cohort was 4 years. The patients included severe (n=2) and attenuated form of MPSI (n=15). Patient demographics and genotypes are described in table 5.1.

<table>
<thead>
<tr>
<th>No.</th>
<th>Age at start of ERT (years)</th>
<th>Sex</th>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>Male</td>
<td>L490P/L490P</td>
<td>MPSI HS</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>Female</td>
<td>C122del/unknown</td>
<td>MPSI HS</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Male</td>
<td>Q380R/T388R</td>
<td>MPSI HS</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>Male</td>
<td>L490P/L490P</td>
<td>MPSI HS</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>Female</td>
<td>Q380R/ Q380R</td>
<td>MPSI HS</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>Male</td>
<td>P533R/P533R</td>
<td>MPSI HS</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>Male</td>
<td>NA</td>
<td>MPS I H</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>Male</td>
<td>A36E/Q70X</td>
<td>MPSI HS</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>Female</td>
<td>L490P/L490P</td>
<td>MPSI HS</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>Female</td>
<td>L490P/L490P</td>
<td>MPSI HS</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>Male</td>
<td>L490P/L490P</td>
<td>MPSI HS</td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>Female</td>
<td>p.R628X/ p.R628X</td>
<td>MPSI HS</td>
</tr>
<tr>
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<td>1</td>
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<td>W402X/W402X</td>
<td>MPSI H</td>
</tr>
<tr>
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<td>5</td>
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<td>L490P/L490P</td>
<td>MPSI HS</td>
</tr>
<tr>
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<td>4</td>
<td>Male</td>
<td>p.Gln380Arg/p.Thr388Arg</td>
<td>MPSI HS</td>
</tr>
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<td>MPSI HS</td>
</tr>
<tr>
<td>17</td>
<td>0.5</td>
<td>Female</td>
<td>L490P/L490P</td>
<td>MPSI HS</td>
</tr>
</tbody>
</table>

Table 5.1 MPSI patient demographics (on long term ERT)
5.3. **Allo-antibodies persist in some patients on long term ERT**

The majority of patients (13/17) on long term ERT had IgG antibodies to Aldurazyme. Seven patients had antibody titre of above 1:1000 and five were positive at the highest limit of the assay. Median antibody titre at the time of last assessment was 1:2048 in those who were positive. In longitudinal series median time to immune response was 35 days and median time to maximum immune response was 90 days. The patients in this series were followed for a median period of 251 Days. All patients in longitudinal data were positive at the time of last assessment as shown in figure 5.1. Two of the five patients in longitudinal series had rising antibody titres at the time of last assessment. One patient (patient 12) had an increase in antibody titre despite plateauing at a level of 1:32000 approximately six months after starting the ERT. The summary of immune response in all patients is shown in table 5.2.
<table>
<thead>
<tr>
<th></th>
<th>Time</th>
<th>Antibody titre</th>
<th>Catalytic inhibition</th>
<th>Uptake inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;2 years</td>
<td>Negative</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>&gt;2 years</td>
<td>256</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>&gt;2 years</td>
<td>256</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>&gt;2 years</td>
<td>4096</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>&gt;2 years</td>
<td>64</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>&gt;2 years</td>
<td>Negative</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>7</td>
<td>&gt;2 years</td>
<td>65500</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>8</td>
<td>&gt;2 years</td>
<td>320000</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>9</td>
<td>&gt;2 years</td>
<td>512</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>&gt;2 years</td>
<td>Negative</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>11</td>
<td>&gt;2 years</td>
<td>Negative</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

| 12 | 0 day | Negative | Yes | Yes |
|    | 21 day | Negative |     |     |
|    | 35 day | 32792   |     |     |
|    | 60 day | 16336   |     |     |
|    | 83 day | 32792   |     |     |
|    | 170 day | 32792  |     |     |
|    | 600 day | 262336 |     |     |
|13 | 180 day | 4096   |     |     |
|    | 210 days | 4096  |     |     |

|14 | 0 day | Negative | Yes | No |
|    | 16 day | Negative |     |     |
|    | 30 day | 262336  |     |     |
|    | 40 day | 262336  |     |     |

|15 | 90 day | 32768   | Yes | Yes |
|    | 120 day | 32768  |     |     |
|    | 251 day | 2048   |     |     |

|16 | >2 years | 131000 | No | Yes |

|17 | 0 day | Negative | Yes | No |
|    | 21 day | 1024    |     |     |

**Table 5.2 The summary of the immune response in MPSI patients (on long term ERT)**
Figure 5.1 Immune response in long term recipients of ERT (longitudinal data).

The figure shows immune response in five patients over a period of time who were treated with ERT only. Patient 12 had a moderate sustained immune response between 3-6 months after exposure to ERT which seems to have escalated on most recent assessment, nearly two years after commencement of treatment. Patients 14 and 15 seem to have a declining immune response which remains above a titre 1:1000. Patients 13 and 17 have a relatively short follow up period and pattern of immune response is not fully established yet.
5.4. Catalytic inhibition

Of those, who had IgG antibodies on ELISA (n=13), 9 patients showed evidence of direct catalytic inhibition (compared to no serum) ranging from 15-60%. A similar pattern of enzyme inhibition was seen when compared to a standard. Similar to the data described in chapter 4, these patients seemed to fall into three categories when catalytic inhibition was assessed in comparison to a standard at low enzyme concentrations as shown in figure 5.2. Four patients (patients 4, 8, 13 and 16) did not show any significant inhibition. Five patients (patients 7, 9, 12, 14 and 17) showed moderate catalytic inhibition ranging from 30-65% at low enzyme concentration (0.45ng/ml). All other patients lie in the intermediate group and appear to show an inhibition of around 20% when compared to a standard. Figure 5.3 shows direct inhibition of catalytic activity of enzyme by patient sera. Similar to the data in chapter 4, direct enzyme inhibition appeared slightly higher particularly at high enzyme concentrations and there were no distinct groups at low enzyme concentrations. All patients except patients 4, 8, 13 and 16 inhibited catalytic activity of enzyme.
Figure 5.2 Percentage catalytic enzyme inhibition compared to a standard (normal serum)

Figure 5.3 Percentage catalytic inhibition (direct) in all patients with positive immune response
5.5. Cellular uptake inhibition

Cellular uptake inhibition was assessed at a single enzyme concentration (93 ng/ml) as per established protocol. Six patients (35%) showed inhibition of cellular enzyme uptake (see figure 5.4). Five patients demonstrated an inhibition of over 60%. Three patients (patients 7, 8 and 16) in this group, inhibited the uptake of Aldurazyme to over 90%. Six patients (patient 2, 3, 4, 5, 14) showed no inhibition of enzyme uptake. All these patients except patient 4 had shown some inhibition of catalytic activity as described previously. All patients who inhibited the cellular enzyme uptake by over 90%, had generated a very high titre immune response (>1:100000) on ELISA.

Figure 5.4 Cellular uptake inhibition in all patients with positive immune response.
5.6. Correlation between ELISA and catalytic inhibition

Correlation between ELISA and catalytic inhibition was assessed by Pearson correlation coefficient using logarithmic values for both percentage catalytic inhibition and ELISA. This analysis showed a poor correlation between the two variables (correlation coefficient 0.18) as shown in figure 5.5.

Figure 5.5  Correlation between ELISA and cellular uptake inhibition
5.7. Correlation between ELISA and Cellular uptake inhibition

In contrast to catalytic inhibition, there appears to be a better correlation between ELISA and cellular uptake inhibition (correlation coefficient 0.49) as shown in figure 5.6. Patient 14 could be identified as an outlier in this analysis. Excluding this patient from analysis, results in a correlation coefficient value of 0.67 suggesting a strong correlation between the ELISA and cellular uptake inhibition. Spearman’s correlation coefficient value, for cellular uptake inhibition, is calculated to be 0.84 which is suggestive of a very strong correlation between these parameters.

![Correlation (ELISA and uptake inhibition)](image)

Figure 5.6 Correlation between ELISA and cellular uptake inhibition
5.8. Discussion

Allo-immune response is widely reported to a number of recombinant human proteins used in clinical practice. The severity, duration and functional nature of immune responses are variable in different clinical disorders. A brief, transient immune response may not have any clinical implications even if the antibodies produced are inhibitory. On the other hand, a weak functional immune response, which is sustained over a prolonged period of time, may cause significant clinical deterioration. This makes it imperative to perform sensitive detection assays followed by highly sensitive functional immune assays. However, if an assay of low sensitivity is used to monitor the patients, there is a risk that low titre immune response may not be detected despite potential neutralization of infused protein by low level antibodies. Similarly, a high titre immune response over a short period of time may not have any clinical consequences for a particular patient. It is, therefore, important to monitor the immune response regularly whilst patient is receiving ERT. The antibody titres should be correlated with biochemical markers and clinical response. As described previously, inferior metabolic response was reported in recipients of ERT with high titre immune response (Wraith, Beck et al. 2007). Wynn et al. in 2009 reported metabolic outcome of the recipients of ERT and HSCT. In the ERT group, outliers were reported who seemed to have suboptimal or minimal immune response whilst on ERT (Wynn, Wraith et al. 2009). Similarly, biomarker studies undertaken by our group showed unexpected fluctuations in patients' biomarker responses during ERT prior to HSCT (Langford-Smith, Mercer et al. 2011). Some of these fluctuations may be explained based on the generation of immune response which is functional in nature.

An important determinant of immune response, in recipients of recombinant proteins, is the presence of CRIM. The patients with attenuated forms of disease are less likely to have high titre immune response due to the presence of residual protein which tolerises patients to the infused recombinant protein.
However, our data show that the majority of patients on long term ERT developed immune responses regardless of their phenotype. In contrast to the patients in chapter 4, the majority of patients in this cohort had an attenuated phenotype. These data also show persistence of allo-immune response, beyond six months, in the majority of patients with MPSI HS (11/15) who were treated with ERT only. This makes it highly unlikely that the generation of immune response is merely due to seroconversion. Patient 12 in longitudinal series had persistent moderate immune response which escalated after a prolonged period of ERT infusion. This is an important observation and suggests that spontaneous tolerance does not always develop in patients with low or moderate levels of immune response and all the patients on long term ERT should have continuous monitoring of their immune response regardless of the severity.

The data in chapter 4 and 5 suggest that the catalytic inhibition of Aldurazyme does not depend upon the severity of immune response i.e. antibody titre. This response appears to be idiosyncratic and becomes more obvious at low enzyme concentrations. As explained in chapter 4, due to a low Cmax and short half-life of Aldurazyme, inhibition at low enzyme concentration may reflect significant inhibition of enzyme (in vivo) by circulating antibodies. However, it is important to remember that the catalytic inhibition of circulating enzyme, outside the cells, may not have any direct clinical consequences for the patient. The enzyme has to be delivered into the cells to become effective which makes cellular uptake inhibition more important. Nonetheless, the ability of antibodies to inhibit the enzyme activity is an important indicator of functional nature of these antibodies.

The data from cellular uptake inhibition assays show a high level of enzyme uptake inhibition in some patients. The majority of patients who showed a high level of enzyme uptake inhibition in this cohort and HSCT recipients (chapter 4) had shown a high titre immune response on ELISA, suggesting that a high level
of antibodies is required to inhibit the uptake of enzyme into the fibroblasts. This is in contrast to the catalytic inhibition assay, where the inhibition is more unpredictable, and severity of catalytic inhibition does not depend upon the level of antibodies in serum. This observation is supported by demonstration of a better correlation between cellular uptake inhibition and ELISA titre when compared to catalytic inhibition and ELISA titre (as measured by Pearson correlation coefficient shown in figures 5.5 and 5.6).

The persistence of high titre, functionally active antibodies in patients on long term ERT has several important implications. It highlights the significance of monitoring quantitative and functional immune response in all recipients of ERT regardless of their disease phenotype. The immune responders should then be closely monitored in terms of biomarker and clinical response. In some patients with attenuated disease type, neutralizing antibodies may make ERT ineffective and hence alternative strategies including immune tolerance induction should be considered. The presence of refractory immune response with deterioration in biomarker and clinical status may warrant different therapeutic approaches including HSCT. Continuation of a highly expensive therapy may not be appropriate in these patients.
6. Allo immune response in other Lysosomal Storage Disorders
6.1. Introduction

As described earlier, the problem of allo-immune response generation was recognised in all LSDs. However the incidence, pattern and functional impact of antibodies appear different in each one of these disorders. Most of the data on the functional nature of antibodies comes from the animal studies and the assays were performed in vitro (Brooks 1999). Unlike quantitative assays, the development of functional immune assays is a great challenge and a number of attempts have been made to develop reliable, robust and sensitive assays. The reported incidence of allo-immune response in Pompe disease and MPSVI is 89% and 97% respectively. In Pompe disease the presence of antibodies have been associated with poor clinical outcome (de Vries, van der Beek et al.; Abbott, Prater et al. 2011; Bali, Goldstein et al. 2012; Banugaria, Patel et al. 2012; Patel, Banugaria et al. 2012). Functionally active inhibitory antibodies have previously been reported in some published studies (de Vries, van der Beek et al.; Patel, Banugaria et al. 2012). However, in MPSVI patients there is generally a lack of compelling evidence about the inhibitory nature of allo-antibodies. White et al. described functional assays to measure the neutralization of enzyme by antibodies. Whilst enzyme neutralization in this study was based on mixing assays, the quantification of true enzyme uptake inhibition by patient fibroblasts was not performed (White, Argento Martell et al. 2008; White, Martell et al. 2008). The described assay used the antibody binding to the calcium-independent mannose-6-phosphate receptor (CIMPR) as a surrogate marker for the cellular enzyme uptake inhibition. This assay provides indirect evidence of cellular uptake inhibition but does not reflect the true uptake inhibition of enzyme by the antibodies. Similarly, there are shortcomings in the functional assays developed by pharmaceutical industry where normal human fibroblasts have been used instead of enzyme deficient patient fibroblasts. The above two approaches may result in overestimation and underestimation of enzyme inhibition respectively. A sensible approach towards developing these assays would be to apply conditions, which closely mimic the in vivo interaction of enzyme and antibodies with minimal manipulation of
patient samples and enzyme. As described previously, the allo-antibodies generated in response to infused ERT are polyclonal in nature which means that the same intensity of immune response (as measured by ELISA assay) in different LSDs may not have same degree of inhibition as assessed by functional assays. This could result in different clinical consequences for same level of immune response in different LSDs. Similarly, within a disease group, immune response in individuals with attenuated disease phenotype may not have same implication as those with severe phenotype. However, recent data suggest that in severe and attenuated forms of LSDs treated with ERT, the presence of antibodies may be equally deleterious and result in adverse clinical consequences (van der Ploeg, Clemens et al. 2010; Abbott, Prater et al. 2011; Banugaria, Prater et al. 2011).

As described in chapter 3, the assays to evaluate the immune response and its functional nature were developed and optimized for Pompe disease, MPS VI and MPSII. An ELISA was performed on all patient samples followed by quantification of catalytic enzyme activity inhibition and cellular uptake inhibition.

6.2. Pompe disease

6.2.1. ELISA

Six patients (10 samples) were tested for the presence of antibodies. Only two patients were found to have IgG antibodies reactive to Myozyme on ELISA assay. Patient 1 was positive at low titre (1:100) but became subsequently negative on a repeat sample six months later during the course of treatment with Myozyme. Patient 3 was found to have persistent high titre immune response (>1:40000), over six months after the start of ERT. All other patients were negative but it is important to note that some patients in this cohort had received prophylactic immune tolerance induction treatment prior to commencement of ERT. The specificity of ELISA was confirmed by western blot analysis as shown in the figure 6.1.
<table>
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<th>ELISA result</th>
<th>Age at start of ERT</th>
<th>CRIM status</th>
<th>Genotype</th>
<th>Clinical presentation</th>
<th>Immunodulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
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<td>4</td>
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<td></td>
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</tr>
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<tr>
<td>Patient 3</td>
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<td>327680</td>
<td>10</td>
<td>Positive</td>
<td>c.123IdelC/unknown</td>
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<tr>
<td></td>
<td>4.5 year</td>
<td>40960</td>
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<td>Patient 4</td>
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<td>Positive</td>
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<td>Infantile</td>
<td>No</td>
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<tr>
<td>Patient 5</td>
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<td>Negative</td>
<td>0.3</td>
<td>Negative</td>
<td>c.525delT/p.Glu176ArgtsX45</td>
<td>Infantile</td>
<td>Yes</td>
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<tr>
<td>Patient 6</td>
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<td>Positive</td>
<td>g.1683G&gt;T/ g.1683G&gt;T</td>
<td>Infantile</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 6.1 Patient demographics (Pompe disease)

**Figure 6.1 Western Blot analysis for Pompe disease**

A western blot analysis for a patient showing the specificity of ELISA for α-glucosidase alpha (Myozyme). Marker region shows the position of IDUA and Myozyme (MZM) bands based on their molecular weight (kDa) according to the ladder. After exposure to ERT, the IgG antibodies in patient serum only bind to MZM and not to IDUA.
6.2.2. Catalytic inhibition assay

Both patients with positive ELISA were tested for catalytic enzyme activity inhibition assay. Patient 1 showed moderate inhibition at low enzyme concentration and enzyme activity was completely blocked at a concentration of 100ng/ml Myozyme. Patient 3, however, showed a relatively low level of enzyme activity inhibition when compared to a standard but remained inhibitory throughout the range of concentrations of enzyme (see figures 6.2 and 6.3). Catalytic inhibition assay was repeated using two samples from the same patient. The sample with antibodies showed inhibition of enzyme activity but the tolerized sample did not show any significant inhibition of enzyme activity when compared to a standard suggesting that the inhibition in serum with antibodies is due to the allo-antibodies to Myozyme (figure 6.4).

Figure 6.2 & 6.3 Catalytic enzyme inhibition in patients with Pompe disease
Patient 1 showed complete inhibition of enzyme activity (direct and compared to a standard) at low enzyme concentration of 0.1Ug/ml. Patient 3 showed low level inhibition at all enzyme concentrations.
Figure 6.4 Specificity of Myozyme catalytic inhibition assay

Pompe disease patient serum with IgG antibodies shows catalytic enzyme inhibition at all enzyme concentrations but the same patient serum (spontaneously tolerized) with no antibodies does not show any inhibition at any enzyme concentration. Data was analysed by 2-way ANOVA using post-hoc Tukey’s test. *** represents P value ≤ 0.001. Error bars refer to standard deviation of the mean.
6.2.3. **Cellular uptake inhibition assay**

Cellular uptake inhibition was performed in all samples which showed IgG antibodies on ELISA. Patient 1 was negative and did not show any quantifiable inhibition of enzyme uptake. Both samples (sample A and B) from patient 3 showed inhibition of cellular uptake of Myozyme. Sample A (high antibody titre) showed above 40% inhibition of enzyme uptake at low (6.25μg/ml) and high (12.5μg/ml) Myozyme concentration whereas sample B (low antibody titre) showed lower uptake inhibition at high Myozyme concentration. The uptake inhibition by sample B increased to 35% when performed at low enzyme concentration (6.25μg/ml).

![Cellular uptake inhibition Pompe disease](image)

*Figure 6.5 Cellular uptake inhibition Pompe disease*
6.3. MPS VI

6.3.1. ELISA

All three patients tested for the presence of IgG antibodies against Nagalzyme showed high titre immune response. Longitudinal samples were available for all patients. Median time to development of immune response was 34 days. Median time to highest immune response was 133 days. The patients were followed for a median period of 503 days. All patients were positive at the time of last assessment, a year or longer after the start of ERT (see figure 6.6). Patient 1 had declining antibody levels at the time of last assessment whereas patients 2 and 3 had stable antibody levels as shown in the figure. Patient 1 developed very high titre immune response during the course of ERT treatment becoming positive at a serum dilution of over 1:250000 about four months after commencement of therapy. Patient 2 and 3 remained moderately positive during their treatment. The specificity of ELISA was confirmed by western blot analysis as shown in figure 6.7.
Immune response in ERT treated MPSVI patients

Figure 6.6 Immune response in patients with MPS VI

Western blot analysis (MPS VI)

Figure 6.7 Western blot analysis for Naglazyme

Western blot analysis shows binding of antibodies in patient serum to Naglazyme only. There is no antibody binding with Myozyme.
6.3.2. Catalytic inhibition assay

There was no direct inhibition of (Naglazyme) seen by any patient serum in this cohort. When compared to a standard, only mild reduction in enzyme activity was observed (about 10%) by patient 1 and 3 sera (figure 6.8 and 6.9). As there were no MPS VI patient samples without any IgG antibodies (all samples positive for ELISA), it was not possible to evaluate the specificity of this catalytic inhibition.

Figure 6.8
Figures 6.8 & 6.9 Catalytic inhibition of enzyme (direct) and against a standard.

Figure 6.9
6.3.3. Cellular uptake inhibition assay

All patients were tested to assess the inhibition of cellular uptake of enzyme at two different enzyme concentrations (160ng/ml and 1.25μg/ml). No inhibition of enzyme uptake was seen by any patient sera at both Naglazyme concentrations as shown in the figures 6.10 and 6.11.

Figure 6.10

Figure 6.11

Figures 6.10 & 6.11 MPS VI Cellular uptake inhibition assay at two different enzyme concentrations
6.3.4. Correlation between antibody titre and biomarker

Longitudinal biomarker data were available for only one of the three patients as shown in figure 6.12. A drop in DS/CS ratio was observed immediately after commencement of ERT. However, despite very high titre antibody response, no significant deterioration in biomarker levels was observed. Unfortunately, biomarker data was not available for comparison in patients 2 and 3.

![Figure 6.12 Correlation between antibody titres and Biomarkers in MPS VI](image)

Figure 6.12 Correlation between antibody titres and Biomarkers in MPS VI

Longitudinal data showing allo-immune response and disease biomarker in MPS VI (patient 1). The biomarker level declines after the start of treatment and remains low despite persistent high titre immune response. There appears to be no correlation between the antibody titre and disease biomarkers in this patient.
6.4. Discussion

The reported incidence of allo-antibodies in Pompe disease and MPSVI is 89% and 97% respectively. The significance of allo-immune response was highlighted initially in CRIM negative patients where a poor clinical response to ERT was attributed to development of immune response (Kishnani, Goldenberg et al. 2010; Abbott, Prater et al. 2011; Bali, Goldstein et al. 2012). The deteriorating clinical response in adult patients after generation of sustained high titre antibody response (de Vries, van der Beek et al. 2010; Patel, Banugaria et al. 2012; Regnery, Kornblum et al. 2012) highlights the significance of immune response in attenuated forms of disease and those who are CRIM positive. There is increasing evidence that the allo-immune response in Pompe disease is detrimental and leads to clinical deterioration. In MPS VI, despite very high incidence of allo-antibodies, the significance of antibodies remains elusive. To date, there is no credible published case series which shows a direct correlation between the allo-immune response and clinical outcome in MPS VI patients treated with Naglazyme. This therapy seems to be well tolerated despite development of persistent allo-immune response.

The incidence of antibodies in this cohort of Pompe disease patients is lower than previously reported literature (2/7). The reported incidence of antibodies (89%) in Pompe disease reflects development of antibody response at any time after exposure of Myozyme, usually within first six months. Due to the unavailability of longitudinal data for the majority of patients with Pompe disease, it was not possible to evaluate the incidence of allo-antibodies post Myozyme exposure. However, the two patients with positive immune response confirm persistence of neutralizing allo-antibodies in long term recipients of Myozyme. Our data show that both patients who developed immune response to Myozyme had inhibitory antibodies. Catalytic inhibition did not appear to correlate with the severity of immune response. Patient 1 with relatively low titres of antibody inhibited the activity of Myozyme completely at low enzyme concentration. Patient 2 had low level of catalytic inhibition despite high titre IgG
immune response. In contrast, the cellular uptake inhibition appeared to be dependent directly on the levels of antibodies present in the serum. The two samples (sample A and B) of patient 3 had different levels of inhibition when tested at two different enzyme concentrations. This could be due to excess enzyme in high enzyme concentration medium, which the low titre antibody serum was unable to neutralize. This uninhibited enzyme was then delivered into the cells, despite the presence of antibodies. The second sample from the same patient (with high titre antibodies) was able to inhibit not only the enzyme at low concentration but also the medium with higher enzyme concentration.

All three MPSVI patients showed high titre IgG ELISA which is consistent with previously published literature. It is very interesting to note that all MPSVI samples, despite presence of a very high level of antibodies, did not inhibit either catalytic activity of enzyme or its uptake by MPSVI fibroblasts. As explained in chapter 3, this may reflect non-inhibitory nature of antibodies. Previously, White et al. described functional inhibition by allo-antibodies in patients with MPSVI who were treated with Naglazyme. However, in the described assays, the actual uptake inhibition was not assessed and the assays were not designed to quantify neutralizing activity of the allo-antibodies. In contrast to the reported assays, our assays did not show any enzyme inhibition. This could also be due to the less sensitive nature of our assays. However, due to a small number of patients in this series it is difficult to conclude either way, but the longitudinal biomarker data which were available for one patient in this series did not correlate with antibody titres, supporting the notion that the antibodies in MPSVI were not inhibitory in nature. At present, based on our data, we can conclude that the antibodies generated in MPS VI patients do not inhibit enzyme activity or cellular uptake and the titre of antibodies does not correlate with biomarkers. This argument is also supported by a lack of credible data which showing a clear correlation between antibodies and either biomarker of disease progression or clinical outcome in MPSVI patients. On the other hand, the presence of antibodies in patient serum may affect the enzyme
activity by a different mechanism e.g. increased clearance of enzyme from circulation. This would require a formal Naglazyme pharmacokinetic study in a patient with high titre antibody. This mechanism of neutralization of enzyme (rapid clearance) could, at least theoretically, be overcome by infusing a higher dose of enzyme and would be influenced by other factors such as renal function. In the absence of a formal PK study, it is difficult to comment any further on the functional nature of allo-immune response in MPSVI.
7. Conclusions and Future direction
7.1. **Aim1: Development of quantitative and functional immune assays for LSDs**

During this project, highly sensitive and specific quantitative immune assays were developed which detected the presence of IgG antibodies in some patient sera, diluted to a titre of over 1:200,000, confirming a high titre immune response. Similarly, highly sensitive ELISA was developed to quantify immune response for Pompe disease, MPSVI and MPSII. In our assays, unavailability of human anti-enzyme antibody necessitated performing these assays with a negative control and positive control for every sample (tested on 16 dilutions for every sample). This makes these assays tedious, expensive and difficult to standardise amongst different laboratories. Availability of human anti-enzyme antibodies would make it possible to develop a single standard curve for a number of specimens, enabling us to quantify antibodies in any given sample regardless of the dilution. This will be an important step towards standardising these assays. This in turn will lead to a reduction in inter laboratory assay variation and make it easy to have a more robust internal and external quality assessment mechanism.

Catalytic enzyme inhibition assays and cellular uptake inhibition assays, developed during this project, were based on currently available protocols for enzyme quantification assays for respective LSDs. Demonstration of catalytic inhibition requires performing these assays across a range of enzyme dilutions including extremely low enzyme concentration. At such concentrations, the actual inhibition is subtle but in comparison to the standard or no serum, the percentage difference becomes more remarkable and easy to quantify. The inhibition at low levels of enzyme concentration is relevant because the pharmacokinetic data available for various ERTs suggest that the serum concentrations of enzyme drop to an almost undetectable level within a few hours of ERT infusion. This makes it imperative to perform these assays at a wide range of concentrations encompassing very low levels. However,
quantification of catalytic inhibition at different enzyme concentrations gives
different levels of inhibition at each of these concentrations which has relatively
limited clinical relevance and this information is unlikely to help clinicians in the
management of patients on ERT. None the less, this assay demonstrates the
functional nature of the antibody and hence forms an important part of the
evaluation of nature of antibodies. From a clinical perspective, however, it may
suffice to report the antibody as demonstrating catalytic inhibition rather than
the percentage inhibition across a range of enzyme concentrations. White et al.
developed catalytic assays by defining a cut off at certain enzyme concentration
for a normal serum (with no antibodies) (White, Argento Martell et al. 2008). Any
inhibition by patient serum (with antibodies) above this level was reported as
showing “catalytic inhibition” by tested serum. A similar method for reporting
could be adopted during further development, optimisation and validation of
these assays for clinical use in future.

In contrast to catalytic inhibition, cellular uptake inhibition assays are more
meaningful and provide clinically relevant information. The use of functional
enzyme assays to elicit cellular uptake inhibition means that these assays
actually demonstrate the cellular uptake inhibition of catalytically active enzyme
incorporating both aspects (catalytic and cellular uptake inhibition) of evaluation
of the functional nature of antibodies. Percentage inhibition and accurate
quantification of inhibition in this context is clinically relevant, as it provides
clinicians, with the basic information on the actual amount of enzyme delivered
into the cells at a given enzyme concentration.
7.2. **Aim 2: To study the incidence and impact of immune response in MPSI patients treated with ERT and HSCT**

The study of immune response in recipients of Aldurazyme, showed that the majority of patients developed a high titre inhibitory immune response to Aldurazyme. Our data showed allo-immune response in all MPSIH patients after exposure to ERT. These data are consistent with previously published studies which show a high incidence of immune response in recipients of Aldurazyme. This immune response was subsequently abrogated by HSCT. However, it is important to remember that all patients in this cohort had severe phenotype of the disease i.e. MPSIH and were expected to have a higher incidence of allo-immune response to ERT. Median time to development of immune response was similar to the previously published data (Kakavanos, Turner et al. 2003). Evaluation of immune response in recipients of ERT showed that the functional immune response persisted in patients beyond six months, in some cases inhibiting cellular uptake to nearly 95%. In view of our data (described in chapter 4), it would be reasonable to initiate a debate on developing a different strategy for the management of patients who generate a neutralizing immune response whilst on treatment with ERT. At present, management strategies particularly the decision whether to offer allogeneic HSCT for patients with MPSI, are predominantly based on the disease severity (phenotype and genotype). Generally, allogeneic HSCT is only offered to patients with severe disease phenotype i.e. MPSIH. Standard of care for attenuated disease type (MPSI HS/MPSI S) is ERT. Whilst HSCT has been a well-established treatment for MPSIH patients for decades, ERT is a relatively new treatment and its limitations are not fully understood. Based on our data, it appears inevitable that some patients, who develop allo-immune response to foreign proteins, will generate a refractory response, making this therapy ineffective. Therefore, when comparing the two treatment strategies i.e. HSCT and ERT, all factors which could influence the outcome of treatment including refractory immune response to ERT, need to be considered. A persistent refractory immune
response to ERT may warrant a different management approach such as immune tolerance induction or HSCT. In this context allogeneic HSCT provides a source of deficient enzyme as well as a mechanism of immune tolerance induction. Significantly improved outcome in paediatric recipients of allogeneic HSCT, makes this a viable therapeutic modality in attenuated type MPSI patients who develop refractory neutralizing immune response.

7.3. Aim 3: To evaluate the nature and incidence of immune response in other LSDs

Utilizing the assays, developed during this project, the functional nature of immune response was studied in all available patient samples. The data, confirms the presence of antibodies in all LSDs analysed, including MPSII, MPSVI and Pompe disease patients. However, this project involved patients from a single centre over a limited period of time and hence the numbers are very small. Whilst significant inhibition was demonstrated by allo-antibodies to Myozyme (in Pompe disease), no inhibition was seen by antibodies in MPSVI patients (IgG antibodies against Naglazyme), despite high titre immune response in all patients with MPSVI. These data may reflect a non-inhibitory nature of antibodies in MPSVI. Previously, data from MPSI, showed that some patients within an LSD group may not have inhibitory antibodies despite high titre antibody response. In MPSI, the incidence of functionally active antibodies was approximately 70% (as shown by the data in chapter 4 and 5). It is possible that the incidence of functionally active antibodies in MPSVI is lower than MPSI and none of the three patients tested in this study had functionally active antibodies. This could be confirmed by testing more MPSVI patient samples. However, this is an important finding and highlights the significance of performing the functional enzyme assays in all recipients of ERT regardless of the disease type, phenotype, disease severity and initial titre of immune response. Collaboration between multiple centres involved in delivering the ERT, could help evaluate the immune response in a larger patient series, which will consolidate the data collected in this project and will provide further vital
information on the incidence and impact of immune response in these rare disorders. Development of functional assays in some disorders e.g. MPSII may not be straightforward, as explained in chapter 3, and may require development of more specific substrates.

7.4. Perspectives and future experiments

In recent years, the focus of management in LSDs has shifted from symptomatic treatment to disease eradication. Some of the available treatments have a potential to cure the disease (HSCT and ERT) whilst others (SRT, CMT and gene therapy) are either experimental or intended to arrest the disease progression. During the last decade, ERT evolved into an established therapeutic modality for a number of LSDs. However ERT is not universally effective, has a very high cost burden and does not correct CNS disease due to its inability to penetrate the BBB. Immune responses to ERT have recently been recognized as a considerable clinical problem. In the past, a lack of standardized assays and reliable biomarkers made it very difficult to assess the therapeutic efficacy of ERT. Our data show that these antibodies may be functionally active and correlate with biomarkers of disease progression in some cases, resulting in reduced efficacy of this treatment. ERT puts a heavy cost burden on health care systems. In the current climate with tightening of health care budgets (Blumenthal and Dixon 2012; Greaves, Harris et al. 2012) it may not be possible to continue delivery of very expensive treatments without restriction. A logical strategy under these circumstances would be to identify the patient groups which will show optimum response to the treatment. Simultaneously, a prudent approach towards rationing the expensive treatments e.g. ERT, is to identify the patients who will have limited or no response to treatment soon after commencing the treatment. Such strategies to try and identify the “therapeutic failures” will inevitably involve developing highly sensitive and reliable biomarkers along with new tests which would predict the
therapeutic efficacy. This will not only help clinicians use resources more effectively, but also refine management of the patients and plan alternative treatments in case of therapeutic failure.

The problem of functional immune response generated against ERT can be addressed by a number of different strategies. A change in structure of the compound, rendering it less immunogenic could abrogate this problem altogether. However, in view of the immunogenic potential of a replaced protein (which is completely deficient in a recipient) and the complexity of human immune response, this may not be possible in all individuals. Another strategy that could be implied, may involve development of immune tolerance induction regimens. The experimental data of immune induction in animal models of LSDs is rather scanty and there is no general agreement on the optimum induction methods. Various immune tolerance induction regimens have been used in recent years which have variable efficacy with unsatisfactory results in some patients. There are substantial risks associated with different immune tolerance induction regimens depending upon their complexity and duration. Development of optimum immune tolerance induction regimens is critical to solving this problem. These strategies have been evaluated in animal models of LSDs. A problem with current approach (Joseph, Munroe et al. 2008) of studying the effects of immune tolerance induction in animals is the lack of a good immune model which closely resembles the human immune system. Published literature is based mostly on experiments in animals (Garman, Munroe et al. 2004) including canine and murine models. However, these models are far from perfect, and a number of novel human immune antibody treatments available in clinical practice, cannot be used in these experiments to evaluate their immune tolerance induction potential. Therefore, development of human immune system is a prerequisite to developing an effective immune tolerance strategy. A NOD/SCID/GammaC null mouse model can develop a human immune system when injected with human CD34+ cells into the liver at a neonatal stage (Ishikawa, Yasukawa et al. 2005; Siapati, Bigger et al. 2005).
These mice when injected with CD34 positive human haemopoietic stem cells from an affected individual, can develop an enzyme naive human immune system in mice. This model can then be used to study the in vivo effect of antibodies on infused IDUA (Myozyme) and evaluate the novel tolerance regimens e.g. human monoclonal anti CD20 and anti CD52 antibodies (Rituximab, campath-1H) in a microenvironment very similar to humans. Other neutralization mechanisms such as rate of clearance of enzyme from circulation can also be performed by serial serum enzyme assays following IDUA infusion in non-tolerized and tolerized mice. This kind of study will provide the crucial data required to run a national or international human trial to explore and optimize the management of this group of patients.

HSCT has a curative potential in patients with high titre immune response by delivering cellular enzyme and inducing immune tolerance. High mortality and significant morbidity make it a more intricate therapeutic option. Recent developments in the field of HSCT have resulted in improved clinical outcome and reduced transplant related mortality. This has made HSCT a viable treatment option for a wider range of LSD patients. This can be used both as a replacement therapy and immune tolerance induction strategy (saif, bigger et al 2012). Based on this evidence, it can be argued that failure of immune tolerance induction should be considered as an indication for HSCT in certain LSDs where the allogeneic HSCT is shown to be an effective therapeutic modality. Due to the rapidity of disease progression in infantile LSDs with high titre immune response, the decision to induce immune tolerance and offer HSCT should be made promptly.

Inability of unmanipulated HSCT to correct disease pathology remains a considerable problem for a number of LSDs e.g. MPSIII. Increasing the copy numbers of delivered enzyme enhances the enzyme delivery and leads to improved outcome in MPSI patients. In murine models, lentiviral vector-mediated \textit{ex vivo} HSC gene therapy has been shown to correct the disease
phenotype (Biffi, De Palma et al. 2004; Biffi, Capotondo et al. 2006; Langford-Smith, Wilkinson et al. 2012). Based on data from animal studies in MLD mouse models, a clinical trial for MLD using lentiviral vector-mediated stem cell gene therapy has been commenced. This clinical trial involves delivery of autologous CD34+ cells transduced with the third generation lentiviral vector (hPGK.ARSA/ MOI=100/ double transduction) using a full intensity conditioning protocol. The initial data from the trial shows excellent donor cell transduction and enzyme levels nearly 13 times the normal levels (Prof. L. Naldini, ASGCT 2011, Dr. A. Biffi, ESGCT 2011). In future, graft manipulation by gene therapy to deliver supra-physiological enzyme levels, HSCT in early age and further decline in transplant related mortality could open doors for extending the indications of HSCT in other LSDs not yet amenable to this treatment.
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Appendix A
Hematopoietic stem cell transplantation improves the high incidence of neutralizing allo-antibodies observed in Hurler’s syndrome after pharmacological enzyme replacement therapy

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ABSTRACT

Background
Mucopolysaccharidosis type I is caused by deficiency of α-L-iduronidase. Currently available treatment options include an allogeneic hematopoietic stem cell transplant and enzyme replacement therapy. Exogenous enzyme therapy appears promising but the benefits may be attenuated, at least in some patients, by the development of an immune response to the delivered enzyme. The incidence and impact of allo-immune responses in these patients remain unknown.

Design and Methods
We developed an immunoglobulin G enzyme-linked immunosorbent assay as well as in vitro catalytic enzyme inhibition and cellular uptake inhibition assays and quantified enzyme inhibition by allo-antibodies. We determined the impact of these antibodies in eight patients who received enzyme therapy before and during hematopoietic stem cell transplantation. In addition, 20 patients who had previously received an allogeneic stem cell transplant were tested to evaluate this treatment as an immune tolerance induction mechanism.

Results
High titer immune responses were seen in 87.5% (7/8) patients following exposure to α-L-iduronidase. These patients exhibited catalytic enzyme inhibition (5/8), uptake inhibition of catalytically active enzyme (6/8) or both (4/8). High antibody titers generally preceded elevation of previously described biomarkers of disease progression. The median time to development of immune tolerance was 101 days (range, 26-137) after transplantation. All 20 patients, including those with mixed chimerism (22%), tested 1 year after transplantation were tolerized despite normal enzyme levels.

Conclusions
We found a high incidence of neutralizing antibodies in patients with mucopolysaccharidosis type I treated with enzyme replacement therapy. We also found that allogeneic hematopoietic stem cell transplantation was an effective and rapid immune tolerance induction strategy.

Key words: immune tolerance induction, hematopoietic stem cell transplantation, Hurler’s syndrome, mucopolysaccharidosis, enzyme replacement therapy, allo-antibodies.


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Introduction

Mucopolysaccharidosis type I (MPSI) is a lysosomal storage disorder (LSD) caused by deficiency of α-L-iduronidase (IDUA). The deficiency of IDUA results in intracellular accumulation of dermatan sulphate (DS) and heparan sulphate and a progressive, multisystem clinical disorder. Recombinant human enzyme replacement therapy (ERT) for LSD first became available in the 1990s and is currently used clinically for MPSI, MPSII, MPSVI, Pompe, Gaucher and Fabry disease at a considerable financial cost (estimated at $150,000-300,000 per patient per annum in MPSI). It is essential that the ERT is functionally active (catalytic activity) and able to target and penetrate enzyme-deficient cells. In MPSI, enzyme is taken up by cells via a mannose-6-phosphate receptor-mediated mechanism.

The formation of allo-antibodies has been reported to occur in all LSD treated with ERT. The clinical importance of antibodies in ERT-treated patients is uncertain but the existence of an allo-immune response to infused exogenous enzyme and recombinant human proteins is well described. In early clinical trials the reported incidence of IDUA-specific antibodies was low (40%) and there was no evidence of immunoprecipitation of enzyme or inhibition of catalytic activity. Subsequently, a prospective, open-label, multinational study revealed a suboptimal biomarker response in the presence of high antibody titters (>1:10,000) when compared to that in patients with no allo-immune response. More recent studies in canine models of MPSI demonstrated up to 90% inhibition of enzyme uptake by MPSI fibroblasts by serum taken from dogs that had high titer anti-IDUA antibodies. There is increasing evidence, mostly derived from animal models of LSD, of an inverse correlation between an observed antibody response and metabolic and clinical outcome. Even though the immune response currently reported in patients with MPSI is 91%, the true incidence of functionally active (neutralizing) antibodies is unknown.

The consequences of a refractory immune response can be serious, ranging from treatment failure to catastrophic rapid progression of disease and high mortality in some LSD. Several clinical protocols for the induction of immune tolerance to exogenous enzyme have been reported for these diseases and others, including hemophiliac disorders; in which there is a similar host immune response against a foreign protein. Allogeneic hematopoietic stem cell transplantation (HSCT) can replace the recipient’s immune system with that of the donor, thereby tolerizing the individual to the replaced enzyme (pharmacological or cellular). The donor’s immune system is naturally tolerized to this protein as the donor possesses normal levels of the enzyme. However, data on allogeneic HSCT as a means of immune tolerance induction are scarce.

In children with severe MPSI (MPSI H, Hurler’s syndrome) the standard of care is allogeneic HSCT. Engrafted donor leukocytes deliver enzyme (cellular ERT) to the brain. Pharmacological ERT is unable to correct neurological disease because of its inability to cross the blood-brain barrier. It has been our practice recently to give ERT to patients with MPSI H in the interval between diagnosis and transplantation in order to improve the somatic (including cardiac) manifestations of the disease and to prepare the child for transplant conditioning therapy.

We have developed quantitative and functional immunological assays and studied the incidence, pattern and impact of the immune response in MPSI patients prior to receiving any treatment and following ERT and HSCT. We have also determined the relationship between these antibody responses and metabolic biomarkers of the disease.

Design and Methods

Patients' samples

Blood samples were collected from 28 MPSI H patients treated and followed up at the Royal Manchester Children’s Hospital over a 4-year period. The samples were collected with informed consent and under permission REC 08/H101063 of the hospital’s ethical commission. The patients’ blood samples were processed within 6 h and the serum was separated and stored at -80°C. One group of patients was followed longitudinally and comprised patients who received ERT prior to HSCT (n=8). The second was a cross-sectional group of patients (n=20) studied at least 1 year after HSCT. One patient (patient 5) rejected the donor cells following HSCT and had autologous cells returned to restore hematologic function. This patient subsequently received a second allogeneic HSCT which was successful. Longitudinal data for this patient during both transplants are included in the results. The demographics, enzyme levels, details of HSCT, immune reconstitution, and immunosuppression for all patients in the longitudinal series are presented in Table 1. The transplant details and information on immunosuppression for all patients in the cross-sectional series are shown in Table 2.

Immunoglobulin G enzyme-linked immunosorbent assay

For the immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA), 96-well plates were coated with recombinant human IDUA enzyme (Aldurazyme) (Genzyme, Framingham, USA) in enzyme-carbonate solution (10 µg/ml in 0.1 M sodium bicarbonate, pH 8.5), overnight and blocked with blocking agent containing 1% human serum albumin. The plates were washed with washing buffer (phosphate-buffered saline (PBS), 0.1% Tween at pH 7) three times and patient’s serum (50 µL) was added (two-fold serial dilutions) in duplicate and incubated at room temperature for 1 h. The plates were then washed three times with washing buffer, and goat anti-human IgG-horseradish peroxidase antibody (1:5000 dilutions in dilution buffer containing PBS, 0.05% Tween, 0.01% human serum albumin) was added and incubated at room temperature for 1 h. Plates were washed again, o-phenylenediamine dihydrochloride solution was added and the reaction stopped with 2.5 M H2SO4. The plates were read at 492 nm immediately. The normal range was determined for each dilution by testing 13 normal sera (from normal volunteers). A positive cut-off value was defined as two standard deviations above the absorbance value for the normal sera at that concentration. To quantify ELISA, the lowest titer with absorbance above the cut-off was reported as a positive serum dilution for that patient’s sample. Positive responses were confirmed by western blotting to show the specificity of the IgG antibody to Aldurazyme.

Catalytic inhibition

Functional enzyme activity was measured using 4-methylumbelliferylα-L-iduronide (4MU) substrate (Glycosynth, Warrington, UK) at 37°C based on previously described methods. We modified the protocol to develop and optimize mixing assays in 96-well plates and assessed inhibition of enzyme activity by patients’ sera. The assay was performed in duplicate on the highest titer
Table 1. Patients’ demographics (longitudinal series of patients).

<table>
<thead>
<tr>
<th>N.</th>
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<th>Genotype</th>
<th>Age at HSCT (months)</th>
<th>Age at ERT (months)</th>
<th>Duration of ERT (days)</th>
<th>Type of stem cells</th>
<th>Source of stem cells</th>
<th>Conditioning</th>
<th>GVHD prophylaxis</th>
<th>Duration of immuno-suppression (days)</th>
<th>CD3 count at assessment x10^6/L</th>
<th>CD19 count at assessment x10^6/L</th>
<th>Enzyme level at diagnosis</th>
<th>Latest enzyme level post HSCT</th>
<th>Donor engraftment (VNTR)</th>
<th>Complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>W402X/ W402X</td>
<td>6.9</td>
<td>4.2</td>
<td>83</td>
<td>MUD</td>
<td>CB</td>
<td>Bu, Flu, ATG</td>
<td>CSA, Pred</td>
<td>184</td>
<td>330</td>
<td>733</td>
<td>0.22</td>
<td>55.5</td>
<td>100%</td>
<td>Autoimmune hemolysis, rhinovirus pneumonia</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>W402X/ W402X</td>
<td>11.5</td>
<td>9.2</td>
<td>64</td>
<td>MUD</td>
<td>CB</td>
<td>Bu, Cy, ATG</td>
<td>CSA, Pred</td>
<td>250</td>
<td>1400</td>
<td>1470</td>
<td>0.22</td>
<td>27.6</td>
<td>100%</td>
<td>VOD (late), skin GVHD, renal tubular acidosis secondary to CSA</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>W402X/ W402X</td>
<td>12.2</td>
<td>8.5</td>
<td>96</td>
<td>MUD</td>
<td>PBSC</td>
<td>Bu, Cy, Campath</td>
<td>CSA, MTX</td>
<td>172</td>
<td>880</td>
<td>68</td>
<td>0.14</td>
<td>49.9</td>
<td>100%</td>
<td>RSV pneumonitis, pneumothoraces</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>Q70X/ Q402X</td>
<td>8.3</td>
<td>4.5</td>
<td>114</td>
<td>MUD</td>
<td>BM</td>
<td>Bu, Cy, Campath</td>
<td>CSA</td>
<td>166</td>
<td>3994</td>
<td>666</td>
<td>0.31</td>
<td>15.7</td>
<td>99%</td>
<td>Cy-induced acute cardiomyopathy</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>Q70X/ W402X</td>
<td>7.5</td>
<td>5.3</td>
<td>87</td>
<td>MUD</td>
<td>CB</td>
<td>Bu, Cy, ATG</td>
<td>CSA, Pred</td>
<td>110</td>
<td>370</td>
<td>160</td>
<td>0.2</td>
<td>38.3</td>
<td>99%</td>
<td>Adenovirus, gastrointestinal &amp; skin GVHD, pneumonia, VOD, CMV reactivation</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>R628X/ R628X</td>
<td>11.5</td>
<td>8.1</td>
<td>112</td>
<td>Sibling</td>
<td>BM</td>
<td>Bu, Cy, Campath</td>
<td>CSA</td>
<td>165</td>
<td>1748</td>
<td>238</td>
<td>0.05</td>
<td>33.8</td>
<td>100%</td>
<td>VOD, CMV reactivation</td>
</tr>
<tr>
<td>7</td>
<td>Female</td>
<td>W402X/ Un-known</td>
<td>13.2</td>
<td>10.2</td>
<td>113</td>
<td>MUD</td>
<td>CB</td>
<td>Bu, Cy, ATG</td>
<td>CSA, Pred</td>
<td>184</td>
<td>854</td>
<td>794</td>
<td>0.09</td>
<td>17.1</td>
<td>100%</td>
<td>Norovirus gastroenteritis</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>R628X/ R628X</td>
<td>5.0</td>
<td>0.9</td>
<td>124</td>
<td>Sibling</td>
<td>BM</td>
<td>Bu, Flu, Campath</td>
<td>CSA, MTX</td>
<td>155</td>
<td>1982</td>
<td>159</td>
<td>0.06</td>
<td>20.1</td>
<td>70%</td>
<td>VOD, adenovirus infection</td>
</tr>
</tbody>
</table>

MUD: matched unrelated donor; CB: cord blood; BM: bone marrow; PBSC: peripheral blood stem cells; CSA: cyclosporine A; Pred: prednisolone; VNTR: variable number tandem repeats; VOD: veno occlusive disease; GVHD: graft-versus-host disease; CMV: cytomegalovirus; Bu: busulphan; Cy: cyclophosphamide; ATG: antithymocyte globulin; RSV: respiratory syncytial virus; Flu: fludarabine.

The percentage inhibition of the catalytic activity for each enzyme dilution was determined as follows:

\[
\% \text{ Inhibition} = \frac{100 - 100 \times \frac{EA_{Ps} - BEA_{Ps}}{EA_{Ns} - BEA_{Ns}}}{EA_{Ps}}
\]

where \(EA_{Ps}\) is the enzyme activity in the presence of the patient’s serum, \(EA_{Ns}\) is the enzyme activity in the presence of normal serum, \(BEA_{Ps}\) is the background enzyme activity in the patient’s serum and \(BEA_{Ns}\) is the background enzyme activity in normal serum.

**Cellular uptake inhibition**

This assay determines the inhibition of mannose-6-phosphate-mediated enzyme uptake and subsequent activity by binding antibodies in non-FCR expressing, IDUA-deficient fibroblasts.

MPSI fibroblasts (MPSI-A171) from a patient with Hurler’s syndrome (genotype W402X/W402X) were established and maintained in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 1% glutamine at 37°C in 5% CO₂. The assays were performed in duplicate. Six-well culture plates were seeded with 1.5×10⁵ fibroblasts/well and grown to 95% confluency. The enzyme was diluted in culture medium (Dulbecco’s modified Eagle’s medium with 1% glutamine) to below half the Km for Aldurazyme (100 ng/mL). Patient’s serum or normal serum was added at a volume of 10 µL (1 in 100 dilutions) to the diluted enzyme solution (1000 µL volume) and incubated for 2 h at room temperature. The culture medium was replaced with the enzyme mix medium and incubated for 1 h in 5% CO₂ at 37°C. The cells were washed with PBS and harvested by trypsinization. The cells were re-suspended and washed twice with PBS and a final suspension was made in homogenizing buffer (0.5 M NaCl/0.02 M Tris pH 7-7.5). Following a cycle of freezing and thawing, the cells were sonicated and centrifuged at 2045 g for 10 min. The supernatant was tested for enzyme activity using the 4MU assay described earlier. The protein concentration was determined.
using a bicinchoninic acid assay, as described elsewhere. No uptake inhibition was observed with normal sera and hence the percentage inhibition was calculated against plain enzyme solution as follows:

\[
\% \text{ Inhibition} = 100 - 100 \times \frac{A_{\text{sample}}}{A_{\text{control}}}
\]

Biomarkers and chimerism analysis

The ratio of DS to chondroitin sulphate (CS), a glycosaminoglycan (GAG) which is not stored in cells in MPSI, is a better marker of disease progression than total urinary GAG. We, therefore, studied DS/CS in our longitudinal series of patients. Total urinary GAG and DS/CS ratio were determined using two dimensional electrophoresis (originally described by Whiteman and LabWorks software (Anachem, Luton, UK)). Donor engraftment was evaluated in our clinical laboratory through analysis of variable number tandem repeats using a UVP gel photography system and LabWorks software.

### Table 2: Patients’ demographics (cross-sectional series of patients).

<table>
<thead>
<tr>
<th>N.</th>
<th>Gender</th>
<th>Type of HSCT</th>
<th>Age at transplant (months)</th>
<th>Source of stem cells</th>
<th>Conditioning</th>
<th>Immunosuppression</th>
<th>Duration of immune suppression (days)</th>
<th>Duration of ERT before HSCT</th>
<th>IgG antibody</th>
<th>Donor engraftment (VNTR)</th>
<th>Complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male MUD</td>
<td>14.4</td>
<td>PBSC</td>
<td>Bu, Cy, Campath</td>
<td>CSA, Pred</td>
<td>154</td>
<td>120</td>
<td>Negative</td>
<td>100%</td>
<td>GVHD (grade I), idiopathic pneumonia, CMV reactivation</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Female MUD</td>
<td>11.0</td>
<td>CB</td>
<td>Bu, Cy, ATG</td>
<td>CSA, Pred</td>
<td>126</td>
<td>90</td>
<td>Negative</td>
<td>100%</td>
<td>GVHD (grade I)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Female MUD</td>
<td>10.7</td>
<td>PBSC</td>
<td>Bu, Cy, Campath</td>
<td>CSA, MMF</td>
<td>122</td>
<td>72</td>
<td>Negative</td>
<td>100%</td>
<td>GVHD (grade I)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Male MUD</td>
<td>15.4</td>
<td>CB</td>
<td>Bu, Cy, ATG</td>
<td>CSA, Pred</td>
<td>270</td>
<td>110</td>
<td>Negative</td>
<td>100%</td>
<td>Chronic skin GVHD (grade I)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Female Sibling</td>
<td>11.0</td>
<td>BM</td>
<td>Treo, Flu</td>
<td>CSA, MTX</td>
<td>90</td>
<td>150</td>
<td>Negative</td>
<td>100%</td>
<td>EBV reactivation</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Male Sibling</td>
<td>17.7</td>
<td>BM</td>
<td>Bu, Cy</td>
<td>CSA, MTX</td>
<td>105</td>
<td>90</td>
<td>Positive (1:256)</td>
<td>100%</td>
<td>No significant problems</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Male MUD</td>
<td>21.5</td>
<td>CB</td>
<td>Bu, Flu, ATG</td>
<td>CSA</td>
<td>122</td>
<td>95</td>
<td>Negative</td>
<td>NA</td>
<td>No significant problems</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Male MUD</td>
<td>25.4</td>
<td>CB</td>
<td>Bu, Cy, ATG</td>
<td>CSA</td>
<td>229</td>
<td>240</td>
<td>Negative</td>
<td>100%</td>
<td>EBV reactivation, neutropenic sepsis, GVHD, VOD, cardiomyopathy</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Female MUD</td>
<td>15.3</td>
<td>BM</td>
<td>Cy, Bu, Campath</td>
<td>CSA, Pred</td>
<td>150</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>100%</td>
<td>GVHD, Sepsis, cardiac arrest</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Male Sibling</td>
<td>7.5</td>
<td>BM</td>
<td>Cy, Bu</td>
<td>CSA</td>
<td>210</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>100%</td>
<td>No significant problems</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Male Sibling</td>
<td>31.3</td>
<td>BM</td>
<td>Bu, Cy</td>
<td>CSA, MTX</td>
<td>115</td>
<td>No prior ERT</td>
<td>Weak positive (1:128)</td>
<td>100%</td>
<td>No significant problems</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Male Sibling</td>
<td>10.8</td>
<td>BM</td>
<td>Bu, Cy, ATG</td>
<td>CSA, Pred</td>
<td>140</td>
<td>No prior ERT</td>
<td>Weak positive (1:164)</td>
<td>90%</td>
<td>Neutropenic sepsis</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Male Sibling</td>
<td>15.2</td>
<td>BM</td>
<td>Bu, Cy, ATG</td>
<td>CSA</td>
<td>188</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>90%</td>
<td>GVHD skin (grade I), neutropic sepsis</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Male Unrelated</td>
<td>16.6</td>
<td>CB</td>
<td>Cy, Melphalan, ATG</td>
<td>CSA</td>
<td>94</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>45%</td>
<td>PTLFD, neutropenic sepsis</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Male MUD</td>
<td>18.8</td>
<td>BM</td>
<td>Cy, Melphalan, ATG</td>
<td>CSA</td>
<td>85</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>100%</td>
<td>CMV reactivation, neutropenic sepsis, EBV reactivation, PTLFD</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Male Sibling</td>
<td>11.8</td>
<td>BM</td>
<td>Bu, Cy, ATG</td>
<td>CSA</td>
<td>154</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>70%</td>
<td>Neutropenic sepsis, Gram-negative sepsis</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Female MUD</td>
<td>22.7</td>
<td>BM</td>
<td>Cy, Bu, ATG, Flu</td>
<td>CSA</td>
<td>186</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>100%</td>
<td>No significant problems</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Female Sibling</td>
<td>13.4</td>
<td>BM</td>
<td>Cy, Bu, ATG, Flu</td>
<td>CSA, Pred</td>
<td>330</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>100%</td>
<td>EBV reactivation, neutropenic sepsis, adenovirus infection, chronic limited skin GVHD</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Male MUD</td>
<td>15.0</td>
<td>BM</td>
<td>Cy, Bu, Campath</td>
<td>CSA</td>
<td>NA</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>NA</td>
<td>No significant problems</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Male MUD</td>
<td>14.9</td>
<td>PBSC</td>
<td>Bu, Cy, Flu, Campath</td>
<td>CSA</td>
<td>243</td>
<td>No prior ERT</td>
<td>Negative</td>
<td>100%</td>
<td>GVHD (grade I), Gram-negative sepsis, EBV reactivation</td>
<td></td>
</tr>
</tbody>
</table>

MUD: matched unrelated donor; CB: cord blood; BM: bone marrow; PBSC: peripheral blood stem cells; CSA: cyclosporine A; Pred: prednisolone; VNTR: variable number tandem repeats; VOD: veno occlusive disease; GVHD: graft-versus-host disease; EBV: Epstein-Barr virus; CMV: cytomegalovirus; Bu: busulphan; PTLFD: post-transplant lymphoproliferative disorder; Cy: cyclophosphamide; ATG: antithymocyte globulin; Flu: fludarabine; MTX: methotrexate.

Results

Pattern of immune response

In the longitudinal series of patients, high titers of IDUA reactive antibodies were seen in 87.5% (n=7) of patients during IgG ELISA. Figure 1A shows the immune response after first exposure to ERT up to the time of HSCT in these patients. One patient (patient 8) had detectable IgG antibodies only at a low titer (1:256). These patients were followed up for a median period of 216 days (range, 155-685). The median time between first exposure to ERT and the first positive IgG antibody result was 38.5 days and while that to the peak response was 55.5 days (range, 17-129). Antibody titers in seven patients (immediate post-transplant data were not available for patient 6 in this group) who received HSCT had dropped to less than 1:10,000 by a median time of 101 days (range, 26-157) despite these patients having a normal level of innate
IDUA. A summary of the immune responses in all these patients is shown in Table 3. The specificity of the ELISA was demonstrated using western blotting, as shown in Figure 1B. This Figure shows that in the absence of antibodies in the pre-ERT sample, there is no antibody binding to Aldurazyme (recombinant IDUA) or Myozyme (recombinant human alglucosidase-α, Genzyme, Framingham, USA). After exposure to ERT, the IgG antibodies in patients’ sera only bound to Aldurazyme and not to Myozyme. ELISA results from all patients over the entire follow-up period are shown in Figure 1C base-lined to the first HSCT. None of the patients had detectable antibodies on the most recent ELISA performed a year or more after the HSCT. It is interesting to note that patient 5 in this series had primary graft rejection with autologous hematologic recovery after infusion of stored autologous cells. This resulted in an escalation of the immune response following subsequent exposure to IDUA which was later eradicated by a second unrelated donor transplant. In this cohort, the median CD3 and CD19 counts were 1140×10⁶/L (range, 330×10⁶/L - 3994×10⁶/L) and 452×10⁶/L (range, 68×10⁶/L - 1470×10⁶/L), respectively, at the time of the last assessment.

Analysis of the sera from patients in the cross-sectional group (n=20) showed that 85% (n=17) of patients tested 1 year or more after HSCT had no detectable antibodies despite normal IDUA levels. Eight of these patients had received ERT prior to HSCT. Three patients in this group had weakly positive IgG antibody responses to IDUA (<1:250). Chimerism data were not available for two patients in this group. The majority of patients (n=14, 78%) had achieved full donor engraftment while 22% (n=4) were chimeric with donor leukocyte engraftment of 45%, 70%, 90% and 90%. The median duration of immune suppression for graft-versus-host disease prophylaxis after HSCT in the cross-sectional and longitudinal series of patients was 150 days (range, 90-330) and 169 days (range, 110-250), respectively (see Tables 1 and 2). The median time to assessment in this cohort was 66 months (range, 12-181).

Enzyme neutralization by antibodies

Figure 2A illustrates the catalytic inhibition in all patients in the longitudinal series. In this series 62.5% of patients demonstrated catalytic inhibition. The inhibition was more pronounced at lower enzyme concentrations. Three patients (patients 4, 5 and 8) did not show enzyme inhibition at any concentration whereas another two patients (patients 7 and 5) showed over 75% inhibition of enzyme activity at concentrations of less than 1 pg/mL. Enzyme inhibition at higher concentrations (7.5-30 ng/mL) was up to 20% in comparison to that of a standard (normal serum). A similar pattern of inhibition was seen by antibodies when human and mouse enzymes were used (data not shown).

To confirm that the inhibition in a patient’s serum was...
due to antibodies, we assessed the enzyme inhibition in the same patient before and after HSCT i.e. the specimen with the highest titer of antibodies and no detectable antibodies on ELISA. The data confirmed that there was no catalytic inhibition in the absence of antibodies (Figure 2B).

**Inhibition of cellular uptake of catalytically active enzyme**

Cellular uptake inhibition was also assessed in the samples with the highest antibody titer for each patient in the longitudinal series. Inhibition of enzyme uptake by MPSI fibroblasts was seen in 75% (n=6) of patients in this series. Figure 3A illustrates the results of the uptake inhibition assay in eight patients in the longitudinal series. Two patients (patients 6 and 8) did not show any inhibition. It is interesting to note that one of these patients (patient 6) had evidence of some enzyme inhibition on catalytic inhibition assays. In contrast the two patients (patients 4 and 5) who did not have any evidence of catalytic inhibition showed 25% and 76% inhibition of enzyme uptake, respectively. Patients 2 and 7 who showed over 75% inhibition of enzyme activity also demonstrated high levels of cellular uptake inhibition (over 70%). There was no correlation between the catalytic inhibition and cellular uptake inhibition in the rest of the patients suggesting that the antibodies are polyclonal. The cellular uptake inhibition assay was repeated in non-tolerized specimens (patient on ERT pre-transplantation and with a high-titer immune response) and tolerized specimens (post-HSCT with no antibodies) from the same patient to confirm that the inhibition was due to the antibodies in the serum (Figure 3B).

**Immune response and disease biomarkers**

Longitudinal biomarker data (DS/CS ratio) were available for six patients in this series. Figure 4 shows the relationship between antibody titers and DS/CS ratio over the follow-up period. Patient 2 showed an immediate improvement in DS/CS ratio after starting ERT. This improvement in biomarkers was halted by a high-titer immune response. This pattern continued even after the HSCT was performed and a further improvement in DS/CS ratio resumed only after the immune response was completely abrogated (Figure 4A). The DS/CS ratio in patient 3 stayed high despite HSCT, with a continuing high-titer immune response. The slight improvement in DS/CS ratio seen in this patient was suboptimal and could be considered as a therapeutic failure at this stage. The DS/CS ratio after resolution of the immune response is not yet available for this patient (Figure 4B). The improvement in biomarker levels in patient 4 (Figure 4C) was significant after ERT was commenced; however, this progress was halted and partially reversed after a high-titer immune response was generated. The subsequent improvement in DS/CS ratio followed resolution of the immune response. Patient 5 rejected his first transplant and was given a second transplant which was successful. His ERT was interrupted during this period. The initial improvement in biomarker levels plateaued during the high-titer immune response despite restarting ERT. After the second HSCT, the biomarker levels showed some deterioration despite ERT and HSCT and improved only after the immune response was completely resolved (Figure 4D). Patient 6 showed a suboptimal biomarker response to ERT during the high-titer immune response (Figure 4E). The DS/CS ratio in patient 7 dropped significantly after ERT but further follow-up data were not available during the immune response and following the HSCT (Figure 4F).

**Discussion**

The immune response to replacement recombinant human proteins has been widely reported. However, the clinical impact of antibodies ranges from no significant effect to almost complete failure of therapy. Hemophilia A and B are examples of congenital disorders in which the immune response (inhibitory antibodies) can make the

<table>
<thead>
<tr>
<th>Table 3. Summary of immune responses in the longitudinal series of patients.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Median time to first positive ELISA test</strong></td>
</tr>
<tr>
<td>38.5 days (range 14-129)</td>
</tr>
<tr>
<td><strong>Median time to highest immune response</strong></td>
</tr>
<tr>
<td>55.5 days (range 17-129)</td>
</tr>
<tr>
<td><strong>Median time to immune tolerance</strong></td>
</tr>
<tr>
<td>101 days (range 26-137)</td>
</tr>
<tr>
<td><strong>Incidence of high titer antibodies</strong></td>
</tr>
<tr>
<td>87.5% (7/8)</td>
</tr>
<tr>
<td><strong>Incidence of catalytic inhibition</strong></td>
</tr>
<tr>
<td>62.5 (5/8)</td>
</tr>
<tr>
<td><strong>Incidence of cellular uptake inhibition</strong></td>
</tr>
<tr>
<td>75% (6/8)</td>
</tr>
<tr>
<td><strong>Immune tolerance 1 year post-HSCT</strong></td>
</tr>
<tr>
<td>100% (28/28)</td>
</tr>
</tbody>
</table>

![Figure 2](image-url) (A) Catalytic enzyme inhibition in eight patients (1-8) compared to a standard (normal serum) across various enzyme concentrations. (B) Inhibition due to tolerized and non-tolerized serum. Non-tolerized serum taken from a patient (patient 3) on ERT before HSCT with high titer antibodies is compared to tolerized serum taken from the same patient a year after HSCT (with no antibodies) which shows no significant inhibition.
Figure 3. (A) Cellular uptake inhibition in all patients (1-8) in the longitudinal series is shown. Patients 6 and 8 showed no cellular uptake inhibition. (B) Tolerized serum (post-transplant with no antibodies) showed no inhibition compared to significant inhibition by non-tolerized serum (prior to transplant) in the same patient.

Figure 4. Correlation between biomarkers (DS/CS ratio) and the antibody titers in six patients in the longitudinal series (patients 2-7). The shaded areas represent the period of ERT and black arrows point to the time of HSCT. (A) Patient 2 showed an immediate improvement in the DS/CS ratio after starting ERT which was halted by high titer anti-IDUA antibodies. The biomarker levels improved after the immune response was abrogated. (B) Patient 3 showed minimal improvement in biomarkers despite HSCT and ERT during the high-titer immune response. (C) The improvement in biomarker levels in patient 4 was significant after ERT was commenced; however, this progress was arrested and reversed after the high titer immune response was generated. The biomarkers improved after resolution of the immune response. (D) Patient 5 rejected his first HSCT and had a second successful transplant. The initial improvement in biomarker levels plateaued during the high-titer immune response despite ERT (note a brief period when ERT was interrupted after the first failed HSCT). (E) Patient 6 showed a suboptimal response to ERT during the high titer immune response. (F) The DS/CS ratio in patient 7 dropped to a significant level but further follow-up data were not available for the period during the immune response and following the HSCT. Biomarker data are not available for patients 1 and 8.
replacement therapy (recombinant human factor VIII and IX) virtually ineffective. It is noteworthy that the true impact of neutralizing antibodies can only be measured accurately using robust functional assays, such as the Bethesda assays for hemophilia inhibitors. It is considerably more challenging to study the impact of antibodies in LSD because of the absence of standardized immune assays and the marked phenotypic heterogeneity in the natural course of a rare illness. While we studied only two mechanisms of enzyme neutralization, it is hypothesized that antibodies against ERT can inhibit the treatment by several mechanisms. The polyclonal nature of the IgG antibodies makes it imperative to develop multiple functional assays to encompass the full range of effects due to antibodies. Despite the heavy financial burden of ERT, there have been no collaborative efforts to develop and standardize quantitative and functional assays. As a consequence, the true incidence and impact of these allo-antibodies remain unknown.

In recent years, the functionally active nature of allo-antibodies and the catastrophic effects of the alloimmune response in some LSD were recognized and led to efforts to develop an effective and quick immune tolerance induction regimen. These strategies had variable outcomes and serious side effects were reported in some cases. There is a risk that intensifying the tolerance induction protocols by combination chemotherapy and immune suppression would result in significantly increased morbidity, as has been reported in hemophilia. Development of immune tolerance with allogeneic HSCT has been reported in various autoimmune disorders and to date over a thousand HSCT have been carried out for this indication. The use of low intensity conditioning regimens, the ability to manipulate the graft and the very low rate of transplant-related mortality have led to the use of allogeneic HSCT as a long-term tolerance induction strategy in some solid organ transplant recipients. The role of HSCT as an immune tolerance induction mechanism in patients with neutralizing allo-antibodies has not yet been established.

All the patients in our study had the severe phenotype of MPSI H (Hurler’s syndrome). Seven patients were homozygous for null mutations, conferring a high risk of allo-immune responses. Consistent with previous reports, we found a high incidence of antibodies in this subgroup. All transplant recipients in the longitudinal series cleared their antibodies to clinically insignificant levels within a median period of 101 days despite normal enzyme levels. None of the 20 patients in the cross-sectional group had a high-titer immune response 1 year or more after HSCT and cessation of immunosuppression. Full donor engraftment was not required for immune tolerance as some patients were tolerated despite achieving only mixed chimerism. These data confirm that allogeneic HSCT is an effective and quick mechanism of inducing immune tolerance. These data also have important implications for CD34 gene therapy. In animal models of LSD and hemophilia, stem cell transplantation after hematopoietic stem cell-based gene therapy tolerated the immune system to the infused protein.

The majority of patients (62.5%) demonstrated evidence of some catalytic inhibition by antibodies. Even though the catalytic inhibition appears weak at high enzyme concentrations, this in vitro inhibition may reflect partial neutralization of infused enzyme due to the very short half-life of Aldurazyme (3 h in the absence of antibodies) and a mean maximum plasma concentration (Cmax) of 1.2-1.7 µg/mL (ALID-014-02: A phase II Open-Label Clinical Trial of Aldurazyme). The inhibition of endogenous (human and mouse) enzyme by antibodies suggests cross-reactivity of anti-IDUA antibodies to endogenous IDUA. This signifies a need for HSCT-induced immune tolerance, as the presence of antibodies at high titer after HSCT would potentially neutralize the cellular therapy (enzyme delivered by allogenic HSCT).

The cellular uptake inhibition can be demonstrated at a much higher enzyme concentration. We used enzyme concentrations of less than half the Km (100 ng/ml) to optimize the uptake inhibition and serum at 1:100 dilution. Corrected for dilution, this is equivalent to just over six times the Cmax (maximum plasma concentration after infusion) of Aldurazyme, making the treatment virtually ineffective in some patients’ with high-titer immune responses. The cellular uptake inhibition at a higher enzyme concentration compared to catalytic inhibition suggests stronger inhibition of the mannose-6-phosphate binding sites (and enzyme uptake) than the catalytic site of recombinant IDUA by anti-IDUA antibodies. Our study looked at the antibody neutralization of enzyme in a specific group of MPSI patients with a greater propensity to develop a high-titer immune response. It is possible that the variable effects and polyclonal nature of anti-IDUA antibodies might have resulted in underestimation of the effects of antibodies in previous studies because of the pooling of data from various groups of patients. It is, therefore, important to assess these patients individually. Analysis of biomarker data and antibody titers show that the recovery is slowed or, in some cases, reversed in the presence of a high-titer immune response. Generally there appears to be a close relationship between the two, although the DS/CS ratio and biomarker responses usually lagged behind antibody responses by several days, confirming the in vivo neutralization of ERT by antibodies.

In conclusion, our data show that the high-titer immune responses in MPSI H patients treated with ERT can neutralize replacement therapy in a significant proportion of patients. In the past, the heterogeneous clinical course of the disease compounded by a lack of robust biomarkers and reliable functional immune assays made it very difficult to evaluate the effect of antibodies in LSD patients treated with ERT. There is now a dire need to standardize quantitative and qualitative immune assays in these patients. Given the remarkable improvement in the outcome of HSCT, this therapy is now a viable therapeutic modality as a mechanism for inducing immune tolerance in patients with refractory immune responses to ERT and other replacement therapies by substituting the enzymatic immune system with that of the donor. The generation of high-titer neutralizing antibodies to ERT prior to HSCT makes it unnecessary to continue ERT infusions in the presence of an immune response, particularly if the transplant is carried out early after the diagnosis and in the absence of any serious co-morbidities. However, inevitably, some clinically unstable patients will benefit from ERT to optimize their clinical status prior to HSCT. Close biochemical and clinical monitoring of the immune response in ERT-treated LSD patients can help to determine the optimum therapy for this group of patients.
Authorship and Disclosures

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