Efficacy of enzyme replacement therapy in α-mannosidosis mice

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Diego Prieto Roces
aus Avilés (Spanien)

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Referent:	Prof. Dr. Kurt von Figura
Korreferent:	Prof. Dr. Hans-Joachim Fritz
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To my family....

Contents

Abbreviations

AA	acrylamide
AV	Autophagic Vacuoles
ADP	adenosine diphosphate
APS	ammonium peroxidisulfate
ATP	adenosine triphosphate
BBB	Brain Blood Barrier
BMT	Bone Marrow Transplantation
bp	basepair(s)
BSA	Bovine Serum Albumin
°C	Celsius grad
ccv	clathrin-coated vesicles
CHO	Chinese Hamster Ovary
Ci	Curie (2.22×10^6 count per minute)
cpm	Counts per minute
dd	double distillate
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTPs	Deoxynucleosidetriphosphate (dATP, dGTP, dCTP, dTTP)
DOL	dolichol-P-P-oligosaccharide
DTT	dithiothreitol
E. coli	Escherichia coli
EDTA	ethylene dinitrilotetraacetic acid
EE	Early Endosomes
ER	endoplasmic reticulum
ERT	Enzyme Replacement Theraphy
EtOH	ethanol
fig.	figure
g	gram
gbw	gram body weight
GTP	guanosine triphosphate

h	hour
^{3}H	tritium
HPLC	High Performance Liquid Chromatography
Kb	kilobase
kDa	kilodalton
l	liter
LAMAN	Lysosomal Acid α-mannosidase
LE	Late Endosomes
Lys	Lysosome
m	milli
M	molar
mA	miliampere
MALDI-TOF	Matrix-assisted laser desorption-ionization time-of-flight
MetOH	methanol
Min	minute
MOPS	morpholinoethane sulfonic acid
M6P	mannose 6-phosphate
MPR	mannose 6-phosphate receptor
nm	nanometer
OD	optical density
PAGE	poly-acrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	negative logarithm of H^{+} concentration
PI	Proteinase inhibitor
PV	Phagocitic Vacuoles
RER	Rough Edoplasmatic Reticulum
rev.	review
RNA	ribonucleic acid
rpm	rounds per minute
Sd	Standard deviation
SDS	sodium dodecylsulfate

SEC	Size Exclusion Chromatography
sec	seconds
TBS	Tris Buffer Saline
TCA	Trichloroaceticacid
TGN	Trans Golgi network
TEMED	N, N, N´, N´-tetramethyl-ethylene diamine
TLC	Thin Layer Chromatography
Tris	tris-(hydroxymethyl)-aminomethane
U	Unit
vol	volumen

1. INTRODUCTION

1.1 Lysosomes

Lysosomes were discovered by de Duve in 1955. The lysosome is a subcellular membrane-surrounded organelle that contains more than 50 acid hydrolases. All sorts of macromolecules are delivered to the lysosome for degradation via endocytosis, phagocytosis, autophagocytosis or through import from the cytosol. Products of degradation are amino acids, monosaccharides, nucleotides, fatty acids, cholesterol and different ions that are cotransported to the cytosol. The lumen is maintained at an acidic pH by an H^+ ATPase in the membrane that acidifies the environment inside the lysosome to a pH under 5 (Mancini *et al.*, 2005). Lysosomal enzymes are active at this low pH, and the membrane of the lysosome constrains the digestive enzymes within the organelle. The cytosol has a pH of about 7.4. Due to their pH-dependent activation the enzymes can not inflict any damage during the transport to the lysosomes.

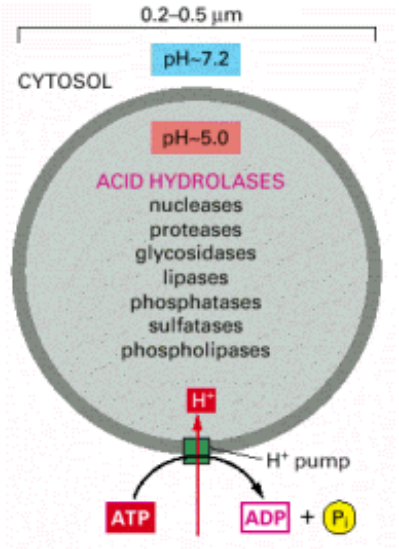

Fig. 1.1: Lysosomes.
The acid hydrolases are hydrolytic enzymes that are active under acidic conditions. The lumen is maintained at an acidic pH by an H^+ ATPase in the membrane that acidifies the environment inside the lysosome.
Reproduced from Alberts *et al.*, 2002, Molecular *Biology of the Cell.*

1.1.1 Lysosomal membrane

The lysosome not only contains a specialized set of enzymes, but also has a specific surrounding membrane. This membrane contains transport proteins that mediate the fusion of lysosomes and other organelles and the transport of degradation products to the cytosol (Eskelinen *et al.*, 2002), where they can be either excreted or reutilized by the cell. Probably to protect themselves from degradation, the lysosomal membrane proteins are highly glycosylated (Eskelinen *et al.*, 2003).

1.1.2 Transport and recognition of lysosomal proteins

Lysosomal enzymes and secretory proteins are synthesized in the rough endoplasmic reticulum (RER) and N-glycosylated (Fig. 1.2) by the transfer of a performed oligosaccharide ($Glc_3Man_9GlcNAc_2$) to asparagines residues from dolichol-P-P-oligosaccharide (Kornfeld & Kornfeld, 1985).

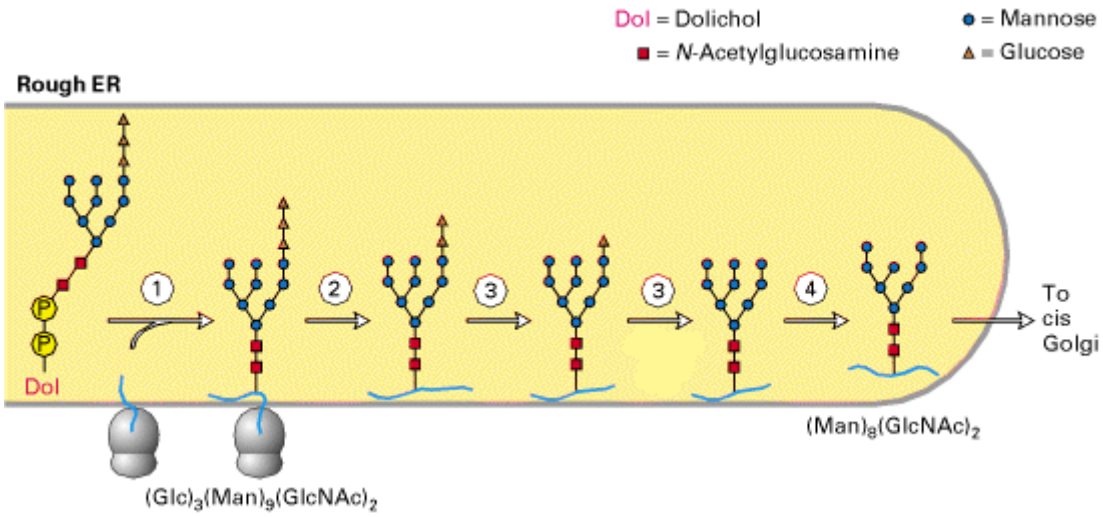

Fig. 1.2: Addition and initial processing of *N*-linked oligosaccharides in the rough ER. The $Glc_3Man_9(GlcNAc)_2$ precursor is transferred from the dolichol carrier to a susceptible asparagine residue in the luminal side of the ER (step **1**). In three separate reactions, first one glucose residue (step **2**), then two glucose residues (step **3**), and finally one mannose residue **4** are removed. The newly made protein is transported in a vesicle to the Golgi for further processing of the oligosaccharide. Reproduced from Lodish *et al.*, 2000.

After specific processing (Fig 1.2) of the oligosaccharide (Kornfeld & Mellman, 1989; Sousa *et al.*, 1995) the proteins are translocated to the cis Golgi. There, the oligosaccharides of secretory proteins are processed to complex-type units and the oligosaccharides of lysosomal enzymes are phosphorylated (reviewed by von Figura & Hasilik, 1986). The diversion of the lysosomal enzymes from the secretory pathway is dependent on the acquisition of the mannose-6-phosphate (M6P) recognition marker (Fig. 1.3).

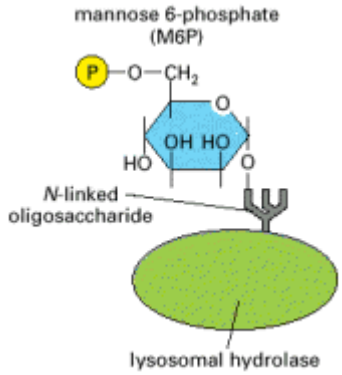

Fig. 1.3*:* The structure of mannose 6-phosphate on a lysosomal enzyme.
Mannose 6-phosphate (M6P) groups, are added exclusively to the N-linked oligosaccharide. Reproduced from Alberts *et al*., 2002.

The sorting system that segregates lysosomal hydrolases and dispatches them to late endosomes works because M6P groups are added only to the appropriate glycoproteins in the Golgi apparatus. This requires specific recognition of the hydrolases by the Golgi enzymes responsible for adding M6P (S. Kornfeld, 1985; Cantor *et al.*, 1992). Since all glycoproteins leave the ER with identical *N*-linked oligosaccharide chains, the signal for adding the M6P units to oligosaccharides must reside somewhere in the polypeptide chain of each hydrolase. Experiments have revealed that the recognition signal is a cluster of neighboring amino acids on each protein's surface (tertiary structure), known as a signal patch (Lang *et al*. 1984).

Two enzymes act sequentially to catalyze the addition of M6P groups to lysosomal hydrolases (Lazzarino et al., 1988). The first is a GlcNAc phosphotransferase that specifically binds the hydrolase and adds GlcNAc-phosphate to one or two of the mannose residues on each oligosaccharide chain (Figure 1.3). A second enzyme, a phosphodiesterase, cleaves off the

GlcNAc residue, leaving behind a newly created M6P marker. Since most lysosomal hydrolases contain multiple oligosaccharides, they acquire many M6P residues, providing a high affinity signal for the M6P receptor.

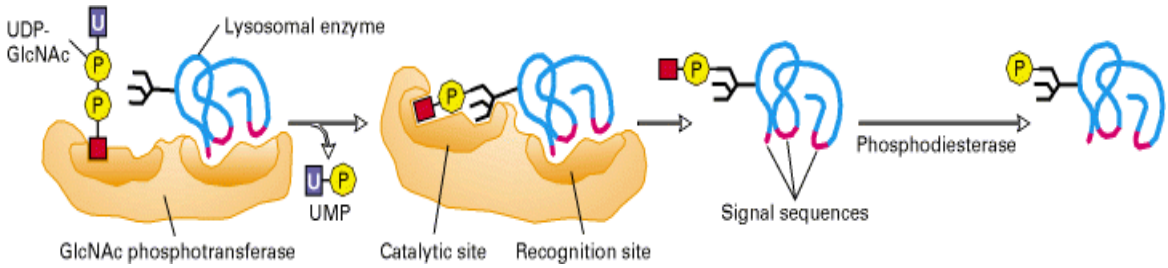

Fig. 1.4: The recognition of a lysosomal hydrolase.
GlcNAc phosphotransferase recognizes lysosomal hydrolases in the Golgi apparatus. The catalytic site binds high-mannose *N*-linked oligosaccharides and UDP-GlcNAc. The recognition site binds to a signal patch that is present only on the surface of lysosomal hydrolases. The GlcNAc is cleaved off by a phosphodiesterase, leaving the M6P exposed. Reproduce from Lodish *et al*., 2000.

The M6P tagged hydrolases moves through the cisternae to the trans Golgi region, where they are recognized by transmembrane mannose6-phosphate receptor proteins (MPRs). Two mannose-6-phosphate-specific receptors are known (Ghosh *et al.*, 2003), one is the 46 kDa cation-dependent receptor (CD-MPR, MPR46) and the other is a 300 kDa cation-independent receptor (CI-MPR, MPR300). CD-MPR exhibits a higher affinity for lysosomal enzymes containing one phosphomonoester, whereas the CI-MPR preferentially binds ligands with two phosphomonoesters (Munier-Lehman *et al.*, 1996).

1.1.3 The trans-Golgi network (TGN)

Lysosomes are part of the highly dynamic endosome / lysosome system (Fig. 1.4), a group of vesicles and vesicular organelles which to a certain extent exchange membrane constituents and contents thus having overlapping propierties.

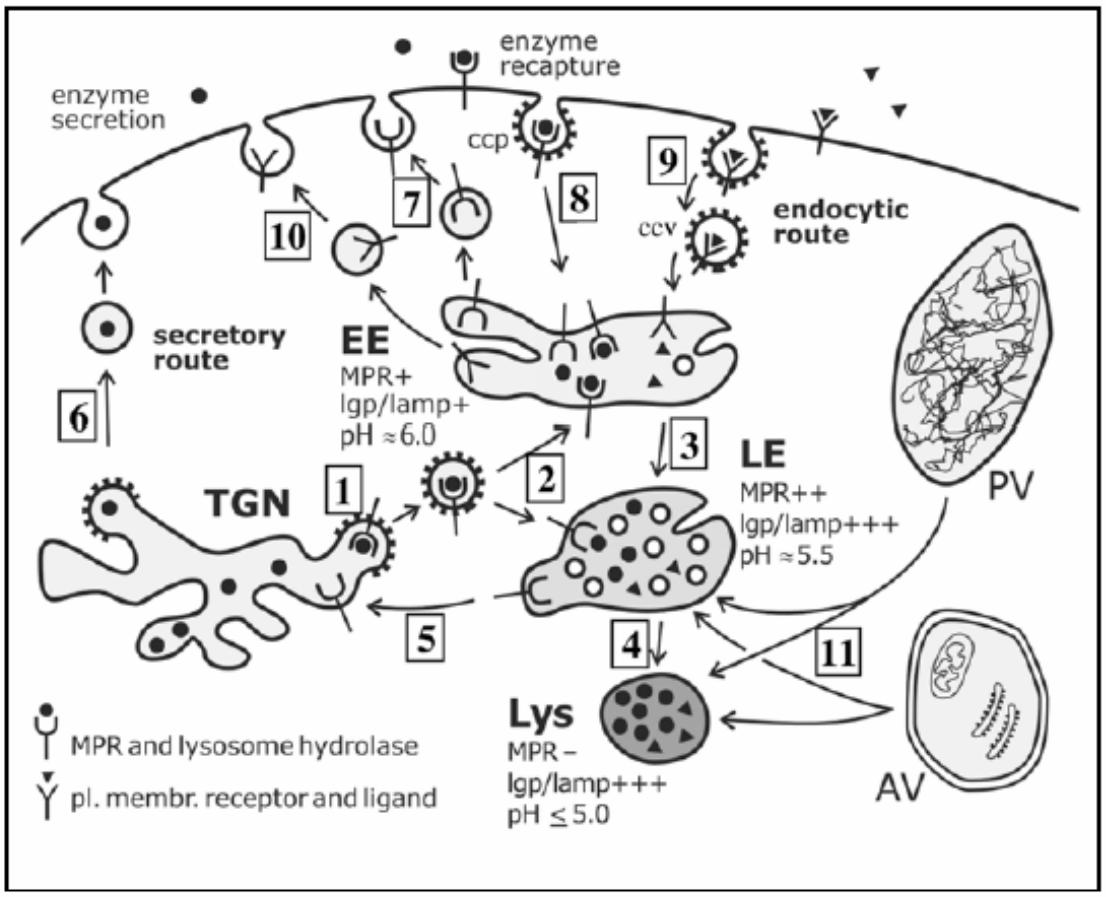

Fig. 1.4: Diagrammatic Summary of the Endosome/Lysosome System

After synthesis in the rER and modification in the Golgi apparatus precursors of soluble lysosomal enzymes decorated with mannose-6-phosphate residues meet the mannose-6-phosphate receptor (MPR) in the trans Golgi network (TGN), are packaged (1) into clathrin-coated vesicles (ccv), and are transported (2) to late endosomes (LE) either directly or indirectly via early endosomes (EE) (3). The process of enzyme transfer (4) from the LE to the lysosome (Lys) is not fully elucidated yet; possibly LE matures to become Lys, or LE and Lys fuse to form a transient hybrid organelle. The MPR is recycled (5) from the LE to the TGN, the lysosome is devoid of MPRs. A minor portion of the enzyme precursors gets into the secretory pathway (6) and is recaptured into clathrin-coated pits (ccp) by MPRs, which may be transferred (7) from the EE to the plasma membrane. Thus the enzyme precursors can reach the lysosome via the endocytic pathway (8) as do endocytic tracer molecules (9), whose receptors are recycled from the tubular extensions of the EE (10). Autophagic vacuoles (AV) and phagocytic vacuoles (PV) acquire lysosomal enzymes by fusion with lysosomes and/or LE (11) to become autolysosomes and phagolysosomes respectively. LE often resemble multivesicular bodies, i.e., they display invaginations of their membrane and internal vesicles budded off the invaginations (or representing cross sections of the invaginations). lgp/lamp, lysosomal membrane glycoproteins/lysosome-associated membrane proteins.
Reproduced from Saftig, 2005, Chapter 1.

In contrast to endosomes, the TGN and the plasma membrane, lysosomes are devoid of mannose-6-phosphate receptors (Johnson & Kornfeld, 1992).
In the TGN, mannose-6-phosphate receptors bind to lysosomal hydrolases on the lumenal side of the membrane and to adaptins assembling clathrin coats on the cytosolic side. In this way, they mediate the packaging of the hydrolases into clathrin-coated vesicles. The vesicles are translocated and fuse directly with late endosomes or indirectly via early endosomes (Griffiths & Gruenberg, 1991; S. Kornfeld, 1992). Early endosomes are less acidic (pH 6.0-6.2) than LEs (pH 5.5-6.0). In the early endosomes the ligand-receptor complexes dissociate and the MPRs cycled back to the plasma membrane. The receptors are retrieved into small vesicles, shuttled back to the TGN and become available for another round of enzyme transfer. Early endosomes can display a few internal vesicles which are formed presumably by budding off from invaginations of the limiting early endosome membrane.
Late endosomes are most probably generated by maturation of early endosomes. The internal vesicles increase in number as the early endosome madures to become a late endosome (Ghosh *et al.*, 2003).

The last steps of enzyme maturation (dephosphorylation and trimming of the oligosaccharides and proteolytic processing) may be initiated in the late endosomes and completed after arrival in the lysosomes (von Figura & Hasilik, 1986).

Not all of the hydrolase molecules that are tagged with M6P for delivery to lysosomes get to their proper destination. Some escape the normal packaging process in the *trans* Golgi network and are transported via the default secretory route to the cell surface, where they are secreted into the extracellular space. Some M6P receptors, however, also take a detour to the plasma membrane, where they recapture the escaped lysosomal hydrolases and return them by receptor-mediated endocytosis to lysosomes via early and late endosomes (Pohlmann *et al.*, 1995; Kasper *et al.*, 1996).

1.2 Lysosomal Storage Disorders

The Lysosomal Storage Disorders (LSD) are a group of about 50 diseases caused by genetic defects that affect one or more of the lysosomal hydrolases. The defect results in the accumulation of undigested substrates in lysosomes, resulting in the formation of large intracellular vacuoles with severe pathological consequences. Many of the diseases that we know as LSD were first described long before the discovery of the lysosome. As the structure and function of this organelle was defined and the different lysosomal proteins identified, the concept of LSD evolved.

Although individually rare, the LSD as a group have a frequency of about 1/8000 live births. As an increased number of patients with the mild form are being identified, the current figures may underestimate the actual frequencies of LSD.

LSDs are mostly caused by the lack of a hydrolase, its activator or a transporter causing accumulation of the hydrolases substrate in the lysosomes for each disorder type. The LSD are inherited in an autosomal-recessive fashion, except Fabry disease (Masson et al., 2004), Hunter disease (MPS II) (Hoopwood et al., 1993) and Danon disease (Danon et al., 1981; Nishino et al. 2000) that are all X-linked recessive. Certain disorders are more prevalent in certain geographic areas or among those of a particular ethnicity.

The lysosomal storage diseases have a broad spectrum of clinical phenotypes. In addition the age of onset, severity of symptoms and central nervous system (CNS) manifestation can vary markedly within a single disorder type. Several LSD such as for example Gaucher (Beutler et al. 2001), Tay Sachs (Gravel et al., 2001) and Pompe (Hirshhorn et al., 2001) have infantile, juvenile and adult.

The severity of a given LSD type will depend partially on the type of storage material product and will also depends on which cells or tissues accumulate the storage material. Other factors that affect the disease outcome are the genetic background and environmental influence. The cells and tissues have certain

thresholds of enzymatic activities below which clinical manifestation occur. This is probably one of the reasons why an infantile and a juvenile/adult form of the same disease can affect different tissues (reviewed by Futerman, 2005)

Most of the patients with a LSD are born apparently healthy and the symptoms develop progressively. The speed and severity of the evolving symptoms depend of many factors, as discussed above. Important from a therapeutic point of view is that lysosomal proteins added to the extracellular space will reach the lysosomes, usually via the mannose 6-phosphate receptor and the mannose receptor (Bijsterbosh et al., 1996; Zhu Y et al., 2004). This concept is known as enzyme replacement therapy, and was first utilised successfully on patients with a mild form of Gaucher disease in the 1990s (Barton *et al.*, 1991)

The most severe form of lysosomal storage disease, however, is a very rare disorder called inclusion cell disease (I-Cell disease). In this disease almost all of the hydrolytic enzymes are absent from the lysosomes and their undigested substrates accumulate in lysosomes. The disease is caused by the defective activity of GlcNAc-phosphotransferase, the enzyme localized in cis Golgi that recognize the lysosomal hydrolases for phosphorylation. Therefore lysosomal enzymes are not phosphorylated, not recognized by M6P receptors and they are carried to the cell surface and secreted by a default pathway (Raas-Rothschild *et al.*, 2000).

1.2.1 Classification of Lysosomal Storage Disorders

There are different classifications of the LSDs. The more frequent is according the chemical nature of the accumulation compound. The LSDs were classified into five different groups (Table 1.1). Defects in glycan degradation (defects in glycoprotein degradation, defects in glycolipids degradation, defects in glycosaminoglycan and defects in glycogen degradation); Defects in lipid degradation; Defects in protein degradation; Defects in lysosomal transporters and defects in lysosomal trafficking.

TABLE 1. Lysosomal Storage Disorders

Protein Defect	Disease	OMIM	Cromosomal Localization
DEFECTS IN GLYCAN DEGRADATION			
Defects in glycoprotein degradation			
α-Sialidase	Sialidosis	608272	6p21.3
Galactosialidosis	Cathepsin A	256540	20q13.1
α -Mannosidase	α -Mannosidosis	248500	19q12
β-Mannosidase	β -Mannosidosis	248510	4q22
Glycosylasparaginase	Aspartylglucosaminuria	208400	4q32
α -Fucosidase	Fucosidosis	230000	1q34
α -N-Acetylglucosaminidase	Schindler	104170	22q13.1
Defects in glycolipid degradation			
A. GM1 Ganglioside			
β -Galactosidase	GM1 gangliosidosis / MPS IVB	230500	3p21.33
β -Hexosaminidase α -subunit	GM2-gangliosidosis (Tay-Sachs	606869	15q23
β -Hexosaminidase β -subunit	GM2-gangliosidosis (Sandhoff)	606873	5q13
GM2 activator protein	GM2 gangliosidosis	272750	5q31
Glucocerebrosidase	Gaucher disease	606463	1q21
Saposin C	Gaucher disease	176801	10q22.1
B. Defects in the degradation of sulfatide			
Arylsulfatase A	Metachromatic leukodystrophy	607574	2q13.31
Saposin B	Metachromatic leukodystrophy	176801	10q22.1
Formyl-Glycin generating enzyme	Multiple sulfatase deficiency	607939	3p26
β -Galactosylceramidase	Globoid cell leukodystrophy (Krabbe)	606890	14q.31
C. Defects in degradation of globotriaosylceramide			
α -Galactosidase A	Fabry	301500	Xq22.1
Defects in degradation of Glycosaminoglycan (Mucopolysaccharidoses)			
A. Degradation of heparan sulphate			
Iduronate sulfatase	MPS II (Hunter)	309900	Xq28
α -Iduronidase	MPS 1 (Hurler, Scheie)	607015	4p16.3
Heparan N-sulfatase	MPS IIIa (Sanfilippo A)	252900	17q25.3
Acetyl-CoA transferase	MPS IIIc (Sanfilippo C)	252930	14
N-acetyl glucosaminidase	MPS IIIb (Sanfilippo B)	252910	17q21
β -glucuronidase	MPS VII (Sly)	253220	7q21.11

TABLE 1. Continued

N-acetyl glucosamine 6-sulfatase	MPS IIId (Sanfilippo D)	252940	12q14
Degradation of other mucopolysaccharides			
N-Acetylgalactosamine 4-sulfatase	MPS VI	253200	5q11-13
Galactose 6-sulfatase	MPS IVA (Morquio A)	253000	253000
Hyaluronidase	MPS IX	601492	601492
Defects in degradation of Glycogen			
α-Glucosidase	Pompe	232300	17q25
DEFECTS IN LIPID DEGRADATION			
Defects in degradation of sphingomyelin			
Acid sphingomyelinase	Niemann Pick type A and B	607808	11p15.2
Acid ceramidase	Farber lipogranulomatosis	228000	8q22
Defects in degradation of triglycerides and cholesteryls ester			
Acid lipase	Wolman and cholesteryl ester storage disease	278000	10q23.2
DEFECTS IN PROTEIN DEGRADATION			
Cathepsin K	Pycnodystostosis	601105	1q26
Tripeptidyl peptidase	Ceroide lipofuscinosis 2	607998	11q15.5
Palmitoyl-protein thioesterase	Ceroide lipofuscinosis 1	600722	1p32
DEFECTS IN LYSOSOMAL TRANSPORTERS			
Cystinosin (cystin transport)	Cystinosis	606272	17p13
Sialin (sialic acid transport)	Salla disease	604322	6q14
DEFECTS IN LYSOSOMAL TRAFFICKING PROTEINS			
UDP-N-acetylglucosamine Phosphotransferase γ-subunit	Mucolipidosis III (I-cell	607838	16
Mucolipin-1(cation channel)	Mucolipidosis IV	605248	19p13
LAMP-2	Danon	309060	Xq24
NPC1	Niemann Pick type C	607623	11q11-12
CLN3	Ceroid lipofuscinosis	607072	16p12.1
CLN 6	Ceroid lipofuscinosis 6	606725	15q21-23
CLN 8	Ceroid lipofuscinosis 8	607837	8pter-p22
LYST	Chediak-Higashi	606897	1q42
MYOV	Griscelli Type 1	160777	15q21
RAB27A	Griscelli Type 2	603868	15q21
Melanophilin	Griscelli Type 3	606526	2q37
AP3 □-subunit	Hermansky Pudliak 2		603401

1.3 Alpha-Mannosidosis

α-Mannosidosis is a lysosomal storage disorder belonging to the group of defects in glycoprotein degradations. It is caused by the deficiency of lysosomal α-mannosidase (LAMAN, EC 3.2.1.24) and is known to occur in man (Öckerman et al., 1967), cattle (Hocking *et al*., 1973), and cat (Burditt *et al*., 1980). The deficiency of LAMAN causes the intralysosomal accumulation of oligosaccharides carrying α1,2-, α1,3- and α1,6-mannosyl residues at their non-reducing termini. These oligosaccharides mainly originate from the intralysosomal degradation of glycoproteins with N-linked oligosaccharides (Fig 1.5).

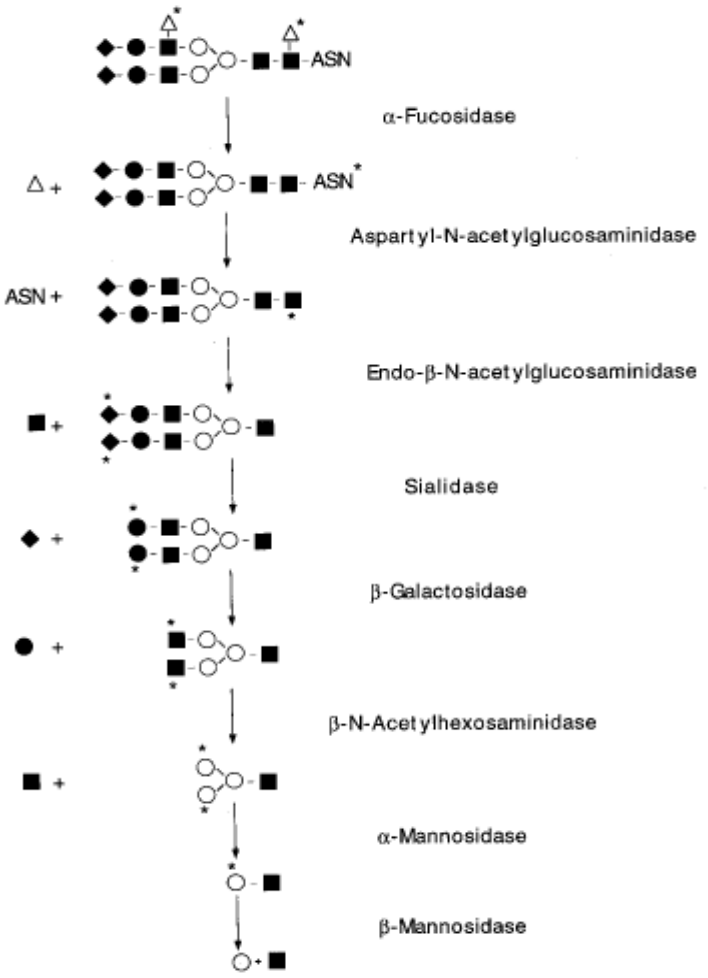

Figure 1.5: Lysosomal degradation pathway of glycoproteins carrying complex-type oligosaccharides. The asterisks indicate the residue targeted for hydrolysis in the next step.

The lysosomal enzymes involved in these steps include, α-fucosidase, β-N-acetylglucsaminidase, sialidase), β-galactosidase, β-N-acetylhexoaminidase, α-mannosidase and β-mannosidase (reviewed by Thomas G., 2001). It should be noted that the stored oligosaccharides originate from the catabolism of dolichol-linked oligosaccharides and from misfolded glycoproteins are redirected to the cytosol for degradation by the proteasome (Hirsch *et al*., 2003; Saint-Pol *et al.*, 1999). These oligosaccharides are trimmed in the cytosol and imported into lysosomes by an ATP-dependent mechanism (Saint-Pol *et al.*, 1999). The lysosomal storage is observed in a wide range of cell types and tissues, including neurons in all regions of the brain. The extent of storage, however, has been shown to be cell type and tissue specific. The clinical phenotype of α-mannosidosis is heterogenous, ranging from severe infantile forms to mild juvenile forms with moderate mental retardation, dysostosis multiplex, coarsening of the face, impaired hearing, recurrent infections and mild hepatosplenomegaly (Thomas G.H., 2001). In its most frequent form, the disease becomes overt within the first year of life. Multiple mutations are found in human α-mannosidosis, but a genotype-phenotype correlation is not apparent (Berg *et al*., 1999).

1.4 Enzyme Replacement Therapy (ERT)

Enzyme replacement and bone marrow transplantation (BMT) are the major therapeutic options in lysosomal storage disorders (for recent reviews see: Neufeld, 2004; Dobrenis, 2004). Enzyme replacement therapy (ERT) is an effective means to improve the clinical manifestations in type I Gaucher disease (Barton *et al*., 1991) and has meanwhile been approved for several lysosomal storage disorders including some involving the brain (Neufeld, 2004). Reports on ERT in α-mannosidosis are so far lacking. The few attempts of BMT in human α-mannosidosis have had variable outcomes, but have indicated that successful engraftment can increase language, social and motor skills (Will *et*

*a*l., 1987; Wall *et al*., 1998; Malm *et al*., 2004). A remarkable success of BMT that included metabolic correction in the brain, has been observed in feline α-mannosidosis (Walkely *et al*., 1994).

Early experiments revealed that the metabolic defect of cultured fibroblasts from mucopolysaccharidosis patients can be compensated by addition of corrective factors which proved to be the wildtype counterparts of the deficient lysosomal enzymes (Fratantoni et al., 1969). The added enzymes are rapidly internalized into the lysosomal compartment where they catabolize the accumulated substrates. Importantly, only 1-5% of the normal cellular activity was required for correction. The detection of this corrective mechanism led to the optimistic prediction that LSDs should be generally treatable by administration of the respective intact lysosomal enzyme, a treatment strategy designated as enzyme replacement therapy (ERT).

The uptake of lysosomal enzymes into the lysosomal compartment of fibroblasts and other cells depends on receptor-mediated endocytosis via a mannose 6-phosphate receptor (MPR), as explained in section 1.1. Due to the requirement of soluble ligands and their receptor-mediated routing to the endosomal/lysosomal compartment, ERT is a therapeutic option for deficiencies of soluble lysosomal polypeptides, but generally not applicable to LSDs caused by the lack of membrane-bound polypeptides and soluble polypeptides residing in compartments not involved into endocytosis.
ERT of animal models for various LSDs such as MPS I (Kakkis *et al.*, 2001), MPS IIIB (Yu *et al*., 2000), MPS VI (Crawley *et al.*, 1996; Byers *et al*,. 2000), MPS VII (Vogler *et al.*, 1999), Fabry disease (Ioannou *et al.*, 2001), Niemann-Pick disease (Miranda *et al.*, 2000) , Pompe disease (Bijvoet *et al.*, 1999, Zhu *et al.*, 2004) revealed that intravenously infused lysosomal enzymes are rapidly internalized by liver, spleen and other peripheral tissues, but usually do not enter the brain parenchyma in therapeutically efficient amounts. As a consequence the visceral, but not the CNS pathology can be improved.

Experimental attempts to overcome the intact blood brain barrier comprise invasive strategies, e.g., intracerebroventricular infusion or temporary disruption of the tight junctions between cerebral endothelial cells by infusing hypertonic solutions (reviewed by Shermann, 2002). The associated risks of infection and neuropathological side effects directed attention to non invasive delivery strategies based on conjugates between blood-brain shuttle vectors and therapeutic enzymes. Though shuttle vectors have been used with some success to target conjugated enzymes to neurons and the brain, it is not yet decided how far the transportation rate is sufficient to achieve therapeutically efficient enzyme levels in the CNS (reviewed by Scherrmann, 2002).

Preclinical and clinical studies demonstrate that ERT can reduce the substrate accumulation in visceral tissues leading to improvement of pathological aspects and gain of life quality. ERT is a reality for Fabry disease (Mignani *et al.*, 2004) and Hurler disease (Wraith *et al.*, 2004). Clinical studies with recombinant human enzymes are ongoing in Pompe desease (Van de Hout et al., 2004; Lachmann *et al.*, 2004), MPS II (Muenzer *et al.*, 2002) and MPS VI (Harmatz *et al.*, 2004). ERT seems to have, however, no effect on the CNS manifestation of neuronopathic LSDs and ameliorates bone and cartilage problems only to a limited extent. Other problems are immune responses to the replacement protein, the need for life-long intervention and the high costs of treatment.

1.5 Alpha-mannosidase knock-out model

To obtain a more accessible model for the study of therapeutic modalities in α-mannosidosis we have generated a mouse model for α-mannosidosis by disrupting the gene for LAMAN (Stinchi *et al*., 1999). A gene targeting vector was constructed to disrupt the α-mannosidase mouse gene and a homologous recombined embryonic stem (ES) cell clone was used to generate chimeras which transmitted the introduced mutation to their offspring. Homozygous

mutant animals exhibit LAMAN deficiency in brain, liver and kidney and elevated urinary secretion of mannose-containing oligosaccharides. Thin-layer chromatography revealed an accumulation of oligosaccharides in liver, kidney, spleen, testis and brain. Electron microscopic examination of α-mannosidase-deficient tissues confirmed a prominent lysosomal storage in different cells of liver, kidney, spleen, pancreas, testis, eye, thyroid gland, smooth muscle, bone and the central and peripheral nervous systems. The morphological lesions and their topographical distribution, as well as the biochemical alterations in mouse α-mannosidosis, closely resemble those reported for human α-mannosidosis.

While the histological and biochemical results underline the similarities between α-mannosidosis in human and mice, the clinical presentation exhibits some differences. In human, α-mannosidosis is presenting as a disease exhibiting a continuum of symptoms ranging from severe to mild forms. In its most frequent form, the disease becomes overt within the first year of life, mostly associated with psychomotor retardation, lens opacities. These clinical symptoms were not observed in α -mannosidase-deficient mice up to the age of 12 months. It therefore appears that the phenotype of murine α -mannosidosis corresponds to a mild form of the human disease. Such attenuation has also been described for other animal models of lysosomal storage diseases (Evers *et al.*, 1996; Hess *et al*,. 1996; Haskins *et al.*, 1979, Yamanka *et al.*, 1994). The reason for this phenotypic attenuation could be related to different rates of storage of oligosaccharides between species and occasionally has been shown to be related to the existence of metabolic bypasses (Phaneuf et al., 1996). It may be that the phenotype of α-mannosidase-deficient mice will worsen with the age and storage material continued accumulation. In fact we have observed a progression of the histological findings with age in the α-mannosidosis mice. In spite of the milder clinical phenotype, the lysosomal storage and excretion of mannose-containing oligosaccharides suggests that murine α-mannosidosis represents a valid mouse model for the human disease.

1.6 Aim of the study

The aim of this study is to analyze the efficacy of Enzyme Replacement Therapy (ERT) experiments with LAMAN of different species (bovine, recombinant mouse and recombinant human) in α-mannosidosis knock-out mouse model.

Groups of α-mannosidosis mice were injected with a single dose of LAMAN and killed at different times after the injection, with the objective of determine: the clearance of the injected enzyme in serum of injected mice; the stability and distribution of the enzyme in different organs (liver, kidney, spleen, heart and brain); and to study if the replacement therapy had a corrective effect on the mannose oligosaccharides storage in the discussed organs.

Once these data was established, groups of α-mannosidosis mice were injected with LAMAN to determine the duration of the treatment effect, and to find the indicated dose to reach the bigger correction of the storage, and the dose interval.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Specific laboratory equipment

Analysis balance 1602 MP and M5P	Sartorious, Göttingen
Electrophoresis camber for agarose gels	Werkstatt Institute
Centrifuges 5402 and 5415C	Eppendorf, Hamburg
Fluorescente Detector RF-10A XL	Shimadzu
Gene quant II, RNA/DNA calculator	Pharmacia Biotech, UK
Intelligent Dark Box II, LAS-1000+	Fuji, Japan
Phosphoimager Fujix BAS1000	Fuji, Japan
HPLC	Waters, USA
(column: Glucosepharose)	Ludger
Ice machine	Ziegra, Isernhagen
Liquid Scintillation Analyzer (1900 TR)	Packard, Canberra
Magnetic Stirrers Ika-Combimag Rer	Janke & Kunkel, Staufen
Mastercycler gradient	Eppendorf, Hamburg
Microplate reader 450	Bio-Rad, München
Multipipet	Eppendorf
pH-Meter	Beckmann, Munich
Photometer UV-VIS Cary 50 Bio	Varian, Darmstadt
Shaker water-bath	Köttermann, Häningsen, Göttingen
Sonicator (W-229F)	Heat Systems Ultrasonic, New York
Spry DC	Camag, Swizerland
Thermal block	Eppendorf, Hamburg

Ultrasonic bath	Bransonic
Ultra-turrax T8	IKA Labortechnik, Staufen
Vacuum concentrator	Bachofer, Reutlingen
Vacuum pump (ALPHA 1-2)	CHRIST
Vacuum pump (ALPHA 1-4)	CHRIST
Vortex-Genie	Bender & Hobein, Zürich
Water Bath Type HOR 7225	Köttermann, Häningen

2.1.2 Chemicals, plasticware and membranes

Chemicals	Boheringen/Roche, Manheim
	Merck, Darmstadt
	Roth, Karlsruhe
	Serva, Heidelberg
	Sigma, Deisenhofen
	Aldrich, Seelze
	Fluka
Autoclave bags	Sarstedt, Nümbrech
Centrifuge tubes	Nalgene, München
Glass microinserts	Macherey-Nagel, Düren
Glass pipettes	Schütt, Göttingen
Glass vials (brown)	Macherey-Nagel, Düren
Microliter syringes	Hamilton Bonaduz, Swizerland
Microtiter plates	Greiner
Needles for syringes	Becton Dickinson, Heidelberg
Nitrocellulose membranes	Schleich and Schüll, Dassel
Parafilm	American National Can™, Chicago
Pasteur Pipettes	Schütt, Göttingen
Pippete tips	Sarstedt, Nümbrech

Plastic tubes 10, 15 and 50 ml	Sarstedt, Nümbrech
Polycarbonate ultracentrifuge tubes	Beckman, Munich
Scisssors	Fine Science Tools, Heidelberg
Spattles	Fine Science Tools, Heidelberg
Sterile filters 0.2 µm and 0.45 µm	Sartorius, Göttingen
Syringes microfines U-100 insulin	Becton Dickinson, Heidelberg
TLC Plates (20x20 Silica gel F60)	Merck, Darmstadt
Tweezers	Fine Science Tools, Heidelberg
Whatman 3MMPaper	Schleich and Schüll, Dassel

2.1.3 Kits, spin columns and reagents

Proteins:

Bio-Rad Protein Assay	Bio-Rad, Munich
Bovine Serum Albumina (BSA)	New England BioLabs
Protease inhibitor cocktail	Sigma, Deisenhofen

Columns:

Sephadex G25 column	Amersham Bioscience

Detergenz:

Triton X-100	Sigma, Deisenhofen

2.1.4 Radioactively labelled substances

[^{3}H]-$NaBH_4$ in 0.1 M NaOH 33 Ci/ mmol	Amerscham, Braunschweig

2.1.5 Enzymes and standards

1-kb DNA marker	Invitrogen
α-glucosidase (from *Bacillus stearothermophilus*)	Sigma, Deisenhofen

α-mannosidase (from Jack bean)	Sigma, Deisenhofen
dNTP mix	Roche
Proteinase K	Roth
Oligonucleotide primer	NAPS, Göttingen
SDS-PAGE protein standard	Bio-Rad, Munich
Taq-DNA polymerase	Roche
Taq-DNA polymerase buffer	Roche

2.1.6 Mice strains

C57 BL/6J, black colour	Charles River, Germany
129SV/J, agouti colour	Charles River, Germany

2.1.7 Antibodies

Primary antibodies:

Rabbit antiserum raised again human LAMAN	Zymanex A/S, Denmark

Secondary antibodies:

Peroxidase conjugated goat anti mouse IgG	Dianova, Hamburg
HRP	Dianova, Hamburg

2.1.8 Primers

Name	Sequence	Description	T_m used for PCR
α-Man2	GCCAGGCAAGGGTTCTACCGCAG	DNA, unmodified	58°C
α-Man3	GAACAGACGCGTGTTGAACAT CA	DNA, unmodified	58°C

2.1.9 Stock Solutions

0.5 M EDTA	181.1 g	Ethylene Diamine Tetra Acetic acid (sodium salt) Dissolved in 800 ml of water, pH was set to 8 with NaOH. Vol. was made up to 1000ml.
Ethidium Bromide	0.2 g	Dissolved in 2m water, stored at 4°C
10x PBS	80 g 1.6 g	Sodium chloride Disodium hydrogen phosphate Dissolved in 800 ml of water, vol. was made up to 1000 ml
20%SDS	20g	Sodium dodecylsulfate Dissolved in 100 ml dd water at 65°C, and sterile flirtier.
10x TBS	1.5 M 0.1 M	Sodium chloride Tris/HCl pH 7.4
1M Tris/HCl	121.1 g	Tris base Dissolved in 800 ml water, pH was set to required value with concentrated HCl Vol. was made up to 1000 ml.
50x TAE	242 g 100 mM	Tris base EDTA To pH 8 with glacial acetic acid Final vol. of 1000ml in water

2.2 Methods

2.2.1 Molecular Biology

2.2.1.1 Preparation of genomic DNA from mouse tail biopsies

lysis buffer:	100 mM	Tris pH 8
	50 mM	EDTA
	0.5 %	SDS
	200 mM	NaCl
proteinase K:	100 µg/ml (stock solution 10 mg/ml)	

At the age of 3-7 weeks, 0.5 to 0.7 cm of tail was cut from the tail-tip of the mice. It was incubated with proteinase K in a total volume of 500 µl in a shaking incubator at 56°C and 700 rpm over night. The lysate was centrifuged for 10 min at 13 000 rpm. After decanting the supernatant into a new cup, the DNA was precipitated by addition of 1 volume isopropanol. The cup was inverted several times during 5-10 min and left standing at room temperature in between until a cloudy precipitate formed. The DNA was captured with a curved pasteur pipet and placed into 500 µl 70% ethanol for 1 min. After that wash, the DNA was air-dried on the pipet and finally dissolved in 50-200 µl of H2O over night at 37°C.

2.2.1.2 Polymerase chain reaction (PCR)

PCR is a technique to exponentially amplify in vitro a small quantity of a specific nucleotide sequence in the presence of DNA template sequence and two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. The reaction is cycled involving 3 steps, which are necessary for DNA amplification:

1. Template DNA denaturation to get single stranded DNA.
2. Primer annealing (binding of oligonucleotide primer to single strands).
3. Extension (synthesis of DNA, starting from bound primers).

PCR is used to amplify the genomic DNA isolated from mouse tail wings, and after separation by Agarose gel electrophoresis (section 2.2.1.3) determine mice genotype.

Genomic PCR:

100 ng-1 µg genomic DNA was used as template DNA. The following primers were used:
Primer 1: α-Man2, sequence GCC AGG CAA GGG TTC TAC CGC AG
Primer 2: α-Man3, sequence GAA CAG ACG CGT GTT GAA CAT CA

PCR program:

1. 5 min 95°C
2. 30 sec 96°C
3. 30 sec 58°C
4. 2 min 72°C
   repeat 2-4 35 times
5. 3 min 72°C

	Stock concentration	Amount per 50 µl reaction
Taq DNA Polymerase	5 U/ml	1 µl
Taq Polymerase Buffer (10x)	100 mM Tris-HCl, pH 9.0 500 mM KCl, 15 mM MgCl2	5 µl
dNTP mix	1.25 mM each dNTP	1 µl
Oligonucleotide primer 1	0,1 nmol/ µl	2,5 µl (1:10)
Oligonucleotide primer 2	0,1 nmol/ µl	2,5 µl (1:10)

To perform several parallel reactions, a master mix was prepared containing water, buffer, dNTPs, primers and Taq DNA Polymerase in a single tube, which was then aliquoted into individual tubes. Template DNA solutions were then added.

2.2.1.3 Agarose gel electrophoresis of DNA

The size and purity of DNA is analyzed by agarose gel electrophoresis. Concentration of agarose used for analysis is inversely proportional to the size of the DNA of interest, that is, the larger the DNA, the lower is the concentration of agarose.

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Agarose concentration (%)	DNA size (kb)
0,6	20 - 1
0,9	7 - 0,5
1,2	6 - 0,4
1,5	4 - 0,2
2,0	3 - 0,1

Gel loading buffer (10x): 0.25% (w/v) Bromophenol blue
40% Saccharose in 1x TAE

Agarose was weighted and dissolved in 1x TAE by boiling in microwave oven. The agarose solution was allowed to cool till about 60 °C and ethidium bromide was added to a final concentration of 0.5 µg/ml. This was poured into the agarose gel cassette and allowed to polymerize completely. The sample DNA was mixed with gel loading buffer and loaded into the lane. The gel electrophoresis was carried out at 100 V. Ethidium bromide is a fluorescent dye which contains a planar group that intercalates between the stacked bases of the DNA. The fixed position of this group and its close proximity to the bases causes the dye to bind to the DNA to display an increased fluorescent yield compared to that of the dye in free solution. Ultraviolet radiation at 254 nm is

absorbed by the DNA and transmitted to the dye; radiation at 302 nm and 366 nm is absorbed by the bound dye itself. In both cases, the energy is re-emitted at 590 nm in the red orange region of the visible spectrum. Hence DNA can be visualized under a UV transilluminator. The gel was photographed using a gel documentation system.

2.2.2 Protein biochemistry

2.2.2.1 Expression and purification of recombinant human LAMAN in CHO cells

Human LAMAN cDNA isolated from HepG2 cDNA library and subcloned into an expression vector carrying a dihydrofolate reductase gene and the LAMAN cDNA under the control of the human CMV-promotor was expressed in Chinese Hamster Ovary (CHO) cells deficient in dihydrofolate reductase. The CHO cells were cultured in a two-compartment CELLine flask (Integra Biosciences Inc.) in serum free ExCell 302 medium (JRH Biosciences) supplemented with 20 nM methotrexate at 37°C in a humidified atmosphere containing 5% CO_2. The medium was diafiltrated using a Pellicon Biomax polysulphone filter with a 100 kDa cut off against 4 volumes 0.02 M Tris-HCl, pH 7.6. Ion-exchange chromatography was performed on DEAE-Sepharose FF (Amersham Pharmacia Biotech AB) using a NaCl gradient in 0.02 M Tris-HCl, pH 7.6. Active fractions were concentrated using an Amicon Centricon Plus-80 centrifugation filter with a 30 kDa cut off and subjected to gel filtration in a HiPrep 26/60 Sephacryl High Resolution column (Amersham Pharmacia Biotech AB) in 0.02 M Tris-HCl, pH 7.6, containing 0.15 M NaCl. After concentration the final preparation had a specific activity of 9-15 U/mg. Experiment was performed by Zymanex A/S, Denmark.

2.2.2.2 Expression and purification of recombinant mouse LAMAN

The cDNA encoding the mouse LAMAN (27) was subcloned in the expression vector pMPSVEH (28). A polyhistidine tail (6 residues) had been added to the C-terminal part of the enzyme. Mouse embryonic fibroblasts deficient in the small and the large mannose 6-phosphate receptors (mpr-/- MEF) (29) were transfected and stably expressing clones were selected with 50 µl/ml hygromycin. For production of recombinant mouse LAMAN the cells were cultured in medium supplemented with 10% FCS in a humidified atmosphere containing 5% CO_2. The secreted recombinant mouse LAMAN was purified from conditioned medium using a three step procedure. In the first step the medium was dialyzed against 20 mM sodium phosphate buffer pH 7,8 containing 500 mM sodium chloride and then loaded onto a Probond column (Invitrogen). The retained enzyme was eluted with a gradient 0 to 0.35 M of imidazole (total volume 80 ml) in 20 mM sodium phosphate buffer pH 6.0 containing 500 mM sodium chloride. The LAMAN containing fractions were dialyzed against 10 mM sodium phosphate, pH 6.0 and loaded onto a DEAE-cellulose. The enzyme was eluted in a 0 to 0.25 M sodium chloride gradient (total volume 80 ml) in 10 mM sodium phosphate buffer. Finally mouse LAMAN was adsorbed to ConA-Sepharose (loading buffer 20mM Tris-HCl, pH 7.4, containing 1 mM $MgCl_2$, 1mM $MnCl_2$, 1 mM $CaCl_2$ and 0.5 M NaCl), and eluted with α-mannopyranoside (0.0-1.0 M) in the same buffer. The final preparation had a specific activity of 17-25 U/mg. Experiment was performed by Chiara Balducci and Tommaso Beccari, University of Perugia, Italy.

2.2.2.3 Purification of bovine LAMAN

Bovine kidney LAMAN was purified from 10 kg of bovine kidneys. Kidneys (10 kg) were cut into small pieces and homogenised in 0.075 M acetic acid/0.15 M NaCl (1:2 mass/vol) using a Wering blendor. The homogenate was centrifuged

at 10000g for 10 minutes. To the supernatant was added ammonium sulphate to to 35% saturation and the mixture was stirred for at least 4 h and centrifuged at 10000g for 10 minutes. Ammonium sulphate was then added to 75% saturation and, after stirring, the solution was centrifuged as described before. The resulting pellet was dissolved in a minimum amount of 0.05 M sodium phosphate pH 7.4, 0.15 M NaCl (NaCl/Pi). The final volume was typically 4 l and the solution was labelled crude extract.

The crude extract was brought to 60 °C for 20 min and the precipitate was removed by centrifugation at 10000g for 10 min. Concavalin-A-Sepharose (Pharmacia) was added to the supernatant. The suspension was mixed for 2 h, the run through a column. The resulting column (2.5 x 10 cm) was washed with NaCl/Pi. Lysosomal α-mannosidase was eluted by 200 ml NaCl/Pi containing 0.2 M α-methylmannoside.

The eluate was applied to a 1.5 x 15 cm column of hydroxyapatite (Bio-gel HTP, Bio-Rad) equilibrated with NaCl/Pi. Elution was carried out with 0.25 M sodium phosphate pH 7.4, 0.15 M NaCl and eluate was dialyzed against 4 l 0.02 M Tris/HCl pH 7.6, with two changes of the medium.

The dialyzate was applied to a Q-Sepharose (Pharmacia) column (2 x 11 cm) equilibrated with l 0.02 M Tris/HCl pH 7.6 at a flow rate of 0.8 ml/min. Enzyme activity appeared in the flow through and was concentrated using a Amicon ultrafiltration unit fitted with YM 30 membrane. The concentrated sample was applied to Superdex 600 (1.5 x 60 cm, Pharmacia) equilibrated with NaCl/Pi at a flow rate of 0.4 ml/min. The fraction containing lysosomal α-mannosidase was collected and concentrated through a Centricon 30 microconcentrator (Amicon). The purity was assessed by SDS/PAGE.

Bovine LAMAN was purified from kidney as described by Olle Tollersrud, university of Tromso, Norway. (Tollersrud *et al*., 1997). The final preparation had a specific activity of 10 U/mg.

2.2.2.4 Preparation of serum

Blood was taken from the mice retroorbital plexus 5 minutes after each LAMAN injection (see section 2.2.4.3). Blood samples were incubated first for 30 min at room temperature, then 60 min at 4°C and centrifuged for 30 min at 4.500 rpm and 4°C. The supernatant was carefully transferred into new tubes. Aliquots were frozen at -20°C.

2.2.2.5 Determination of protein concentration using the BIORAD reagent

Bovine Serum Albumin (BSA) stock solution 1 mg/ml
Concentration range: 0.02 – 0.16 mg/ml

To determine the protein concentration of the three different LAMAN species, Bio-Rad protein assay was employed which is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomasie Brillant Blue G-250 shifts from 494 to 595 nm when binding to a protein occurs. A standard curve was made using BSA in the range of 0,02 - 0,16 mg/ml. 5 µl of the sample (diluted 1:20 in TBS) was used for the protein estimation. In a 96-well microtiter plate, 100 µl of each standard dilution and the samples to be measured were pipetted with 225 µl of the BIORAD reagent. The samples were incubated for 15 minutes at room temperature and optical density was measured at 595 nm in a microplate reader.

2.2.2.6 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

4x stacking gel buffer: 0.5 M Tris/HCl pH 6.8, 0.4% SDS

4x resolving gel buffr:	1.5 M Tris/HCl pH 8.8, 0.4% SDS, 0.4% APS
acrylamide (AA) solution:	30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide
ammonium peroxodisulfate (APS):	10% solution in water
5x sample buffer:	0.225 M Tris-Cl pH 6.8, 50% glycerol, 5% SDS, 0.05% bromphenol blue, 0.1M DTT
5x anode buffer:	50 mM Tris base, 192 mM glycine
5x cathode buffer:	0.5% SDS in anode buffer

SDS-PAGE is a biochemical technique where proteins are separated based on their molecular weight. The gel is discontinuous; it has a stacking gel which is present on top of the resolving gel. SDS-PAGE can be used to separate proteins based on their native size (native SDS-PAGE) or their subunit size (reducing SDS-PAGE). Two clean glass plates were fixed together with a spacer of appropriate thickness (0.75- 1.5 mm) using holders or clips. The resolving gel was poured and isobutanol was layered on top of it. The gel was allowed to polymerize for about 30 min and isobutanol was thoroughly washed off using double distilled water. The stacking gel was poured on top of the resolving gel and a comb, of appropriate size and appropriate number of wells, was placed in the stacking gel and left undisturbed for about 30 min. After polymerisation of the stacking gel, the combs were removed and the wells were cleaned with double distilled water to wash off any unpolymerized acrylamide.
Samples were mixed with sample buffer to a 1x concentration, incubated at 95°C for 5 min and cooled to room temperature before loading onto the gel. The gel was placed in a tank with either 1x cathode buffer (for a one-buffer-system) or 1x cathode/anode buffer (for a two-buffer-system) and run at 30-40 mA until the dye front passed the bottom of the gel. After removal of the stacking gel, proteins were either stained with Coomassie or Silver Staining or the gel was processed by Western Blotting.

	Resolving Gel (30 ml)	*Stacking Gel (10 ml)*
4x Buffer	7,5 ml	2,5 ml
Acrylamid (AA)	9,8 ml	1,3 ml
APS	250 µl	100 µl
Temed	25 µl	10 µl
Water	12,6 ml	6,1 ml

Glass plates were cleaned with 70% EtOH before use. Discontinuous gel system was used, were a non-restrictive large pore gel, called a stacking gel, is layered on top of a separating gel called a resolving gel. Polymerising solutions were prepared as follows for one gel 10% SDS-polyacrylamide.

2.2.2.7 Staining of Polyacrylamide gels.

2.2.2.7.1 Silver Staining

Fixative:	30% (v/v) Ethanol
	10% (v/v) Acetic acid
Sodium thiosulphate solution:	0.5 M Sodium acetate
	30% (v/v) Ethanol
	0.5% Gluteraldehyde
	0.2% Sodium thiosulphate
Silver nitrate stain:	0.1% (w/v) Silver nitrate
	0.02% (v/v) Formaldehyde (37%)
Developer:	2.5% (w/v) Sodium carbonate
	0.01% (v/v) Formaldehyde (37%)
Stop solution:	0.05 M Glycine

All the solutions were made in double distilled water with final volume of 250 ml. for one gel.

The gel was first incubated in fixative for 90 min at RT and then in Sodium thiosulphate solution for 1hour at RT or overnight at 4 °C. After 3 washes with dd water for 20 min each, the gel was stained with Silver nitrate solution for 1hour at RT and washed with water for 2 min. The gel was washed for 1 min with half of the Developer which was then discarded, and again washed for 1-4 min with rest of the solution. Developing was stopped with Stop solution, and then the gel was washed with dd water.

2.2.2.7.2 Coomassie Blue staining

Coomassie blue stain:	0.5% (w/v) Coomassie blue
	50% (v/v) Methanol
	10% (v/v) Acetic acid in dd water
Destaining solution	50% Methanol and 10% glacial acetic acid
	dissolved in dd water.

The gel was stained in Coomassie blue solution at RT for 1-2 hours and destained using the destaining solution overnight. After complete destaining the gel was washed with double distilled water for 15 min and then dried in a gel drier.

2.2.2.8 Determination of phosphorylation of LAMAN

MPR binding buffer:	50 mM imidazole HCl, pH 6.5
	0.15 M NaCl
	5 mM sodium β-glycerophosphate
	10 mM $MgCl_2$
	2 mM EDTA
	0.2% Sodium azide
Washing buffer:	5 mM glucose 6-phosphate
	In MPR binding buffer

Elution Buffer: 5 mM mannose 6-phosphate
In MPR binding buffer

500 µl of the three different LAMAN preparations (all diluted 1:300 in PBS / 2% FCS pH inactivated) was mixed with equal volume of binding buffer and incubated overnight at 4°C with an Affigel-10-based-affinity matrix (Bio-Rad) to wich a 1:1 mixture of MPR46 / MPR300 affinity matrix (2.5 mg/ml) purified from goat was inmovilized (Koster *et.al.*, 1993). The MPR46 / MPR300 was a kind of gift of Siva Kuma, Hyderabad, India. Unspecific bound material was removed by washing the column successively with MPR binding buffer (3x 1 ml) and with washing buffer (2x 1 ml) of 5 washing buffer. M6P-containing proteins ere eluted with elution buffer (5x 1 ml). Fractions of 1 ml were collected
LAMAN activity was determined (see section 2.2.2.10) in the unspecific bound material fractions and in the fractions eluted with 5 mM mannose 6-phosphate.

2.2.2.9 Preparation of organ extracts

TBS/PI Buffer: 10mM Tris/HCL pH 7,4
150 mM NaCl
1 mM PMSF (in isopropanol)
1 mM iodoacetamide
5 mM EDTA

Mice organs (liver, spleen, kidney, heart and brain) were collected at killing time (section 2.2.2.4) and frozen in liquid nitrogen for storage at -80°C. Organs extracts were homogenized for the determination of LAMAN activity.
An appropriate amount of tissue (50-70 mg) of collected was cut into mm-sized pieces were homogenized with an ultra turrax at 4°C in 9 volumes (per weight) of TBS/PI. Triton X-100 was added to a final concentration of 0.5% w/v (1% for liver) and after incubation for 30 minutes on ice, the samples were sonicated (3 times 20 sec) and then centrifuged for 15 minutes at 13000 g (4°C). The

supernatant was immediately used for LAMAN activity determination, or stored at -20°C.

2.2.2.10 LAMAN activity assays

Buffer:	0.2 M sodium citrate pH 4.6
	0.008 % NaN_3
	0.4 % BSA
	0.9 % NaCl
Substrate:	10mM p-nitrophenyl-α-mannopyranoside (in buffer)
Stop solution:	0.4 M Glycin/NaOH pH 10.4

For determination of LAMAN activity in the organ extracts (section 2.2.2.9) and in serum (section 2.2.2.4), 10-50 µl of enzyme sample was added to 50 µl buffer and incubated with 50 µl substrate for 0.5-5h at 37°C. Volume was made up to 0.2 ml with 0.9% NaCl (see table).

One ml of the stop solution was added, samples were centrifuged at 13000 rpm for 10 minutes and absorbance was read at 405 nm (ε = 18500 $M^{-1}cm^{-1}$). All the determinations were done in duplicate and with the appropriate blanks (enzyme and substrate blanks).

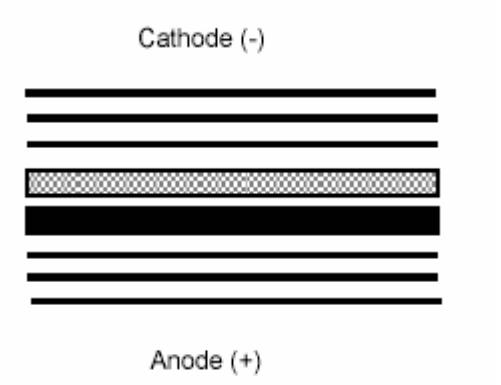
Probes	*Enzyme Blank (B_E)*	*Substrate Blank (B_S)*
50-10 µl sample	50-10 µl sample	50-10 µl water
50 µl substrate	50 µl water	50 µl substrate
50-90 µl 0.9% NaCl	50-90 µl 0.9% NaCl	50-90 µl 0.9% NaCl
50 µl buffer	50 µl buffer	50 µl buffer

2.2.2.11 Western Blotting

cathode buffer:	40 mM ε-aminocaproic acid
	20 mM Tris/HCl
	20% (v/v) methanol
	(pH 9 was adjusted with free Tris base)
anode buffer:	75 mM Tris/HCl
	20% (v/v) methanol
	(pH 7.4 was adjusted with HCl)
TBS:	10 mM Tris-Cl pH 7.4, 150 mM NaCl
TBST:	0.1% (v/v) Tween-20 in TBS
blocking buffer:	5% milk powder in TBST

For Western blotting of human LAMAN 20-40 µg of protein was separated on a 10% SDS-polyacrylamide gel. After SDS-PAGE electrophoresis, the proteins were transferred to PVDF membranes using a semi-dry blotting system. Transfer efficiency was checked with Ponceau staining.

Six pieces of 3 mm Whatman paper and one piece of nitrocellulose membrane were cut to the size of the SDS gel. Gel and membrane were equilibrated for 5-15 min in cathode buffer. The blot was assembled without air bubbles according to the following scheme:



For transfer, the current was set to 1 mA/cm2 gel size for 45-60 min. The membrane was then briefly washed with TBST and incubated in blocking buffer for one hour at room temperature. Decoration with the primary antibody (rabbit antiserum raised against recombinant human LAMAN, diluted 1:50000) diluted in blocking buffer occurred overnight at 4°C. After washing three times 10-15 min with TBST, the blots were incubated with horseradish peroxidase (HRP) coupled secondary antibodies, diluted 1:20000 in blocking buffer, for one hour at room temperature. The blot was washed three times 10-15 min with TBST and incubated with chemiluminescence substrate solution.
Signals were visualised using the ECL-Detection System (Amersham, Freiburg, Germany).

2.2.2.12 ELISA Assays

Covering Buffer:	10 mM Tris-HCl pH 7.5
	150 mM NaCl
Blocking solution:	3%BSA in PBS
Washing Buffer 1:	10 mM Tris-HCl pH 7.5
	150 mM NaCl
	0.05% Tween 20
Washing Buffer 2:	10 mM Tris-HCl pH 7.5
	150 mM NaCl
Substrate buffer:	50 mM Citrate-Phosphate buffer pH 4.3
Peroxidase substrate:	250 µl 40 mM ABTS
	6.5 µl 3% H_2O_2
	10 ml substrate buffer

Enzyme-linked Immunosorbent Assays (ELISAs) combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme. ELISAs can provide a useful measurement of antigen or antibody concentration. There are two main variations on this method: The ELISA can be used to detect the presence of

antigens that are recognized by an antibody or it can be used to test for antibodies that recognize an antigen. An ELISA is a five-step procedure: 1) coat the microtiter plate wells with antigen; 2) block all unbound sites to prevent false positive results; 3) add antibody to the wells; 4) add anti-mouse IgG conjugated to an enzyme; 5) reaction of a substrate with the enzyme to produce a colour product, thus indicating a positive reaction.

ELISA assays were done to determine the amount of IgG present in mice serum (see section 2.2.2.4) through ERT with LAMAN.

1 µg antigen (human LAMAN) in 50 µl covering buffer was incubated over night at 4°C in a 96-well microtiter plate. After washing 3 times with 200 µl washing buffer 1, 200 µl blocking buffer was added and the microtiter plates were incubated 1 h at room temperature. Plates were washed again 3 times with washing buffer 1, and 100 µl mice serum (diluted 1:10000) was added. After 3 h incubation at 37°C and washing another 3 times with washing buffer 1, 100 µl of peroxidase-conjugated goat anti mouse IgG (1:500 in washing buffer 1) was added and the plates were incubated 2 h at room temperature. The plates were washed 3 times with washing buffer 1 and 2 times with washing buffer 2. Peroxidase substrate (100 µl) was added and after 30 min incubation at room temperature, the optical density was measured at 405 nm. Water was used as blank and serum from mock injected mice as control.

2.2.2.13 Immunoprecipitation with pansorbin

Washing buffer:	PBS
PANSORBIN	Heat killed and inactivated *Staphylococcus aureus* cell suspension in PBS

Immunoprecipitation is a procedure by which antibody-antigen complexes are removed from solution by addition of an insoluble form of an antibody binding protein such as Pansorbin.

Immunoprecipitation assays were performed to determine if IgG antibodies were forming complexes with human LAMAN through ERT.

Pansorbin aliquots of 0.5 ml are washed with 1 ml PBS and centrifuged 10 min at 13000 rpm (4°C). 1ml of supernatant was removed, and pansorbin was washed 2 times more.
Mice serum (3 µl) from blood taken 5 minutes after each injection was added to 150 µl Human LAMAN in PBS / 1 mg/ml BSA and incubated over night on a rotating wheel at 4°C. Pre-washed pansorbin (60 µl) was added and after 1 h incubation at 4°C, samples were centrifuged 10 min at 13000 rpm (4°C). Supernatant was collected and pellet was 3 times washed with 1 ml PBS and resuspended in 210 µl PBS. LAMAN activity was measured in supernatant and pellet (see section 2.2.2.10).

2.2.2.14 LAMAN inactivation assays

Alpha-mannosidosis mice developed IgG antibodies titters through ERT. To ensure if these antibodies were inactivating the human LAMAN, the next experiment was performed.
Mice serum (2 µl) from blood was taken 5 minutes after each injection and was incubated overnight with 198 µl Human LAMAN (5-8 U/mg) in PBS / 1 mg/ml BSA on a rotating wheel at 4°C. Samples were prepared in duplicate and LAMAN activity was measured before and after incubation (section 2.2.2.10).

2.2.3 Oligosaccharides chemistry

2.2.3.1 Isolation of oligosaccharides

Tissue samples (50-60 mg) collected from mice at killing times (section 2.2.4.4) were cut into small pieces and homogenized with 0.6 ml H_2O (HPLC grade) at 4°C. After freezing (-20°C) and thawing twice, samples received ultrasonic

treatment (3 times 10 sec) and proteins were precipitated by the addition of 2.6 ml methanol of 100%. After vortex and centrifugation 3 min at 4500 rpm (4°C), supernatants were collected with Pasteur pipettes and 1.3 ml 80% methanol was added to pellets. Samples were sonicated (10 sec), vortexed, centrifuged 5 min at 4500 rpm (4°C), and supernatants were pooled with these from the first extraction. For lipids extraction, 0.9 ml chloroform was mixed with the supernatants and after centrifugation 2 min at 3300 rpm (4°C), 2.73 ml water (HPLC grade) was added. Samples were vortexed, centrifuged 10 min at 4000 rpm (4°C) and supernatants were desalted by incubation for 1 h at 4°C with 0.25 g mixed-bed ion-exchange resin (AG 501-X8, 20-50 mesh). The unbound material was lyophilized and resuspended in HPLC water to a final concentration of 1 mg tissue per µl.

2.2.3.2 Separation of neutral oligosaccharides by thin layer chromatography (TLC)

Running Buffer 1:	n-Butanol / acetic acid / H_20 (100:50:50)
Running Buffer 2:	n-propanol / nitromethan / H_20 (100:80:60)
Staining solution:	0,2% orcinol in H_2S0_4 (20% in water)

TLC plates (20x20 Silica-gel F60, Merk) were previously heated 15-20 min at 110°C, and neutral oligosaccharides extracted from equal aliquots of tissue were loaded (10 µl, in 4 times) as a line at 1.5 cm from the edge of the plate and with a wide of 1.5 cm for each sample. After drying the plates at room temperature for ~ 1h, the oligosaccharides were separated by developing overnight with running buffer 1. After drying (~ 1h at room temperature and then 5 min at 110°C), the plates were developed for 4 h in running buffer 2. The plates were dried for 1 h at room temperature and then 5 min at 110°C, Sprayed with the staining solution, and heated at 110°C for 5-10 minutes, until desired

stained level. The size of the oligosaccharides was determined by MALDI-TOF (see section 2.2.3.6).

2.2.3.3 Densitometric analysis of TLC plates

TLC plates were placed into the Intelligent Dark Box II LAS-1000+ (Fuji) and photographed with Phosphoimager Fujix BAS1000 (Fuji). Images were analysed for densitometry quantization with the help of bioinformatics software (AIDA Image Analyzer V3.10.039).

2.2.3.4 Digestion of oligosaccharides with α-glucosidase or jack bean α-mannosidase

To avoid the interference with glycogen derived oligosaccharides, 20 µl of the oligosaccharide extracts (2.2.3.1) from liver and heart were incubated overnight at 37°C with 40 U/ml **α-glucosidase** from *Bacillus stearothermophilus* in 20 mM phosphate, pH 6.8. The incubation mixture was heated at 96°C to denature the proteins. After centrifugation 10 min at 13000 g, the supernatant was desalted by incubation 1h at 4°C with a ion-exchange resin (AG 501-X8, 20-50 mesh), lyophilized and resuspended in 20 µl of water, and then separated by TLC (2.2.3.2).

To verify the nature of the oligosaccharides, 20 µl of the oligosaccharide extracts (2.2.3.1) were incubated overnight at 37°C with 30 U/ml **α-mannosidase** from Jack Bean in 0.1 M sodium acetate pH 5.0, containing 2 mM $ZnCl_2$. After incubation the oligosaccharides were prepared as described for samples digested with α-glucosidase and separated by TLC (2.2.3.2).

2.2.3.5 Quantitative analysis of neutral oligosaccharides in organs (HPLC)

Labelling Buffer:	0.34 M 2-anthranilamide
	1 M $NaBH_3CN$
	in DMSO / acetic acid (7:3), freshly made
Sample Buffer:	Acetonitrile / 80 mM Ammoniumformiat, pH 4.4 (65:35)
HPLC Buffer A:	Acetonitril
HPLC Buffer B:	Amoniumformiat 80 mM, oH 4.4

2.2.3.5.1 Preparation of labelling buffer

Labelling buffer was prepared in brown glass vials. 4.7 mg of 2-anthranilamide was dissolved with 100 µl DMSO:Acetic Acid (7:3). DMSO and Acetic Acid should be dehydrated (with Na_2SO_4). These 100 µl were used to dissolved 6.28 mg of $NaBH_3CN$ previously weight under a N_2 atmosphere in a brown glass vial.

2.2.3.5.2 Labelling of samples

The neutral oligosaccharides (0.3 µl) prepared from mice tissues (2.2.3.1) were mixed in glass microinserts with 220 pmol (10 µl) of a decasaccharide which served as an internal standard and had the composition $GlcNAc_4Man_3Gal_3$. The mixture was lyophilized and resuspended in 5 µl of the labelling buffer. After incubation for 2 h at 65°C, the samples were purified by paper chromatography.

2.2.3.5.3 Paper Chromatography

The samples (5 µl) were load onto Whatman Paper (3x15 cm), 3 cm from the origin. The starting point was marked, and the chromatography was developed in ethyl acetate for about 40-50 min. After drying at room temperature, the

oligosaccharides, which remain at the starting point, were extracted by sonicating the paper in 2 ml of HPLC water (2 times 1 ml). Samples were lyophilized and dissolved in 300 µl of sample buffer.

2.2.3.5.4 HPLC

The oligosaccharides were loaded onto a Gluco-Sepharose column (Ludger) and eluted with the acetonitrile / ammonium formiate buffer (65:35) at a flow rate of 0.4 ml/min during 72 min. Afterwards the column was washed following the schema:

Time (min)	*Flow (ml/min)*	*% Buffer A*	*% Buffer B*
0	0.40	65	35
72	0.40	47	53
75	0.40	0	100
77	1.00	0	100
92	1.00	0	100
95	1.00	65	35
100	0.40	65	35
105	0.00	65	35

Fluorescence (excitation 350 nm, emission 450 nm) was recorded (Shimadzu, RF-0A XL) and the mass of the oligosaccharides determined by MALDI-TOF (2.2.3.6).

2.2.3.6 MALDI-TOF

Mass spectrometry is an important tool for analyzing and characterizing large biomolecules of varying complexity. The matrix assisted laser desorption/ionization (MALDI) technique has enabled the analyses of large

biomolecules by mass spectrometry to become easier and more sensitive. An attractive feature of the time-of-flight (TOF) mass spectrometer is its simple instrumental design. TOF mass spectrometers operate on the principle that when a temporally and spacially well defined group of ions of differing mass/charge (m/z) ratios are subjected to the same applied electric field and allowed to drift in a region of constant electric field, they will traverse this region in a time which depends upon their m/z ratios.

MALDI-TOF was used for the analysis of neutral mannose oligosaccharides in the tissues of α-mannosidosis mice (2.2.3.1).

Samples from HPLC fractions (2.2.3.5) were lyophilised and dissolved in 2-3 µl water. Water extracts from TLC plates (2.2.3.2) were derivatized with 1-phenyl-3-methyl-5-pyrazolone and dissolved in 2-3 µl water. 2,5-dihydroxybenzoic acid (DHB, 5 mg/ml in water) was used as matrix. DHB (0.5 µl) and 1 µl of sample were spotted onto the Anchorchip target (Bruker Daltonik), and dried under room temperature. Mass spectrometric analysis was performed on a Reflex III MALDI-TOF (Bruker Daltonik) with a 337nm UV laser.

2.2.3.7 Reduction of liver mannose oligosaccharides with ^{3}H-$NaBH_4$

Reducing agent:	^{3}H-$NaBH_4$ in 0.1 M NaOH (27.6 mCi/ml; 33 Ci /mmol)
Buffer:	1.0 M Borate Buffer pH 8

To verify that the correction of the storage of neutral mannose oligosaccharides was real, and not due to a loss of sugars during the reparation procedure, ^{3}H labelled oligosaccharides were prepared and used as spike-in control for the isolation of oligosaccharides of brain.

Neutral oligosaccharides isolated from 2.8 g liver of α-mannosidosis mice (2.2.3.1) were isolated (approx. 8.4 mmol in HPLC grade water), lyophilized and

dissolved in 400 µl buffer. Reducing agent (400 µl ≡ 333 mmol, 11mCi) was added slowly and pH was set to 8.1 with 55 µl 1M HCl (amount was previously calculated with pilot experiments). After incubation over night at room temperature, 0.5 mmol (18 mg) of unlabeled $NaBH_4$ was added, and after 3 hours incubation at room temperature, the mixture was slowly acidified to pH 5 with 42 µl acetic acid, to destroy the excess of borohydride. Sample was incubated 1 hour at room temperature.

2.2.3.8 Gel filtration chromatography

Elution buffer: 0.2 M Pyramidin acetate, pH 5.5

Gel filtration chromatography is a technique used for separation of proteins, peptides, oligonucleotides and other biomolecules on the basis of size. Molecules move through a bed of porous beads, diffusing into the beads to greater or lesser degrees. Smaller molecules diffuse further into the pores of the beads and therefore move through the bed more slowly, while larger molecules enter less or not at all and thus move through the bed more quickly. Both molecular weight and three dimensional shape contribute to the degree of retention. Gel Filtration Chromatography may be used for analysis of molecular size, for separations of components in a mixture, or for salt removal or buffer exchange from a preparation of macromolecules.

The ^3H labelled liver oligosaccharides (2.2.3.7) were fractionated by gel chromatography.
Sephadex G25 column was equilibrated with elution buffer and 300 µl ^3H-labelled mannose oligosaccharides were added slowly with the help of a Pasteur pipette. Sample was eluted and fractions of 500 µl fractions were collected. Fractions radioactivity was determined using a scintillation counter and plotted. Selected fractions for re-chromatography were pooled, lyophilized, dissolved in the appropriate amount of 0.2 M Borate Buffer pH 8.0, and further

analyzed until obtaining the desired sample containing all the ^{3}H labelled mannose oligosaccharides.

2.2.4 Animal manipulation and treatment

Animal experiments were approved and are registred under AZ: G 46.04 (Bezirksregierung Braunschweig).

2.2.4.1 Housing of mice

Mice were kept in the animal rooms of the biochemistry II department. Conditions were according to conventional standards, with a temperature of 20±2°C and 50±15% air moisture. The “day phase” lasted from 6-18 h, and midnight was generally assumed as time of mating.

The animals were housed in polypropylene cages with stainless steel mesh tops and solid bottoms with wood shavings as bedding material. Cages (267 x 207 x 104 mm or 425 x 266 x 188 mm) were changed once a week. The cages were supplied with a polycarbonate water bottle (capacity 250 ml) with a stainless steel nozzle and Durethane cap. Water bottles were filled as necessary and changed/washed once weekly.

Once a week, the floor of the animal room was swept and then mopped with a disinfectant solution (0.5% Tego 2000).

2.2.4.2 Injection of the mice

LAMAN was injected into the tail vein of 8-14 week old α-mannosidosis mice (final volume up to 5.3 µl/g body weight). Mock-injected mice received the same volume of 10 mM phosphate, pH 7.4 in 0.15 M NaCl (PBS). In a single experiment the mice originating from up to three litters did not differ in age. Five

min after injection, blood was taken from the retroorbital plexus to control for the amount of injected enzyme. Serum was prepared and stored at -20°C (2.2.2.1).

In the last performed experiment (see section 3.9), mice were also anaesthetized by intra peritoneal injection prior to LAMAN injection (section 2.2.4.3) with a dose of 75 µl per 10 gram body weight, and mice were kept at 37°C until recovery (about 30 minutes).

2.2.4.3 Perfusion of mice and collection of organs at killing time

perfusion solution:	PBS
anaesthetic:	10 mg/ml Ketavet (contains ketamin-Cl)
	2 mg/ml Rompun (contains xylazine-Cl)
	in 0.15 M NaCl
fixative solution:	0.1 M phosphate buffer, pH 7.4
	6% glutardialdehyde

Mice were anaesthetized by intra peritoneal injection with 20-25 µl. After several minutes, the mouse should be unable to move. Its pain sensitivity was tested by pinching the tail or the feet and by fixing the feet with needles. If the mouse still reacted to one of these treatments, another 50% dose of anaesthetic was injected. Increasing amounts of the agent may lead to an early death of the mouse, which is directly preceeded by gasping.
A strip of about 1 cm of the ventral skin and muscle was cut to access the peritoneum. Lifting the sternum, the diaphragm was dissected from the ribs and the thorax was opened and bent aside. A winged perfusion set, connected to a 20 ml syringe with 0.9% NaCl, was introduced into the left ventricle, at about 0.5 cm depth and without damaging the inner wall. A small cut was applied to the right atrium of the heart as an exit for the blood. The mouse was perfused by manual injection at about 1-2 ml/min flow. Dark-red blood should exit the heart and the mouth, liver and kidney should become pale.

Organs (liver, spleen, kidney, heart and brain) were excised from mice and immediately either frozen in liquid nitrogen for storage at -80°C.

For further microscopical analysis by Prof. Lüllmann-Rauch (University of Kiel), small sections of liver, kidney and brain were cut with a razor blade and immersed in crystal vials filled with fixative solution (see section 2.2.5)

2.2.4.4 Breakdown of Brain Blood Barrier (BBB)

Evans Blue 1% in PBS (100 µl) was injected into the tail vein of 8 week old α-mannosidosis mice and wild type mice. Evans Blue is a fluorescence chemical that can cross within minutes a damaged BBB. The mice were sacrificed 1 hour after injection, and to wash out Evans blue from circulation, the mice were perfused through the left cardiac ventricle with 50 ml PBS (see section 2.2.3.4).
Brains were collected, and one hemisphere was homogenized with the ultra turrax at 4°C in 1 ml PBS. After centrifugation 30 minutes at 3300 rpm (4°C), supernatants were collected and an equal amount of TCA was added for protein precipitation. The samples were incubated overnight at 4°C, centrifuged 30 min at 3300 rpm (4°C), supernatants collected, and after dilution 1:3 in EtOH, fluorescence was measured (Ex: 620 nm, Em: 680 nm) with Fluorescente Detector RF-10A XL, Shimadzu.
To know the amount of Evans blue present in each hemisphere a standard curve was made using Evans Blue in the range of 1 - 500 ng.

2.2.5 Histological examinations

For electron microscopy, small sections of liver and kidney collected at killing time were immersed in fixative solution 0.1 M phosphate, 6% glutardialdehyde pH 7.4. Tissue samples were post-fixed with 2% osmium tetroxide, dehydrated and embedded in Araldite. Semi-thin sections were stained with toluidine blue. Ultrathin sections were processed according to standard techniques. For histochemical investigations, the sections were inmersed with Bouin's solution diluted 1:4 in 10 mM phosphate pH 7.4, 0.15 M NaCl. Embedding was performed in low melting point paraffin (Wolff, Wetzlar, Germany). Serial sections (7 µm) were cut and mounted on glass slides covered with Biobond (British Biocell, London, UK). Central sections of each series were stained with haematoxylin and eosin for standard light microscopy.

3. RESULTS

Purified LAMAN of different species (bovine, recombinant mouse and recombinant human), were used to assess the efficacy of Enzyme Replacement Therapy (ERT) experiments in a generated model of α-mannosidosis knock-out mice. Reports on ERT in α-mannosidosis are so far lacking. The aim of the experiments described in this study was to determine the distribution of the enzyme in liver and peripheral tissues (spleen, kidney, heart, brain), its clearance in the serum, and the appropriate treatment dose and frequency to achieve a maximal correction of the storage in different organs.
Human recombinant LAMAN for the studies was provided by the company Zymanex (Stockholm, Sweden), mouse recombinant LAMAN by Tomasso Beccari (Perigia, Italy), and LAMAN purified from bovine kidney by Ole Tollersrud (Tromso, Norway).
Knock-out α-mannosidosis mice generated as described in Stinchi *et al*., 1999, were bred for the ERT-experiments in the animal facilities of the biochemistry department, Göttingen.

3.1 Characterization of the LAMAN preparations

3.1.1 Separation of the LAMAN preparations by SDS-PAGE

LAMAN purified from three different species (see sections 2.2.2.1; 2.2.2.2; 2.2.2.3) was used for enzyme replacement in α- mannosidosis mice. As separated by 10% SDS-PAGE (2.2.2.6) and stained with Coomassie Blue (2.2.2.7), the LAMAN from bovine kidney (Fig 3.1, lane 1) occurs as a mixture of polypeptides (11-48 kDa) generated by limited proteolysis from a common precursor (Tollersrud *et al*., 1997), while the LAMAN from recombinant mouse (Fig 3.1, lane 2) and recombinant human (Fig 3.1, lane 3) occur as a precursor of 130 kDa and polypeptides of ~70 and ~55 kDa.

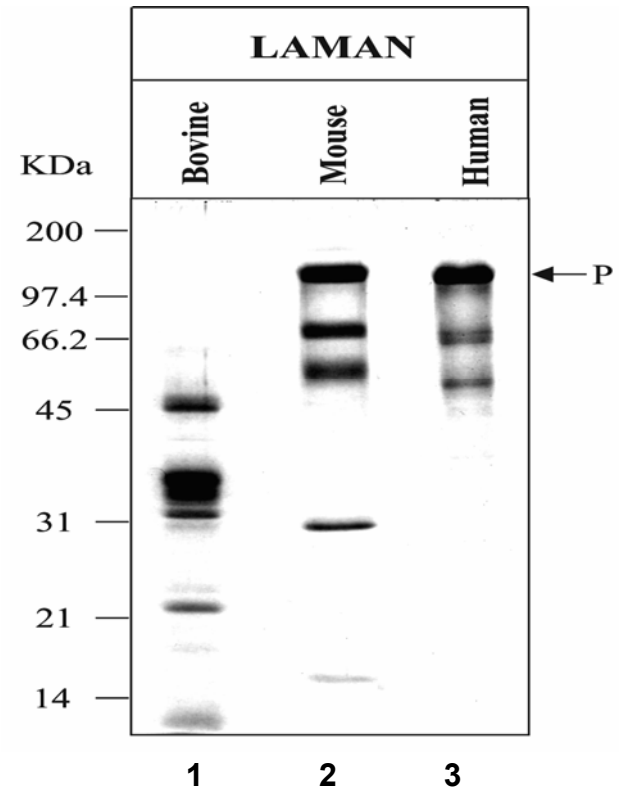


Fig. 3.1: Polypeptide pattern of bovine, mouse and human LAMAN.

10 µg of LAMAN purified from bovine kidney or the secretions of cell overexpressing mouse or human LAMAN were separated by 10% SDS-PAGE (2.2.2.6) and stained with Coomassie Blue (2.2.2.7). The 130 kDa polypeptides seen in mouse and human LAMAN correspond to the precursors (P) and the polypeptides ranging from 11 to 70 kDa to proteolytically processed forms of LAMAN. The 46-48 kDa polypeptides in bovine LAMAN represent partially processed intermediates.

3.1.2 Phosphorylation of the LAMAN preparations

The uptake of the different LAMAN species by the lysosomes is dependent on the presence of mannose-6-phosphate (M6P) groups. A high content of M6P groups will provide a high affinity signal to the M6P receptor (see section 1.1), helping the uptake of LAMAN by the lysosomes. To test for the content of M6P groups, a Man6P-receptor affinity matrix was used (see section 2.2.2.8).

The three LAMAN species separated in section 3.1.1 were incubated with the affinity matrix and after elution of bound material LAMAN activity was determined (see section 2.2.2.10) in the bound and unbound fractions.

The LAMAN from bovine kidney, as expected for a lysosomal hydrolase purified from tissue (2.2.2.3) and thereby having been exposed to endosomal / lysosomal phosphatase activity, exhibited a low content of Man6P-recognition marker. When incubated with a Man6P-receptor affinity matrix, 6.4% of the LAMAN activity bound to the matrix in a Man6P-dependent manner (Table 3.1).

The recombinant mouse and human LAMAN were isolated from the secretions of Man6P-receptor deficient mouse fibroblasts (2.2.2.2) and of CHO cells (2.2.2.1), respectively. The enzymes were largely isolated in their precursor forms, but contained a variable fraction (5-35%) of proteolytically processed forms of about 55 and 70 kDa (Fig. 3.1).

The mouse LAMAN had a higher content of Man6P-recognition marker which mediated binding of 73.6% of the activity to the affinity matrix. Of the human LAMAN only 4.2% bound in a Man6P-dependent manner to the Man6P-receptor affinity matrix, indicating that the content of Man6P-recognition marker is as low as for the bovine LAMAN (Table 3.1).

LAMAN species	*Total activity*	*Bound activity*	*% Binding*
Bovine	117.12	7.4	6.4
Mouse	105.62	77.74	73.6
Human	181.47	7.62	4.2

Table 3.1: MPR affinity chromatography.
The values give the activity (mU/µl) in the bound fraction measured in percent of total activity (bound and unbound fractions).

3.2 Corrective effect of a single intravenous injection of mouse LAMAN

As shown in section 3.1.2, the mouse LAMAN had a higher content of Man6P-recognition marker (73.6%) as compared to LAMAN of the other two species. Due to the high content of M6P groups, a higher uptake of the enzyme by the lysosomes was expected. Therefore mouse LAMAN was selected for the first replacement therapy with α-mannosidosis knock-out mice.

In order to study the short and the long term effect of a single dose of LAMAN on the storage of neutral oligosaccharides, eight α-mannosidosis mice at the age of 9 weeks received 100 mU mouse LAMAN per gram body weight (3.6 µl/g body weight) intravenously (2.2.4.2), and the mice were killed 2 h to 16 days after injection (2.2.4.3). Mouse LAMAN pattern (see section 3.1.1), was composed of about 90% precursor and 10% proteolytically processed forms. One α-mannosidosis mouse was injected with PBS and used like mock-control.

Analysis of alpha-mannosidase activity (2.2.2.10) in the serum (2.2.2.4) of blood taken 5 minutes after injection revealed that in the mice killed after 8 h and 1 day the greater part of the enzyme had been injected paravenously. The analysis was therefore restricted to mice killed after 2 h, 4 h, 10 h, 4 days, 8 days and 16 days.

3.2.1 Clearance in serum

In order to control for the amount of injected enzyme and its clearance from the circulation blood was taken from the retroorbital plexus 5, 30 and 60 minutes after injection (2.2.4.2) and LAMAN activity was determined (2.2.2.10). Five min after injection the LAMAN activity varied by less than 6% indicating that the mice had received comparable amounts of enzyme. The enzyme was cleared

from circulation with a half life of 19.4 minutes and a standard deviation of 1.5 minutes (Table 3.2).

Mouse killed after (h)	**LAMAN activity in serum (mU/ml)**		
	5 min	30 min	60 min
2h	1928	616	65
4h	1849	827	303
10h	2068	840	414

Table 3.2: LAMAN activity in serum of α-mannosidosis mice after injection of 100 mU mouse LAMAN per g body weight.

3.2.2 Stability and distribution of the enzyme

Mouse LAMAN injected α-mannosidosis mice were anaesthetized and killed by intracardial perfusion with PBS 2, 4 and 10 h after injection (2.2.4.3) and organ extracts were prepared for determination of LAMAN activity (2.2.2.10). Uptake of mouse LAMAN was seen into all organs except brain.

The maximum activity of LAMAN in the organs was observed 2-4 h after injection (Table 3.3). In liver the activity was 6-7 times higher than in control mice, but also in spleen and heart the maximum values exceeded those of controls. In kidney at maximum one fourth of the activity in control mice was reached. The small activity seen in brain may be due to enzyme located in the vascular system. The data from table 3.3 demonstrate that the LAMAN activity persists in brain at a constant level for at least 10 hours. As the LAMAN activity in the blood rapidly decreases with time, this observation suggests that the LAMAN activity in brain extract is not blood derived.

Genotype	**Mouse LAMAN injected (100mU/ g body weight)**	**LAMAN (mU/g wet weight)**				
		Liver	**Spleen**	**Kidney**	**Heart**	**Brain**
(+/+)* (n)	-	136,7±29,1 (19)	116,3±44,5 (3)	168,4±34,5 (9)	12,6±3,1 (3)	25±1 (2)
(-/-)* (n)	-	2,9±2,5 (21)	2,8±2,1 (4)	2,5±1,1 (10)	0,6±0,5 (3)	0,4±0,3 (3)
(-/-)$^{+}$	2h^{+}	1057	185	26	22	2,9
	4h^{+}	883	183	40	5	1,4
	10h^{+}	197	36	10	2	1,0

Table 3.3: LAMAN activity in tissue extracts of control and α-mannosidosis mice before and after injection of 100 mU mouse LAMAN per g body weight.
* +/+ refers to control mice, -/- to α-mannosidosis mice, and n to the number of animals investigated.
$^{+}$ All values were corrected for the mean α-mannosidase activity in serum at t_{zero} (2350 mU/ml serum). The correction factors were 0,98, 1,07 and 0,96 for 2, 4 and 10h respectively.

Between 4 and 10 h after injection the enzyme activity decreased rapidly in liver, spleen and kidney with an apparent half life of 2.8, 2.6 and 2.9 hours respectively. The immunoblot showed in Fig 3.2 demonstrates that the internalised precursor of LAMAN (130 kDa) was rapidly processed in liver to mature forms. 2 and 4 hours post injection only proteolytically processed forms of ~70 kDa and ~35 kDa (not seen in mock injected mouse) were detectable.

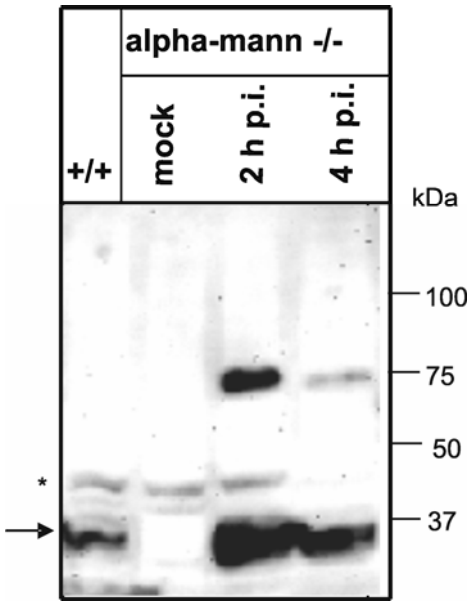


Fig. 3.2: Western Blot (WB) of mouse liver.
20 µg proteins from mouse liver were separated by 10% SDS-PAGE (2.2.2.6) and transferred to PVDF membranes (2.2.2.7). Rabbit antiserum raised against recombinant human LAMAN, (1:50000) was used as primary antibody and horseradish peroxidase (HRP) (1:20000) as coupled secondary antibody.
→ Endogenous alpha-mannosidase
* Non specific signals

3.2.3 Short effect of the treatment

Organ extracts were also prepared for the determination of neutral oligosaccharides (2.2.3.1). The hallmark of α-mannosidosis is the storage of neutral oligosaccharides in a wide variety of tissues (see Fig. 3.3, lane 1 and lane 2; Stinchi *et al.*, 1999)). As determined by mass spectrometry (2.2.3.6) and sensitivity to jack bean α-mannosidase (2.2.2.4, see outcome in Fig 3.10), the oligosaccharides M2 to M9 contain 2 to 9 mannose residues and a single N-acetylglucosamine residue at the reducing terminus, and therefore result from the action of an endoglucosaminidase. The amount of neutral oligosaccharides quantified by densitometry (2.2.3.3) in liver and spleen decreased progressively with time to 15% and to 7% of that observed in mock-injected animals, while in kidney and heart the storage was reduced only to about 49% and 74% (Fig. 3.3).

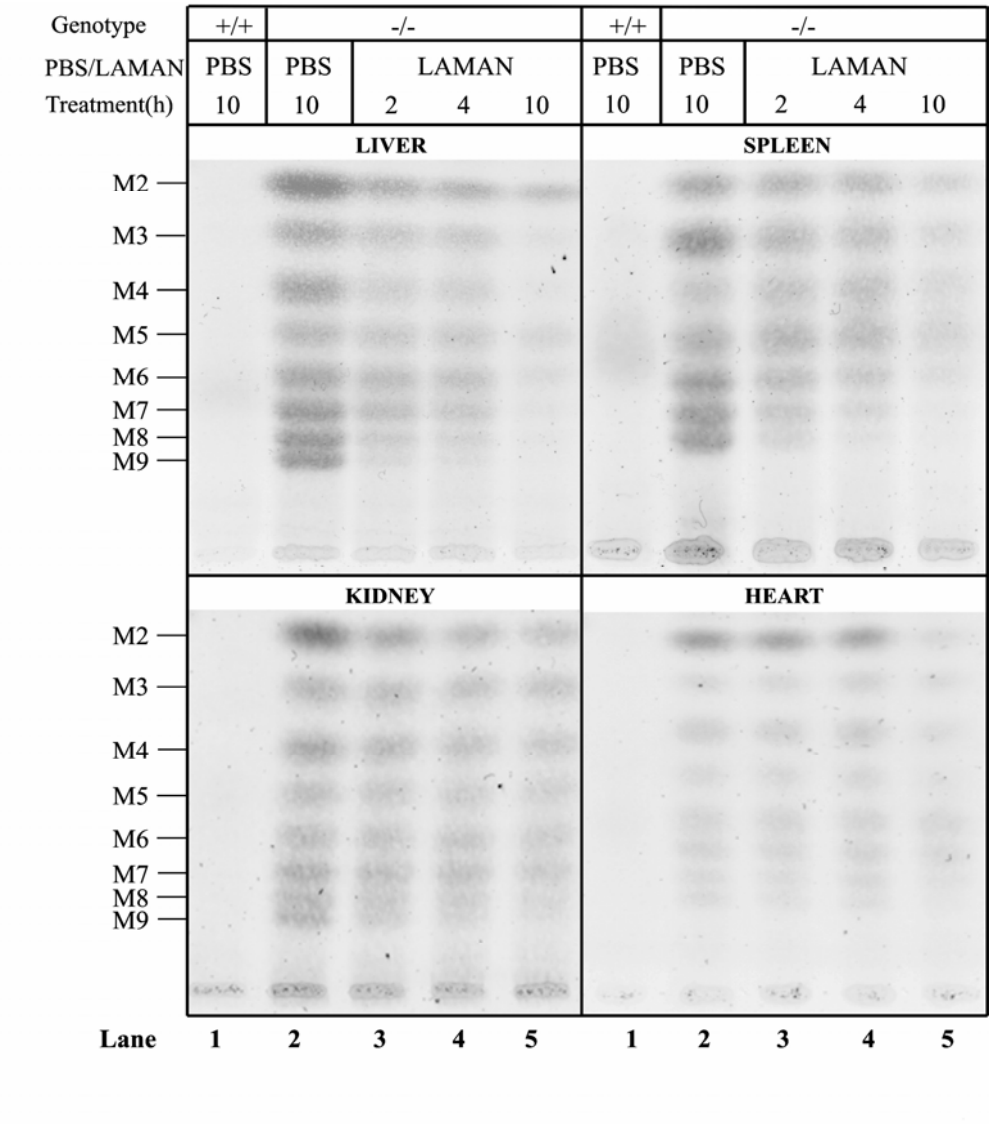


Fig. 3.3: Thin-layer chromatography of neutral oligosaccharides in liver, spleen, kidney and heart.

The fraction of neutral oligosaccharides from equal amounts of tissues was separated by thin-layer chromatography (2.2.3.2). Lane 1 contains the sample from mock-injected control mice (+/+), lane 2 from mock-injected α-mannosidosis mice (-/-), lane 3, 4 and 5 the samples from α-mannosidosis mice injected with 100 mU mouse LAMAN per g body weight and killed after 2, 4 and 10 h, respectively (see also Table 3.3). Oligosaccharides were detected with orcinol/ sulphuric acid (2.2.3.2) and quantified by densitometry (2.2.3.3). For calculation of the storage the amount of neutral oligosaccharides in α-mannosidosis mice was corrected for that in mock-injected control mice. The storage in LAMAN injected α-mannosidosis mice was expressed as percentage of that in mock-injected α-mannosidosis mice.

3.2.4 Duration of the treatment effect

To determine how long the corrective effect of a single dose of mouse LAMAN persisted, three α-mannosidosis mice were examined 4, 8 and 16 days after the injection of 100 mU mouse LAMAN per g body weight. At all time points the LAMAN activity in the organs (spleen, kidney, heart, brain) was in the range of non-treated or mock-injected α-mannosidosis mice (see table 3.3), except for liver, where 4 days after injection the LAMAN activity (15.1 mU/g wet weight) was still about 5-fold higher than in mock-injected mice.

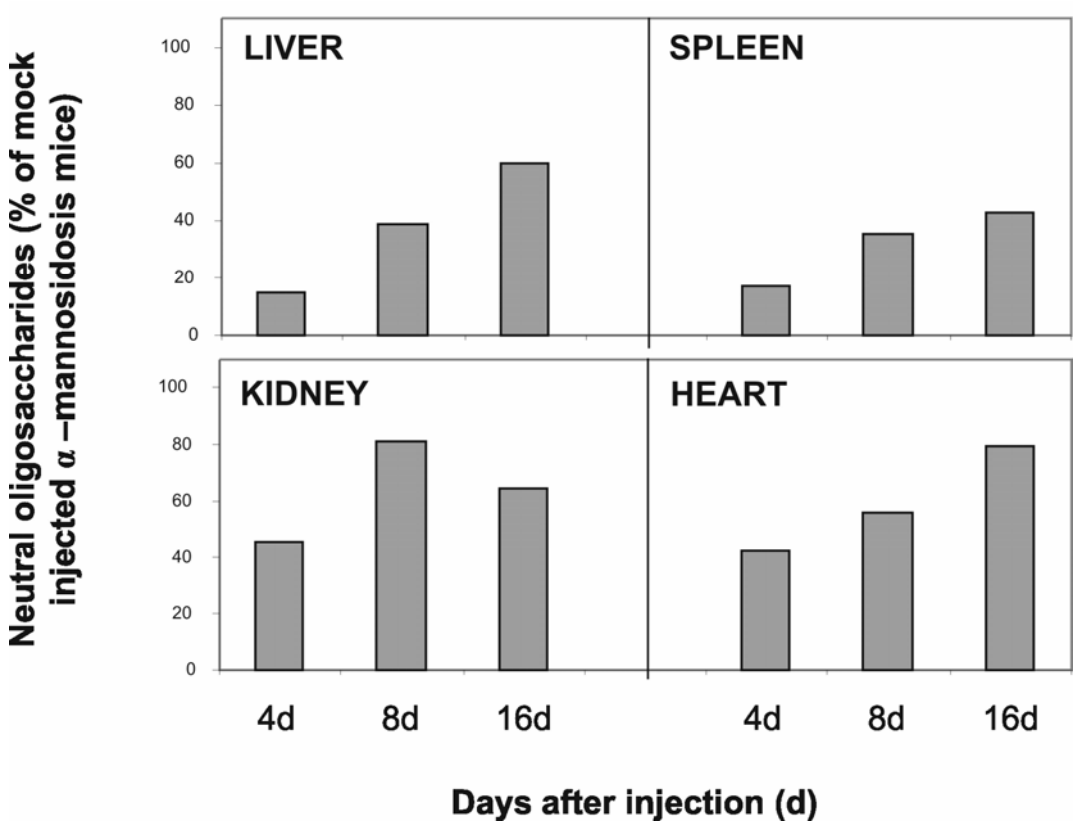


Fig. 3.4: Neutral oligosaccharides in tissue extracts of α-mannosidosis mice injected with 100 mU mouse LAMAN per g body weight.

The mice were killed 4, 8 and 16 days after injection and the neutral oligosaccharides in extracts of liver, spleen, kidney and heart were separated by TLC (2.2.3.2) and quantified by densitometry (2.2.3.3).

The storage of neutral oligosaccharides (as calculated in section 3.2.3) in liver, spleen and kidney 4 days after injection was in the range observed 10 h after injection (compare Fig. 3.3 and Fig. 3.4). In heart storage had decreased from 74% after 10 h to 42% 4 days after injection. This indicates that the corrective effect seen after 10 h persisted for about 4 days in spite of the fact that little or no LAMAN activity is detectable 10 h after injection in organs such as kidney or heart. After 4 days the storage of oligosaccharides clearly began to increase again. The increase observed between day 4 and day 16 after injection corresponded to 20-40% of the storage seen in mock-injected α-mannosidosis mice (Fig. 3.4).

3.3 Comparison of the clearance and corrective effect of bovine, mouse and human LAMAN

To compare the corrective effect of the LAMAN preparations from the three different sources of LAMAN, mice were injected with a dose of LAMAN that was expected to yield a partial correction. We therefore injected 50 mU LAMAN per gram body weight (0.88, 0.91 and 0.55 µl per gram body weight from bovine, mouse and human LAMAN respectively). LAMAN preparations were the same as characterized in section 3.1.1.

In the present study six α-mannosidosis mice at the age of 8 weeks were intravenously injected (2.2.4.2) with the dose discussed above. Two mice were injected with each enzyme source, and were killed 48 hours after the injection. One α-mannosidosis mouse was injected with PBS and used like mock-control.

3.3.1 Clearance in serum

In order to control for the amount of injected enzyme and its clearance from circulation, blood was taken from the retroorbital plexus 5, 15, 30, 35 and 65 min after injection, depending on the species of injected LAMAN (Table 3.4). These times were selected due to the expected half life times in serum.

The bovine and the human LAMAN were rapidly cleared from the circulation with half lifes of 4 min and 8 min, respectively (Fig. 3.5). The clearance of the highly phosphorylated mouse LAMAN was slower and at least biphasic. About 85% of the enzyme was cleared with an apparent half time of 12 min, while the apparent clearance of the remaining fraction was about 47 min (Fig.3. 5).

Two mice injected with	**LAMAN activity in serum (mU/ml) after**									
	5 min		**15 min**		**30 min**		**35 min**		**65 min**	
	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd
Bovine LAMAN	268.5	16.5	36.5	7.5	4.5	0.1	_		_	
Mouse LAMAN	484.0	37.0	_		_		101.5	7.4	69.5	5.3
Human LAMAN	476.0	54.0	229.5	37.5	53.5	6.5	_		_	

Table 3.4: LAMAN activity in serum of α-mannosidosis mice after injection of 50 mU/g LAMAN body weight from the three different sources. Mean and standard deviation (Sd) of two animals is shown.

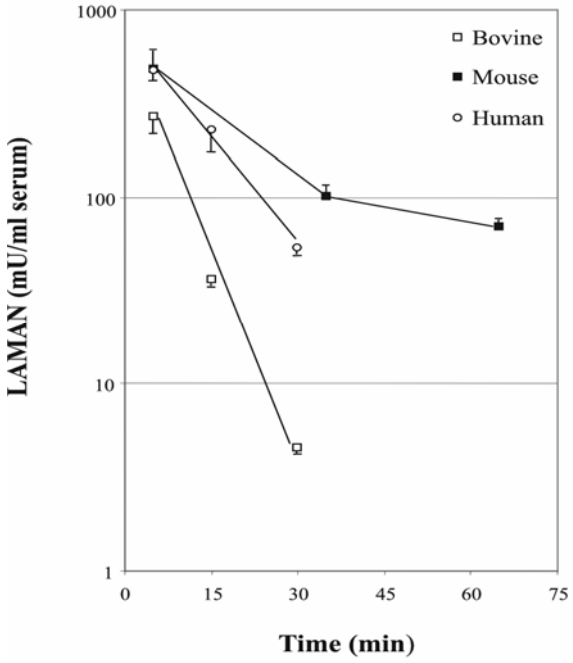


Fig. 3.5: Clearance of LAMAN from serum.
LAMAN of bovine (□), mouse (■) or human (○) origin, 50 mU/g body weight, was injected into the tail vein of 8 weeks old α-mannosidosis mice. Blood was collected from the retroorbital plexus 5 to 65 min after injection. Each value represents the mean of two animals. The range is indicated by bars where it exceeds the size of the symbols.

3.3.2 Corrective effect of the treatment

The mice were killed 2 days after injection and extracts from liver, spleen, kidney and heart were examined for neutral oligosaccharides (Fig. 3.6). With the exception of liver, the corrective effect was highest for the human LAMAN and lowest for the bovine LAMAN. The corrective effect of the mouse enzyme was intermediate. Only in liver was the corrective effect of the bovine enzyme (4% of the storage remaining) more pronounced than that of the human enzyme (14% of the storage remaining). In liver the corrective effect of mouse LAMAN was weakest (23% of the storage remaining).

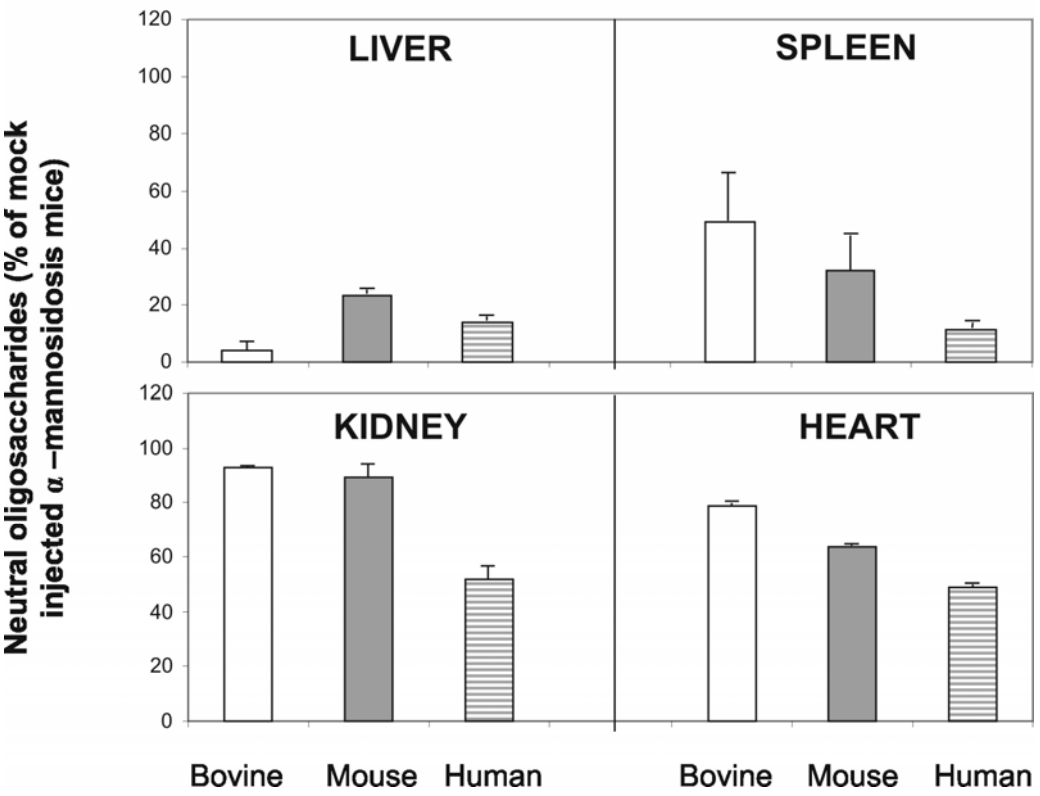


Fig. 3.6: Corrective effect of bovine, mouse and human LAMAN on the storage of neutral oligosaccharides 48 h after injection.

Mice received 50 mU LAMAN per g body weight (see Fig. 3) 2 days prior tissue analysis. Neutral oligosaccharides were separated by TLC (2.2.3.2) and quantified by densitometry (2.2.3.3). The bars indicate the range observed in two independent mice.

3.4 Corrective effect of human LAMAN at high dose

The comparison of the bovine, mouse and human LAMAN indicated that the poorly phosphorylated human LAMAN had a relatively higher corrective potential in kidney and heart, two organs which are more resistant to metabolic correction than liver and spleen.

To evaluate the corrective potential of the human enzyme, seven α-mannosidosis mice at the age of 14 weeks were injected with a single dose of 250 mU LAMAN/g body weight (1.6 µl/g body weight). Human LAMAN pattern (see section 3.1.1) was composed of about 70% precursor and 30% proteolytically processed forms. One α-mannosidosis mouse was injected with PBS and used like mock-control.
Mice were analysed 4 hours to 12 days after injection. To obtain insight in the half life of enzyme in tissues mice were killed after 4h, 8h, 16h and 24h. To follow the correction of storage and the persistence of the effect, mice were killed after 3d, 6d and 12d after injection. Five min after injection the LAMAN activity had a mean of 3729 mU/ml in 9 injected mice and a standard deviation of 103 mU/ml, indicating that the mice had received comparable amounts of enzyme.

3.4.1 Stability and distribution of the enzyme

Mice were anaesthetized and killed by intracardial perfusion with PBS at the selected times (2.2.4.3). The enzyme activity (2.2.2.10) was determined in extracts of liver, spleen, kidney and brain. Uptake of human LAMAN was seen into all organs except brain. In liver, the LAMAN activity 4 h after the injection was approximately 20 times higher than in control mice. In spleen and heart the activity exceeded these of controls (1/5 and 1/2 respectively) while in kidney only 1/2 of the LAMAN activity in control mice was reached.

The human LAMAN activities recovered 24 h after injection were much higher than expected from the experiments with mouse LAMAN. In liver the human LAMAN activity was still 6 times higher than in control liver. In spleen and kidney it accounted for 10-15% of that in control (Table 3.5)

Hours after injection	**LAMAN (mU/g wet weight)***				
	Liver	**Spleen**	**Kidney**	**Heart**	**Brain**
(+/+)* (n)	136,7±29,1 (19)	116,3±44,5 (3)	168,4±34,5 (9)	12,6±3,1 (3)	25±1 (2)
4	2774	157	81	21	2,1
8	2639	65	45	8	1,7
16	1549	31	26	10	2,7
24	796	24	16	6	3,0

Table 3.5: LAMAN activity in tissue extracts of α-mannosidosis mice 4-24 h after injection of 250 mU of human α-mannosidosis per g body weight.
+ All values were corrected for the mean LAMAN activity in serum at t_{zero} (5012 mU/ml serum).The correction factors varied between 0,903 and 1,222.
* +/+ refers to control mice and n to the number of animals investigated.

To follow the uptake and distribution of human enzyme, organs were weight and LAMAN activity was determined in tissue extracts prepared 4, 8, 16 and 24 h after injection of 250 mU human LAMAN per g body weight (Table 3.5). Less than 20% of the injected LAMAN was recovered after 4 h in the tissues examined (liver 18%, kidney 0.4%, spleen 0,12% and heart 0,04%).

When compared to the uptake of mouse LAMAN and taking into account that a 2.5 fold higher amount of human LAMAN was injected, the activity recovered in liver, kidney and heart 4 h after injection was comparable for both enzyme preparations (compare Table 3.3 and Table 3.5). Uptake by spleen appeared to be 2-3 times less efficient for the human enzyme. The biggest difference between the human and mouse enzyme was the higher stability of the human LAMAN internalized by liver, kidney and spleen. While the activity of the human LAMAN had decreased in these organs after 24 h to 17-33% of the 4 h value (see Table 3.5), that of the mouse enzyme had decreased to 20-26% of the 4 h value already after 10 h (see Table 3.3).

Like in the previous experiment with the mouse LAMAN, immunoblot analysis demonstrated that the internalised precursor of LAMAN (130 kDa) was processed rapidly to mature forms in liver and spleen (Fig 3.7).

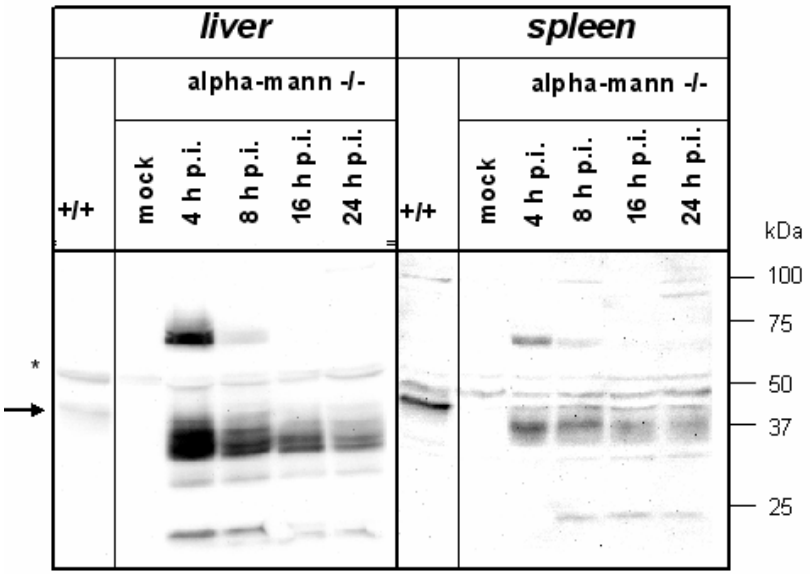


Fig. 3.7: Western Blot (WB) of mouse liver and spleen.
20 µg proteins from liver and spleen of treated mice were separated by 10% SDS-PAGE (2.2.2.6) and transferred to PVDF membranes (2.2.2.7). Rabbit antiserum raised against recombinant human LAMAN, (1:50000) was used as primary antibody and horseradish peroxidase (HRP) (1:20000) as coupled secondary antibody.
→ Endogenous alpha-mannosidase
* Non specific signals

4 hours post injection and proteolytically processed forms of ~70 kDa, ~35 kDa and ~20 kDa (not seen in mock injected mouse) were detectable in liver and spleen. Processed form of ~70 kDa was also detectable 8 hours post injection, but 16 hours post injection only proteolytically processed forms of ~35 and ~20 kDa were observed.

3.4.2 Corrective effect of the treatment

Organ extracts from α-mannosidosis mice killed 1 to 12 days after injection were also prepared for determination of neutral oligosaccharides (2.2.3.1) and analysed as described in section 3.2. In liver storage was fully corrected 1 and 3 days after injection. After 6 and 12 days neutral oligosaccharides started to accumulate again, but reached only about 30% of the storage level before treatment at day 12 (Fig. 3.8).

In spleen and kidney, the storage of neutral oligosaccharides decreased to 12 and 18%, respectively. It is noteworthy, that in spleen and kidney the maximum of correction was observed only after 3 and 6 days, respectively. In both organs neutral oligosaccharides started to reaccumulate 6 and 12 days after the injection (see Fig. 3.8). The neutral oligosaccharides in the brain of α-mannosidosis mice were apparently not affected by the treatment.

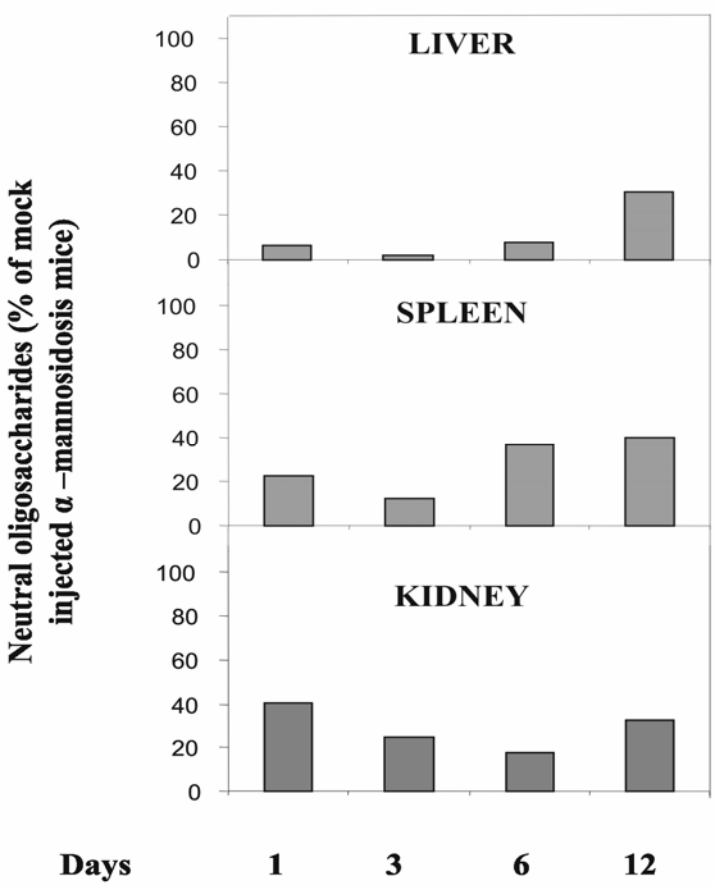


Fig. 3.8: Neutral oligosaccharides in tissue extracts of α-mannosidosis mice after injection of a single dose of 250 mU of human α-mannosidase per g body weight

The mice were killed 1, 3, 6 and 12 days after injection. The neutral oligosaccharides in the tissue extracts of liver, spleen, kidney and heart were separated by TLC (2.2.3.2) and quantified by densitometry (2.2.3.3).

Small sections of liver were cut with a razor blade at the killing time (2.2.4.3) and immersed in crystal vials filled with fixative solution for light microscopical examination (2.2.5). The liver of α-mannosidosis knock-out mice injected with 250 mU human LAMAN per g body weight 1 day after the injection, revealed the almost complete disappearance of storage vacuoles. In untreated α-mannosidosis mice, storage vacuoles were prominent in sinus endothelial cells, Kupffer cells and hepatocytes. Vacoules reappear 12 days after injection (Fig. 3.9).

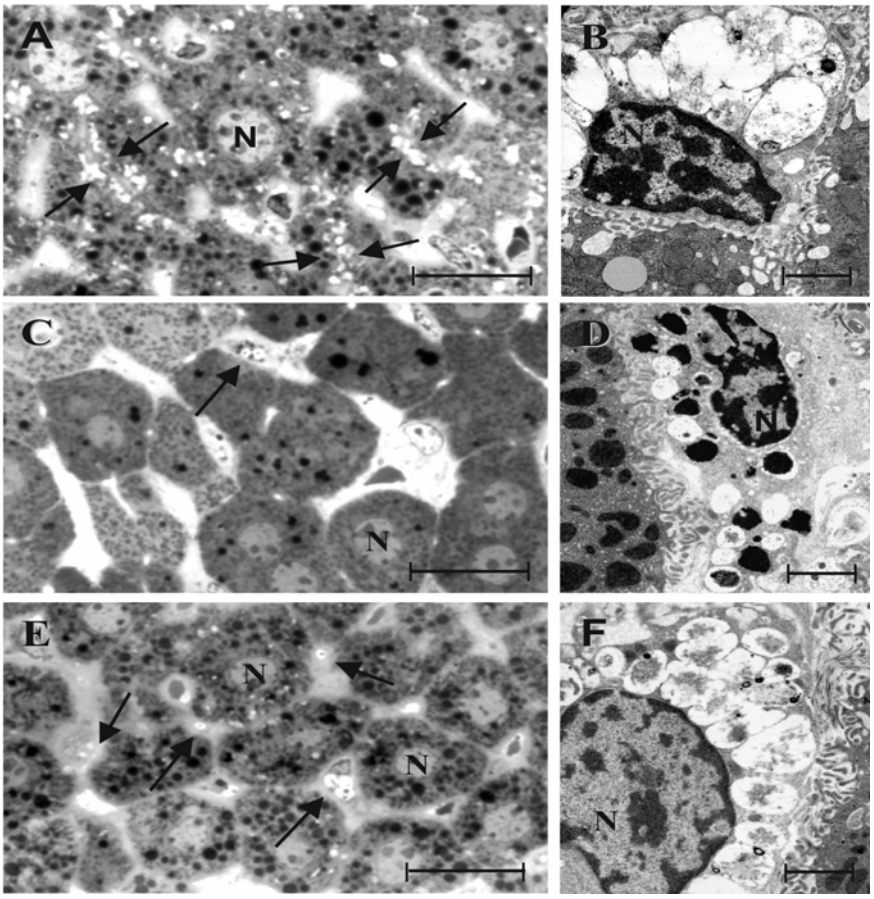


Fig. 3.9: **Disappearance and reappearance of storage vacuoles in liver.** A, C, E, light microscopy (semithin sections); B, D, F, ultrastructure of Kupffer cells; A, B, mock-injected α-mannosidosis mouse (age 14 weeks). Hepatocytes show clear vacuoles along the bile canaliculi (A, arrows). The heavily vacuolated sinus wall cells are not clearly discerned at the light microscopic level (A) because of the extremely narrow cytoplasm bridges between the vacuoles as seen in (B). C, D, α-mannosidosis mouse, 1 day after injection of 250 mU human LAMAN per g body weight. Vacuoles have disappeared from hepatocytes (C). Very few vacuolated sinus wall cells are seen (C, arrow). Vacuoles in Kupffer cells are smaller than in the mock-treated animal and can contain an electron-dense matrix (D). E, F, α-mannosidosis mouse, 12 days after injection of 250 mU human LAMAN per g body weight. Vacuoles have reappeared in sinus wall cells (E, arrows) and less so in hepatocytes. In Kupffer cells (F) the vacuoles qualitatively resemble those in mock-injected mice. The dense inclusions in the hepatocytes shown in A, C, and E represent lipid droplets, which are equally encountered in the corresponding wild type mice (not shown). Bars represent 20 µm and 2 µm, respectively.

3.5 Corrective effect of human LAMAN after two repeated injections of high doses

The corrective effect of the mouse (see Fig. 3.4) and human LAMAN (see Fig. 3.8) was only transient. Neutral oligosaccharides started to reaccumulate 3 to 6 days after injection. Increasing the amount of injected enzyme would be a means to delay the reaccumulation. The corrective effect of a given dose of LAMAN is expected to be higher when administered as two half doses separated by an appropriate interval than as a single dose. Rather than increasing the amount of LAMAN to 500 mU/g body weight we administered two times 250 mU human LAMAN per g body weight each at day 0 and 3.5 prior to the analysis.

A group of 4 α-mannosidosis mice at the age of 9 weeks were intravenously injected (2.2.4.2) with the discussed LAMAN dose (4.5 and 5.3 µl/g body weight, at day 0 and 3.5 respectively). The pattern of the human LAMAN preparation, characterized like in section 3.1.1, was of about 50 % precursor and 50 % proteolytically processed forms. Two α-mannosidosis mice were injected with PBS and used like mock-controls. All mice were killed for analysis 7 days after the first injection.

Blood was taken 5 min after the first and second injection. The α-mannosidase activity in serum varied between 4178-4443 mU/ml (less than 4%, indicating that the mice had received comparable amounts of enzyme) which was in the expected range.

3.5.1 Corrective effect of the treatment

Mice were anaesthetized and killed by intracardial perfusion with PBS 7 days after first injection (2.2.4.3). Organ extracts were prepared for determination of neutral oligosaccharides (2.2.231), separated by TLC (2.2.3.2) and quantified by densitometry (2.2.3.3).

The experiment resulted in a full correction of the storage in kidney and heart and the residual storage in spleen was less than 20% of that in mock-injected α-mannosidosis mice (Fig. 3.10).

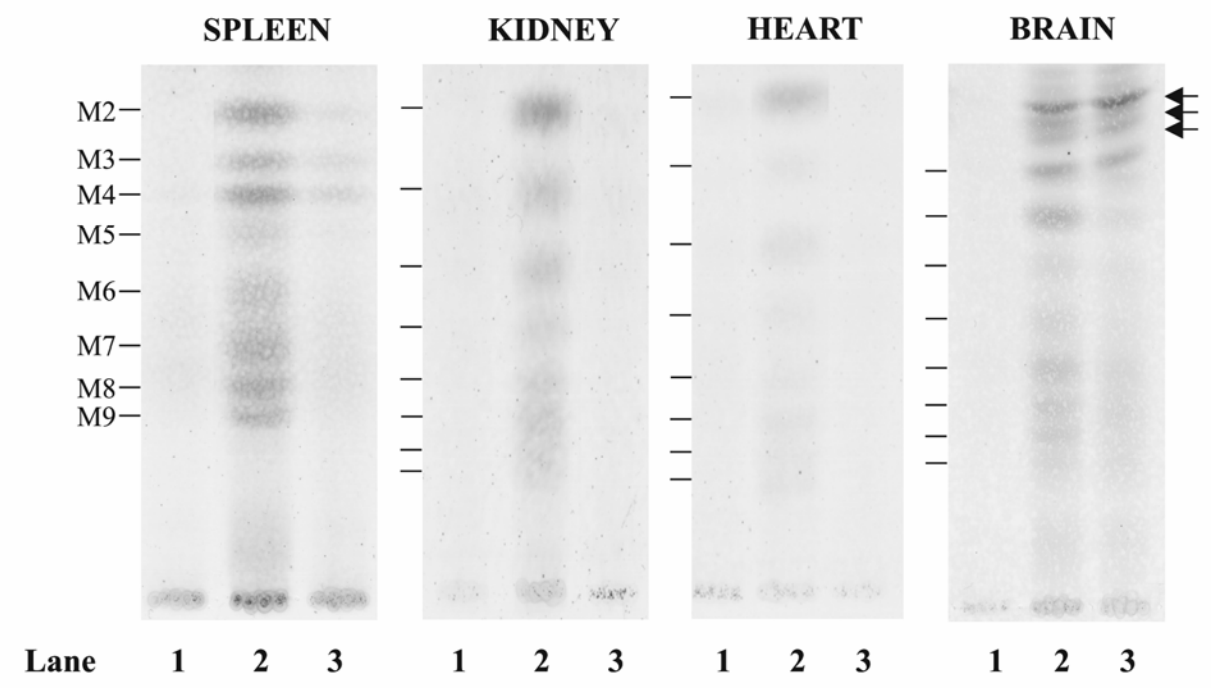


Fig. 3.10: Neutral oligosaccharides in tissue extracts of α-mannosidosis mice twice injected with 250 mU human LAMAN per g body weight.

Control (lane 1) and α-mannosidosis mice (lane 2 and 3) were injected with PBS (lane 1 and 2) or 250 mU human LAMAN per g body weight (lane 3) 0 and 3.5 days prior analysis. The migration of oligosaccharides composed of 2 to 9 mannose residues and a single N-acetylglucosamine residue (M2 to M9) is indicated. Arrows mark orcinol-positive material insensitive to LAMAN (2.2.3.4). The results in a duplicate set of mice (not shown) varied by less than 15%.

Small sections of liver and kidney were cut with a razor blade at the killing timesand immersed in crystal vials filled with fixative solution for light microscopical examination (2.2.5). That demonstrated the absence of storage vacuoles in liver (Fig 3.11) and in tubular epithelia of kidney (Fig. 3.12)

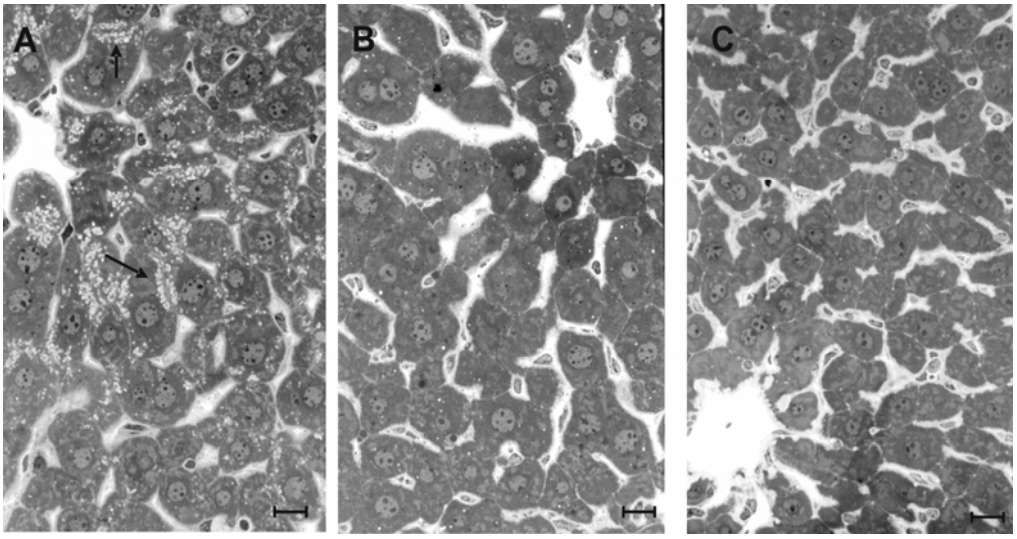


Fig. 3.11: Light microscopy of liver in α-mannosidosis mice

α-Mannosidosis mice were injected with PBS (A) or 250 mU human LAMAN per g body weight (B) 7 and 3.5 days prior killing. Wild type control mice (C) were injected with PBS. **A**, Hepatocytes show clear vacuoles along the bile canaliculi (A, arrows). **B**, After the injection of human LAMAN, the vacuoles have disappeared from hepatocytes. **C**, wild type mouse no vacuoles are seen in hepatocytes. Bars represent 20 µm.

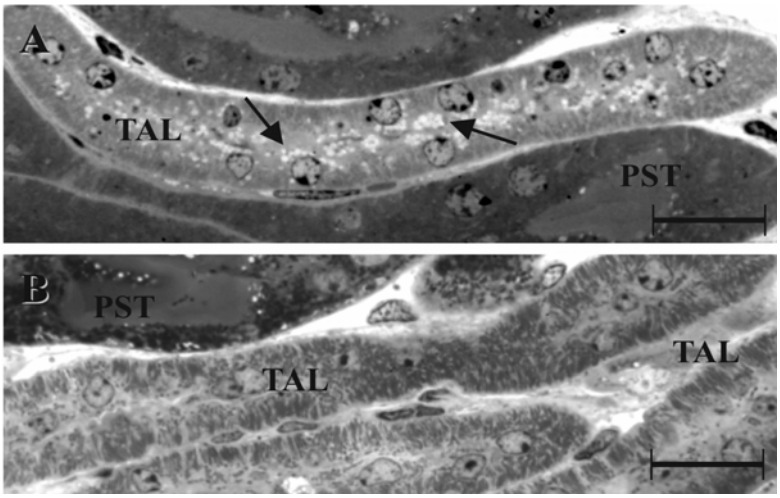


Fig. 3.12: Light microscopy of kidney in α-mannosidosis mice.
α-Mannosidosis mice were injected with PBS (**A**) or 250 mU human LAMAN per g body weight (**B**) 7 and 3.5 days prior killing. The outer stripe of the outer medulla is shown. Numerous clear vacuoles are seen in the thick ascending limb (TAL) of Henle's loop of the mock-injected mouse (A, arrows) in contrast to the LAMAN-treated mouse (B). The proximal straight tubules (PST) are free of pathological vacuoles in either animal. Bars represent 20 µm.

3.5.2 Correction of the mannose oligosaccharides storage in brain

Most notably, in brain the level of neutral oligosaccharides as quantified by TLC was only half of that in the brain of mock–injected α-mannosidosis mice (see Fig. 3.10). To quantify the neutral oligosaccharides on a molar basis the neutral oligosaccharides extracted from the brain of the mock- and LAMAN-injected α-mannosidosis mice were derivatized with 2-anthranilamide, to introduce a fluorescent tag at the reducing terminus of the oligosaccharides (2.2.3.5.1).

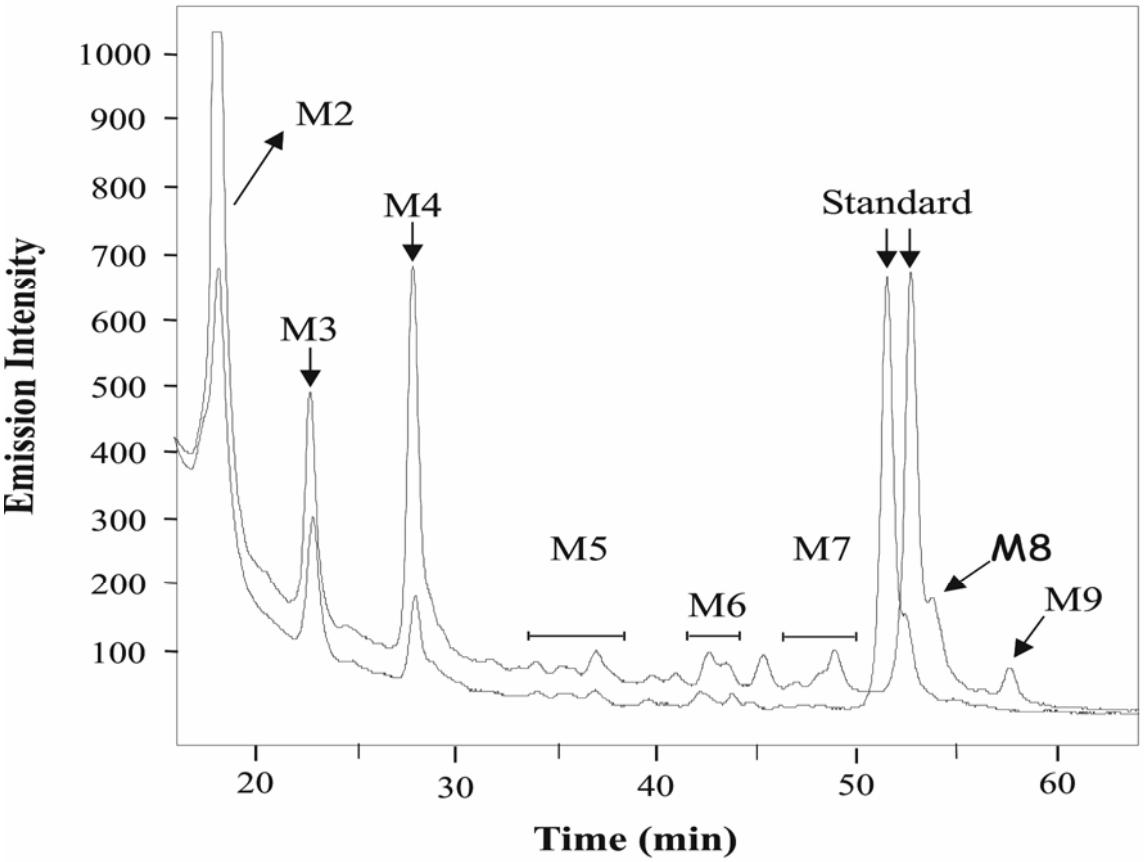


Fig. 3.13: HPLC-separation of 2-anthranilamide derivatized neutral oligosaccharides from brain

Neutral oligosaccharides from brain of α-mannosidosis mice injected with PBS (upper scan) or 250 mU human LAMAN per g body weight (lower scan) 7 and 3.5 days prior killing were mixed with 220 pmol $GlcNAc_4Man_3Gal_3$ as an internal standard, derivatized with 2-anthranilamide and separated by HPLC. The position of the $GlcNAc_4Man_3Gal_3$ standard and of oligosaccharides composed of 2 to 9 mannose residues and a single N-acetylglucosamine residue (M2 to M9) are indicated.

The fluorescently labelled oligosaccharides were separated by HPLC (2.2.3.5.3) and quantified by comparison with an internal standard oligosaccharide ($GlcNAc_4Man_3Gal_3$) added prior to the derivatization with 2-anthranilamide (Fig. 3.13).

The major oligosaccharide species in brain of α-mannosidosis mice contain 2, 3 or 4 mannose residues. They are present at concentrations of 319, 87 and 164 pmol/g tissue, respectively, and account for 61% of all neutral oligosaccharides in the brain. In the brain of α-mannosidosis treated mice the concentration of the neutral oligosaccharides decreased to 26% of mock-injected mice. The corrective effect was noted for each of the oligosaccharide species but was relatively highest for the species with 9, 7 and 4 mannose residues (see Fig. 3.13).

3.6 Preparation of ^{3}H labelled liver oligosaccharides

To verify that the correction of the storage of neutral mannose oligosaccharides found in brain was real, and not due to a loss of sugars during the preparation procedure, ^{3}H labelled oligosaccharides were prepared (2.2.3.7) to be used as spike-in control for the isolation of oligosaccharides from brain.

To obtain ^{3}H-labelled control-oligosaccharides, neutral sugars were prepared from 2.8 g liver of α-mannosidosis mice (2.2.3.1). Approximately 8.4 mmol in water (HPLC grade) were obtained. The sample was lyophilized, dissolved in 1.0 M Borate Buffer pH 8.0 and reduced with ^{3}H-$NaBH_4$ in 1.0 M Borate Buffer pH 8. The sample was next acidified with acetic acid, to destroy the excess of borohydride (2.2.2.17). ^{3}H labelled reduced oligosaccharides were chromatographed on a column of Sephadex G-25 (2.2.2.18).

3.6.1 Gel Filtration Chromatography

To purify the desired spike-in control sample containing all the reduced ^{3}H mannose oligosaccharides (M2 to M9), reduced oligosaccharides (300 µl) were fractionated onto a Sephadex G25 column equilibrated with 0.2 M Pyramidin acetate buffer pH 5.5 (see purification strategy in table 3.6). The flow-through fractions containing ^{3}H-labeled polysaccharides were collected (30 fractions) and the radioactivity was determined using a scintillation counter (Fig 3.14).

A maximum of radioactivity (in cpm) was observed between fractions 6 and 20. High radioactivity levels were assumed to be due to a high content of free radioactivity in the sample. To obtein the longer mannose reduced oligosaccharides, which are characterized by a faster migration, three pools of fractions (I, fractions 6 to 9; II, 10; III11 to 12) were lyophilized and dissolved in 150 µl, 100 µl and 150 µl 0.2 M Borate Buffer pH 8.0 respectively. At this initial step, fractions 13 to 20 were not analyzed further, and will be included in the purification of the ^{3}H oligosaccharides if not all the mannose residues (M2 to M9) are included in the final preparation.

Chromatography	Fractions selected for re-chromatography			
1 st	6 to 10	11	12	
	I	II	III	
2 nd	7 to 11	7 to 11	8 to 16	17 to 18
	A	A	B	C
	Sample 1			*Sample 2*

Table 3.6: C**hromatography-purification strategy.**
From the first chromatography of the reduced mannose oligosaccharides, three pools of samples were selected for rechromatography (I, II and III). These three pools were rechromatographed. Fractions 7 to 11 from the rechromatography of pools I and II were selected and pooled (pool **A**). From the rechromatography of pool III, fractions 8 to 16 (pool **B**) were selected and pooled with pool **A** (*Sample 1*), and fractions 17 and 18 were also selected and pooled (*Sample 2*).

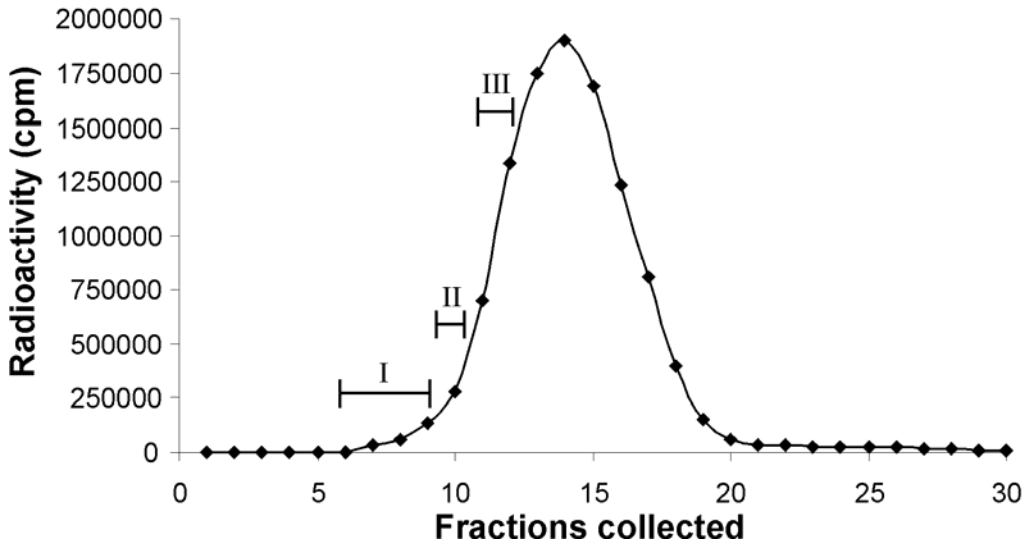


Fig 3.14: **Gel Filtration Chromatography of mannose oligosaccharides reduced with ^{3}H-NaBH$_4$.**
Oligosaccharides in 1.0 M Borate buffer pH8 (300 µl) were separated on Sepharose G25 and 0.2 M Pyrimidin acetate pH 5.5 was used as eluant. The fraction size was 500 µl and radioactivity of each fraction was measured and plotted. The fractions indicated with the bar were pooled and re-chromatographed on the same column.

The three selected pools (I, II and III, see Fig 3.14) were re-chromatographed, 25 fractions of each were collected, and radioactivity was measured. From the rechromatographed pool I (Fig 3.15), fractions 7 to 11 were selected (**A**), assuming that they should contain the longer reduced mannose oligosaccharides. Fraction 12 was initially discarded, assuming that the high radioactivity level was due to a high content of free radioactivity in the sample. From the rechromatographed pool II (Fig 3.15), fractions 7 to 11 (**A**) were also selected.

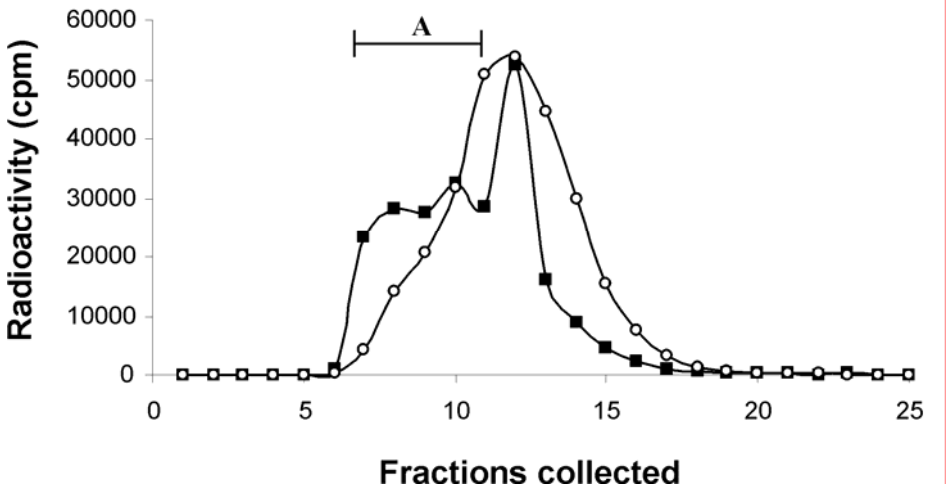


Fig 3.15: Rechromatography of pooled samples I and II.
Pools of samples I (■) and II (○) from the first gel chromatography were loaded on Sepharose G25 with 0.2 M Pyrimidin acetate pH 5.5 as eluant. The fraction size was 500 µl and radioactivity of each fraction was measured and plotted. The fractions indicated with the bar were pooled.

In the rechromatographed pool III (Fig 3.16), two peaks of radioactive material were observed. Presuming that the reduced oligosaccharides were present in the first peack, fractions 8 to 16 (**B**) were selected, pooled, lyophilized dissolved in 100 µl 0.2M Borate Buffer pH 8.0 and applied again onto the Sepharose G25 column, to ensure that the smaller reduced mannose oligosaccharides are also included in the final preparation. Twenty fractions of 500 µl each were collected (Fig. 3.17).

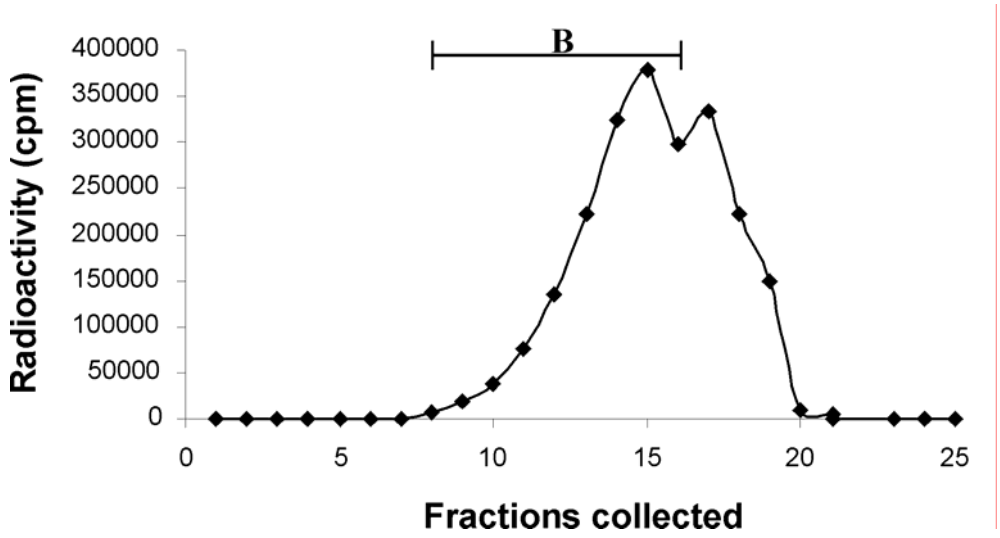


Fig 3.16: Rechromatography of pooled samples III.
Pool of samples III from the first gel chromatography were loaded on Sepharose G25 with 0.2 M Pyrimidin acetate pH 5.5 as eluant. The fraction size was 500 µl and radioactivity of each fraction was measured and plotted. The fractions indicated with the bar were pooled.

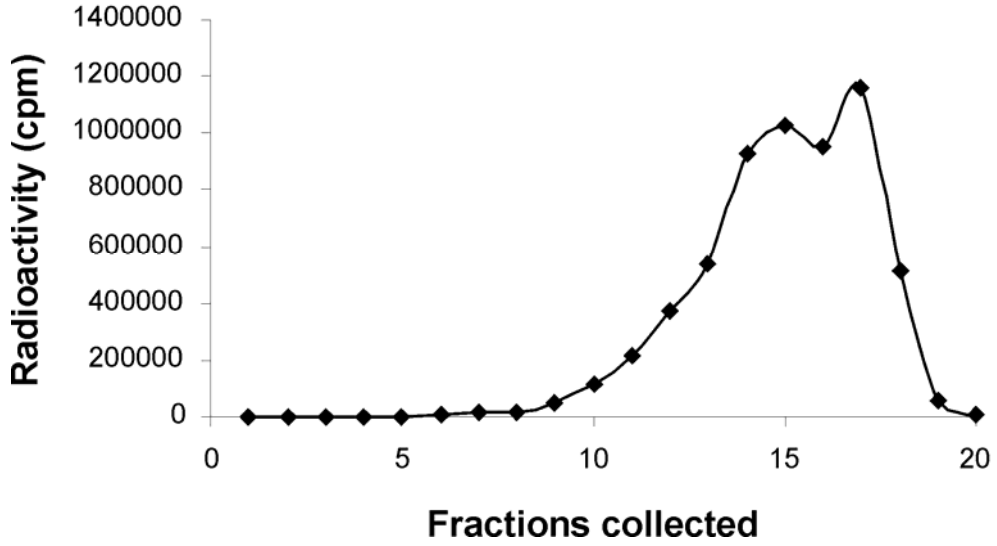


Fig 3.17: Rechromatography of pooled samples B.
Pool of samples **B** from the rechromatographed pool III were loaded on Sepharose G25 with 0.2 M Pyrimidin acetate pH 5.5 as eluant. The fraction size was 500 µl and radioactivity of each fraction was measured and plotted.

The chromatography profiles presented in figures 3.16 and 3.17 are similar. As we were not sure whether the nature of the second peak of the chromatogram showed in Fig. 3.16 included the smaller reduced oligosaccharides, we prepared a separate pool of fractions 17 and 18 (pool **C**).

In samples **A** and **B**, we supposed that the bigger and middle size oligosaccharides were included. Therefore this samples were pooled (*Sample 1*), lyophilized and dissolved in 1 ml water. Sample **C,** were we supposed the small sized oligosaccharides to be included, was also lyophilized and dissolved in 1 ml water (*Sample 2*).
The salts were eliminated from the recovered fractions by further lyophilization, until a constant value of radioactivity (cpm) was reached. The final radioactivity values were:
Sample 1: 274.5 x 10^6 cpm
Sample 2: 79.5 x 10^6 cpm

3.6.2 Thin Layer Chromatography (TLC)

To ensure that all the types of ^{3}H labelled oligosaccharides (M2 to M9) were included in the selected chromatographed samples, samples 1 and 2 were loaded onto a TLC plate (0.5 x 10^6 cpm from each sample) and mixed with a preparation of liver oligosaccharides (10 µl). The different reduced mannose oligosaccharides (M2 to M9) were separated by TLC (2.2.3.2), stained, scraped with a scalpel, and dissolved over night in 500 µl water (HPLC grade). Radioactivity was determined using a scintillation counter (Fig 3.18).

All the mannose oligosaccharides (M2 to M9) could be found in the selected chromatographed samples. Therefore both samples were pooled, lyophilized and dissolved in 1 ml water. The measured radioactivity was 276.294 x 10^6 cpm.

These ^{3}H labelled mannose oligosaccharides were used as spike-in controls for the preparation of brain oligosaccharides in the replacement therapies described later in sections 3.8 and 3.9.

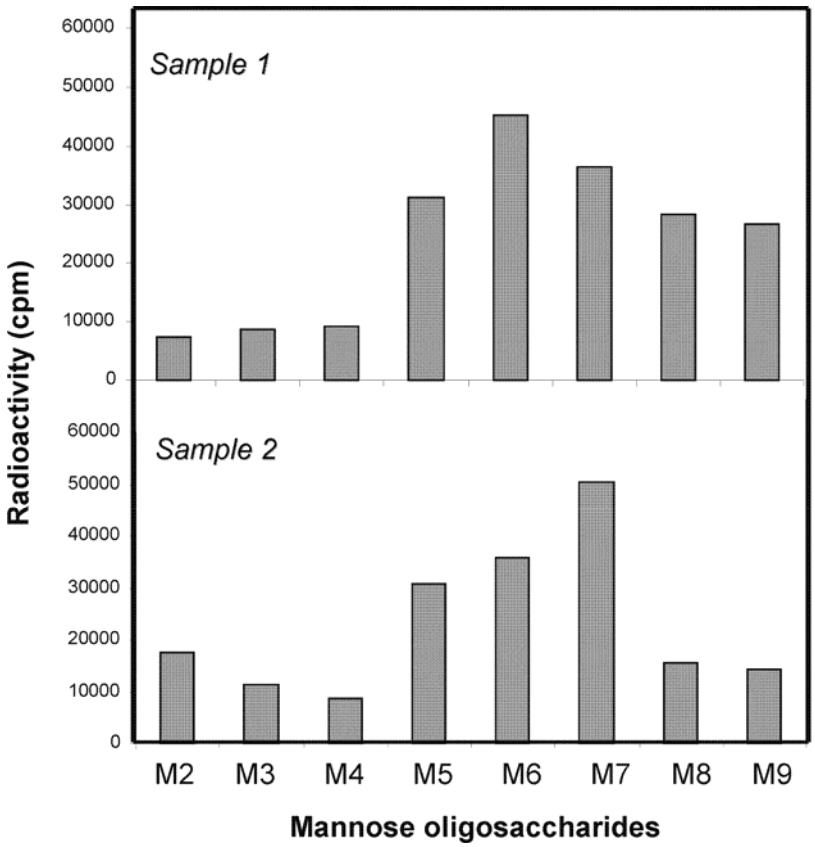


Fig 3.18: Radioactivity of ^{3}H mannose labelled oligosaccharides after separation by TLC.
Radioactivity of mannose reduced oligosaccharides (M2 to M9) from chromatographed samples 1 and 2 after separation by TLC and scintillation quantization.

3.7 Analysis of the Blood Brain Barrier (BBB) function in α-mannosidosis mice

To exclude that the reduction of storage material in the brain of α-mannosidosis mice (section 3.5.2) was due to a dysfunction of the blood brain barrier, the following experiment was performed.

Control and α-mannosidosis mice, at the age of 8 weeks, were injected into the tail vein with 1% Evans blue. The mice were killed 1 hour after injection. To wash out the Evans blue dye from the circulation, the mice were cardially perfused with 50 ml of PBS. One brain hemisphere was homogenized in PBS, and Evans blue was quantified by fluorometry (section 2.2.4.4) (Jeyakumar *et al*., 2003).

The amount of Evans Blue in the homogenate of control and α-mannosidosis mice was indistinguible (Table 3.7)

	Evans blue (ng / hemisphere)
Control mice (n=4)	11.2 ng ± 4.7
α-mannosidosis mice (n=4)	12.0 ng ± 4.8

Table 3.7: Analysis of BBB function
The values give the ng of Evans blue found in one brain hemisphere of mice killed 1 h after the injection of Evans blue 1% in PBS.

These data indicate that in the α-mannosidois mice, there is no dysfunction of the Blood Brain Barrier. These data was confirmed by immunohistochemical staining of immunoglobulins in the brains of α-mannosidase knock-out mice. No immunoglobulins were observed (data not shown) suggesting an intact BBB.

3.8 Corrective effect of a long term experiment with human LAMAN injections once a week

As shown in sections 3.2, 3.3, 3.4 and 3.5, the corrective effect on the storage of neutral mannose oligosaccharides after ERT was time-, tissue- and dose-dependent. After a single dose of human LAMAN the maximal corrective effect was observed between 3 and 6 days after injection. Thereafter neutral oligosaccharides started to accumulate again. The injection of two times 250 mU/g body weight at an interval of 3.5 days was sufficient to clear liver, kidney and heart from neutral oligosaccharides within one week. Also in brain the storage of neutral oligosaccharides decreased by more than 70%. This data clearly underline the efficacy of a ERT for the correction of storage in α-mannosidosis and suggests that ERT can substantially decrease storage also in the brain.

With the goal of finding the best therapeutic strategy of the ERT, long term experiments were performed using different doses of human LAMAN and administrating the drug at different intervals.

It was already shown (section 3.4) that after a single dose of human LAMAN, the maximal corrective effect was observed between 3 and 6 days after injection. After 6 days oligosaccharides start to accumulate again, but 12 days after the injection the storage is still 60-70 % of that in mock injected mice. Therefore we wanted to test whether the injection of the mice with the same high dose every 7 days, when the storage is still not as pronounced as compared to that in mock injected mice, a progressive weekly improvement of the storage could be achieved.

The first long term experiment was performed with a group of 19 α-mannosidosis mice at the age of 7 weeks. The mice received weekly 250 mU human LAMAN per g body weight (4.5 – 8.5 µl/g body weight) intravenously

(2.2.4.2). Human LAMAN pattern (section 3.1.1), was composed of about 70% precursor and 30% proteolytically processed forms. Two mice were killed 1, 2, 4, 8, 12, and 16 weeks after the initial injection. Two mock injected α-mannosidosis mice were also used as controls at every killing time point.

In order to control for the amount of injected enzyme blood was taken from the retroorbital plexus 5 minutes after each injection. If an animal did not receive the full dose, the missing amount was estimated from the blood level and injected to the animal on the same day. Five min after injection the LAMAN activity (2.2.2.10) varied by less than 10% indicating that the mice had received comparable amounts of enzyme.
Not all the mice injected with human LAMAN survived the therapy, 6 mice died during the experiment for unknown reasons (Fig 3.19).

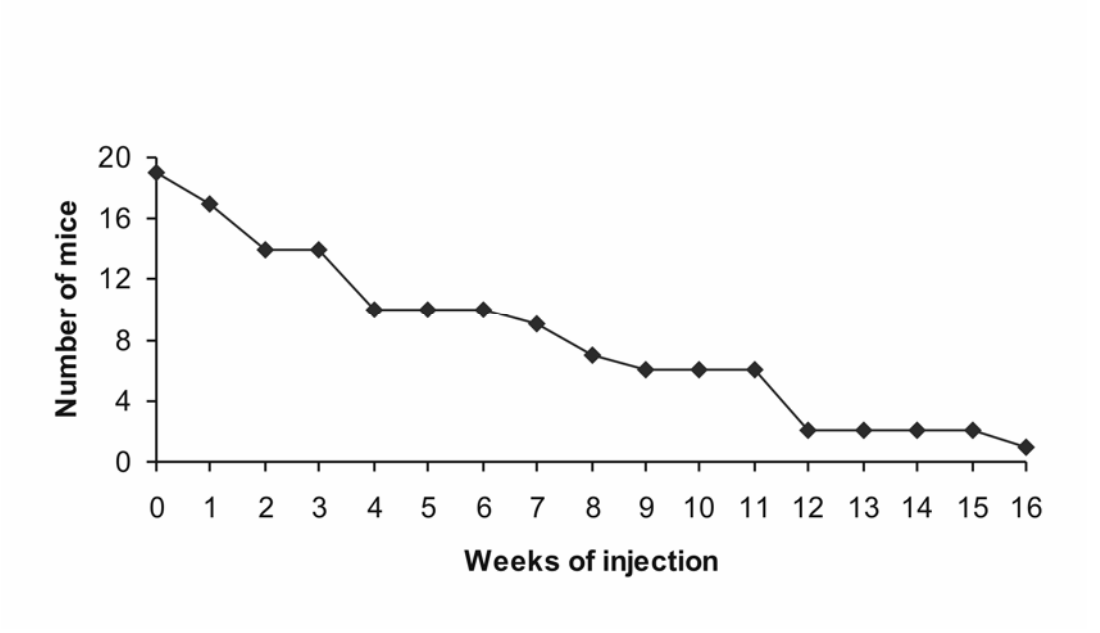


Fig 3.19: Mice mortality during ERT experiment with human LAMAN injections once a week.
From 19 mice at the start of the experiment, 2 mice were killed after 1, 2, 4, 8, 12 and 16 weeks, while 6 mice died during the 4 months therapy. Mortality rate was of 31%.

3.8.1 Stability and distribution of the enzyme

Mice were anaesthetized and killed by intracardial perfusion with PBS at the selected killing times (2.2.4.3) and organ extracts were prepared for the determination of LAMAN activity (2.2.2.10). Activity was only measurable in liver but not in spleen and kidney (Table 3.8). The activity in liver was 5-8 folds of that in mock injected mice. As already seen in section 3.4, one week after the injection of 250 mU human LAMAN per gram body weight, LAMAN activity was only measurable in liver, while in spleen, kidney and heart, the activity was comparable to that of mock injected mice (see table 3.3)

Weeks after first injection	LAMAN (mU/g wet weight)*	
	Liver	
	Mean	Sd
1	24.5	1.6
2	11.5	2.5
4	12.5	0.3
8	16.5	0.3
12	25.5	1.0
16	24.0	0.7

Table 3.7: LAMAN activity in liver extracts of α-mannosidosis mice 1-16 weeks after weekly injection of 250 mU of human α-mannosidosis per g body weight.
Mean and standard deviation (Sd) of two animals is shown.

3.8.2 Corrective effect of the treatment

Organ extracts were also prepared for determination of neutral oligosaccharides (2.2.3.1). Neither in kidney nor in spleen a total correction of the storage was observed, but the storage remained at a constant level throughout the 16 weeks of therapy (Fig 3.20)

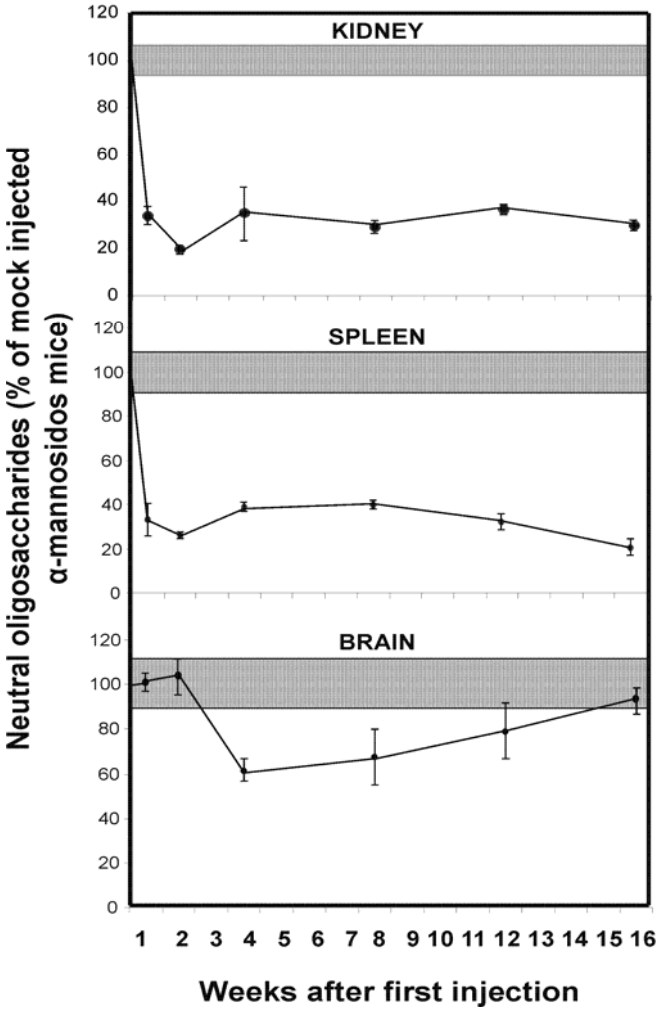


Fig. 3.20: Neutral oligosaccharides in tissue extracts of α-mannosidosis mice after weekly injection of 250 mU of human α-mannosidase per g body weight.
Mice were killed 1, 2, 4, 8, 12 and 16 days after injection. Neutral oligosaccharides in the tissue extracts of kidney, spleen and brain were separated by TLC (2.2.3.2) and quantified by densitometry (2.2.3.3). The over frame represent the storage range in mock injected mice. Standard deviation obtained from duplicates.

A residual storage of about 25-40% of that determined in mock-injected mice was seen in kidney and spleen, while in spleen the storage was reduced to 20% of that in mock injected mice after 16 weeks of treatment (Fig 3.20).

Most notably, in brain the level of neutral oligosaccharides as quantified by densitometry after separation by TLC was again notably reduced (around 45% of correction) after 4 weeks of treatment, and also in one mouse treated for 8 weeks, and one mouse treated for 12 weeks (Fig 3.20). After 16 weeks of treatment, no correction of the storage was observed in the brain of treated mice..

To be sure that this reduction of the oligosaccharides storage in brain was not due to a loss of material during preparation, ^{3}H labelled mannose oligosaccharides (see section 3.6) were used as spike-in control. In the preparation of neutral oligosaccharides from brain, 0.5 x 10^{6} cpm of the ^{3}H labelled oligosaccharides were added to the sample in the initial step. Radioactivity values before and after oligosaccharides preparation showed that the recovery of brain oligosaccharides was 55 % varying less than 9% in the 24 prepared samples.

3.8.3 Immune response

To asses the immune status of mice during the treatment with weekly injections of human LAMAN, the general humoral immune response was determined measuring the IgG level with an ELISA (2.2.2.12). Antigen (human LAMAN) was coated into a microtiter plate and mice serum (diluted 1:10000) was added. After 3 h incubation at 37°C peroxidase-conjugated goat anti mouse IgG was added and reaction was developed by addition of peroxidase substrate.

Antibodies against hLAMAN were first observed after the 3rd injection and reached a maximum after the 4th-5th injection. The titers dropped continuously

after the 5th injection indicating that the mice developed a tolerance (Fig 3.21). No immune response was observed in two of the treated mice.

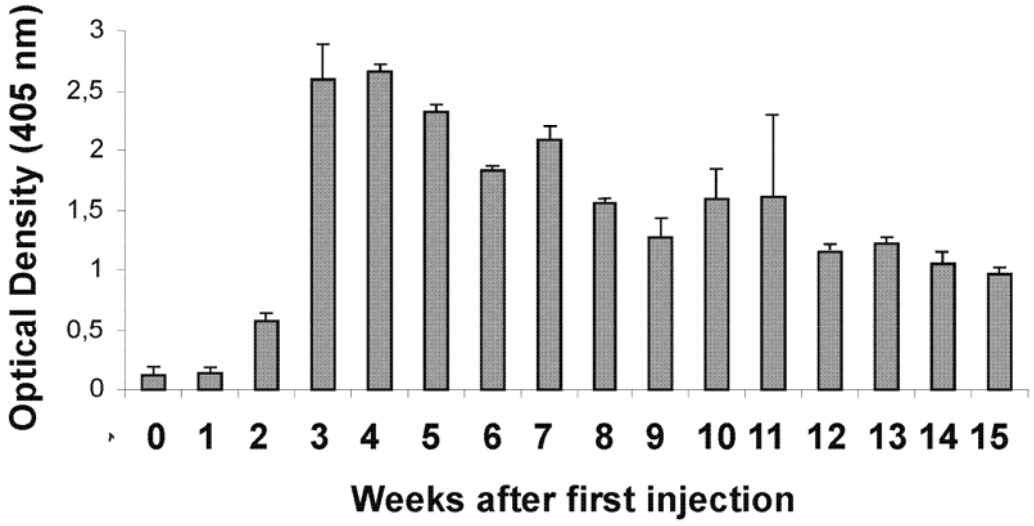


Fig 3.21: Development of antibodies (ELISA) after one weekly injection.

IgG levels after each single injection of mice treated through 16 weeks were determined by ELISA (2.2.2.12). Standard deviation obtained from duplicates of two mice.

Immunoprecipitation assays with Pansorbin (2.2.2.13) were performed with the serum obtained 5 minutes after each single injection and no LAMAN activity was detectable in the pellet, while 93% of the LAMAN activity determined prior to IP was detectable in the supernatant. These observations indicated that in no case the antibodies did precipitate human LAMAN. Therefore, the antigen against why IgG antibodies are secreted, did not seem to be necessary related to human LAMAN.

To test if the IgG titers affect LAMAN activity towards a synthetic substrate (2.2.2.14), mice serum of injected mice obtained 5 minutes after each single injection was incubated over night at 4 °C with human LAMAN. Activity was measured before and after incubation. LAMAN activity varied by less than 10% before and after incubation, therefore the IgG titers did not apparently inactivate human LAMAN.

3.9 Corrective effect on a long term experiment with human LAMAN injections twice a week

The goal of this long term therapy is to achieve a complete correction of the storage in the peripheral tissues, and see if the correction of storage observed in brain (section 3.5 and 3.8) is reproducible.
In a first attempt for a long term LAMAN treatment experiment, in which the mice received human LAMAN at a high dose once a week, a complete correction of the storage was not observed in peripheral tissues (see section 3.8.2). A residual storage of about 25-40% of that in mock-injected was observed in spleen and kidney, while a partial correction of the storage in the brain of some of the α-mannosidosis injected mice was also observed (see sections 3.5 and 3.8).
With the aim of a total correction of storage in kidney and spleen, and state if the effect of multiple LAMAN injections in the storage of brain is reproducible, the dose interval for the next long therapy was increased to one injection every 3.5 days, maintaining the same high dose.

In this experiment 17 α-mannosidosis mice at the age of 7-8 weeks were injected with human LAMAN (3.0 – 3.7 µl/g body weight) at an interval of 3.5 days, which was sufficient to clear within one week liver, kidney and heart from neutral oligosaccharides (see section 3.5). Human LAMAN pattern (see section 3.1.1), was composed of about 60% precursor and 40% proteolytically processed forms respectively. Four mice were killed 1, 2, and 4 weeks after the initial injection and four mock injected α-mannosidosis mice were also used as control at each sacrification time.

Since we had the impression that in previous long ERT-experiment (section 3.8) some mice died under the restraint stress during tail vein injection and the blood sampling (2.2.4.2), mice were anaesthetized prior to injection. The anaesthesia

dose was of 70 µl per 10 gram body weight of anaesthetic (2.2.4.2). Mice were kept at 37°C until recovery from anaesthesia (about 30 minutes).
Animals spontaneously dying during the therapy (5 of 17 mice, see Fig 3.22) were immediately cooled at 4°C and subjected to a macroscopic post mortem examination at the Tierärztliches Institut, Göttingen. Different organs seemed to be lightly inflamed, but the exact cause of death was not found.

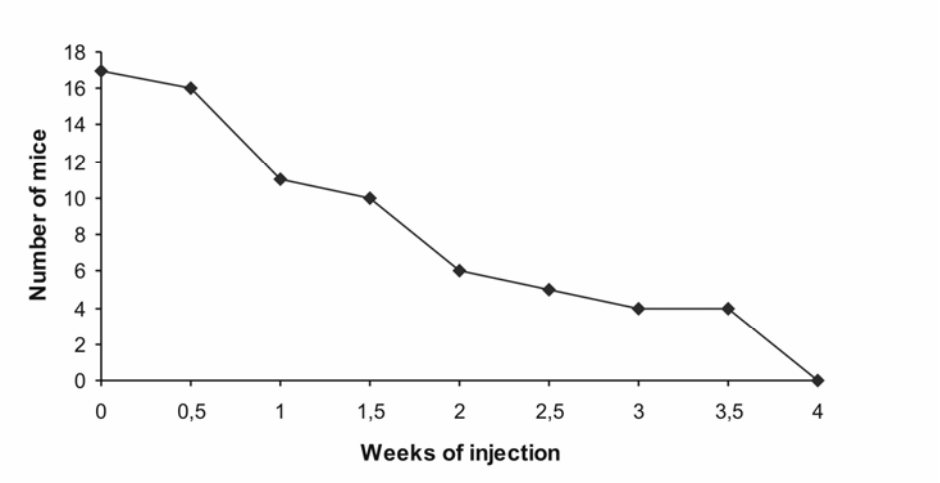


Fig 3.22: Mice Mortality during ERT experiment with human LAMAN injections twice a week.
From 17 mice at the start of the experiment, 4 mice were killed after 1, 2, and 4 weeks, while 5 mice died during the 4 weeks of therapy. Mortality rate was of 29%.

To control for the amount of injected enzyme blood was taken from the retroorbital plexus 5 minutes after each injection. If an animal did not receive the full dose, the missing amount was estimated and given to the animal on the same day. Five min after injection the LAMAN activity varied by less than 8% indicating that the mice had received comparable amounts of enzyme.

3.9.1 Stability and distribution of the enzyme

Organ extracts from liver, spleen and kidney were prepared to determine the LAMAN activity (2.2.2.10). After a single injection of 100 mU per gram body weight of mouse LAMAN, activity was still detectable after 4 days (see section

3.2.4), Therefore, after multiple injections each 3.5 days of human LAMAN at a higher dose, an increase of LAMAN activity was expected. The outcome of this experiment with repeated LAMAN injections showed that LAMAN activity decreased progressively with the weeks of treatment (Table 3.9).

Weeks after first injection	LAMAN (mU/g wet weight)*		
	Liver	Spleen	Kidney
(+/+)* (n)	136,7±29,1 (19)	116,3±44,5 (3)	168,4±34,5 (9)
(-/-)* (n)	2,9±2,5 (21)	2,8±2,1 (4)	2,5±1,1 (10)
1 (n)	152.0 ± 14.0 (4)	26.5 ± 2.4 (4)	32.5 ± 6.4 (4)
2 (n)	88,5 ± 6,3 (4)	12.0 ± 4.0 (4)	18.5 ± 2.3 (4)
4 (n)	66.7 ± 11.6 (4)	4.7 ± 1.4 (4)	3.0 ± 1.2 (4)

Table 3.9: LAMAN activity in tissue extracts of α-mannosidosis mice 1-4 weeks after first injection of 250 mU of human α-mannosidosis per g body weight.
* +/+ refers to control mice, -/- to α-mannosidosis mice, and n to the number of animals investigated.

In liver, LAMAN activity reached the levels of control mice 1 week after the first injection, and thereafter decreased progressively with the weeks of treatment. In spleen and kidney, LAMAN activity 1 week after the first injection was 9 and 13 times of that compared to mock injected mice, respectively. Thereafter it decreased also with the weeks of treatment.

3.9.2 Corrective effect of the treatment

Organ extracts were also prepared for determination of neutral oligosaccharides (2.2.3.1). Neither in kidney nor in spleen a total correction of the storage was reached. Again a residual of about 22-36% of that in mock-injected mice was observed, remaining at a constant level throughout the 4 weeks of treatment (Fig 3.23).

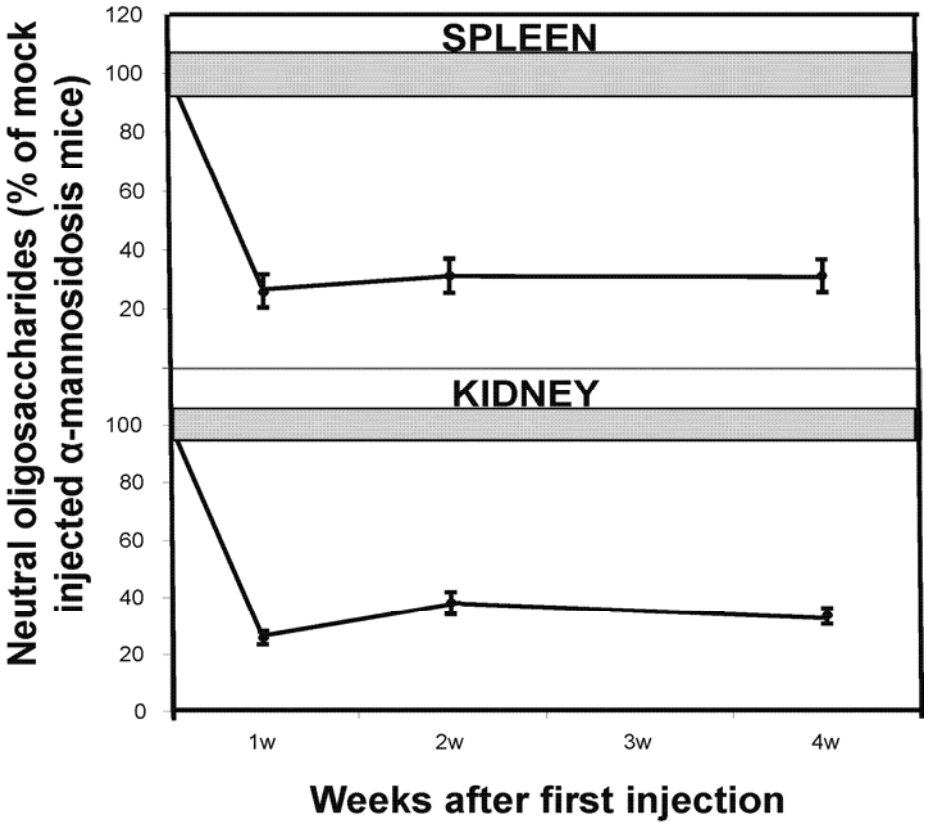


Fig. 3.23: Neutral oligosaccharides in tissue extracts of α-mannosidosis mice after injecting twice a week 250 mU of human α-mannosidase per g body weight

The mice were killed 1, 2, and 4 weeks after injection. The neutral oligosaccharides in the tissue extracts of spleen and kidney were separated by thin-layer chromatography (2.2.3.2) and quantified and quantified by densitometry (2.2.3.2). The over frame represent the storage range of mock injected mice. Standard deviation obtained from duplicates.

^{3}H labelled mannose oligosaccharides (see section 3.6) were used again as spike-in control. In the preparation of neutral oligosaccharides from brain, 0.5 x 10^{6} cpm of the ^{3}H labelled oligosaccharides were added to the sample. Radioactivity values before and after oligosaccharides preparation showed that the recovery of brain oligosaccharides was 54 % varying less than 8% in the 18 prepared samples.

As quantified by densitometry (2.2.3.3) after separation by TLC (2.2.3.2), storage in brain was again reduced to 40% of that in mock-injected mice in two of the mice killed 2 weeks after the first injection. About 50% of residual storage was also observed in one mouse treated throughout 4 weeks, and about 60% in one mouse treated throughout 2 weeks. All other analyzed mice showed a small correction of about 15-20% (Fig 3.24).

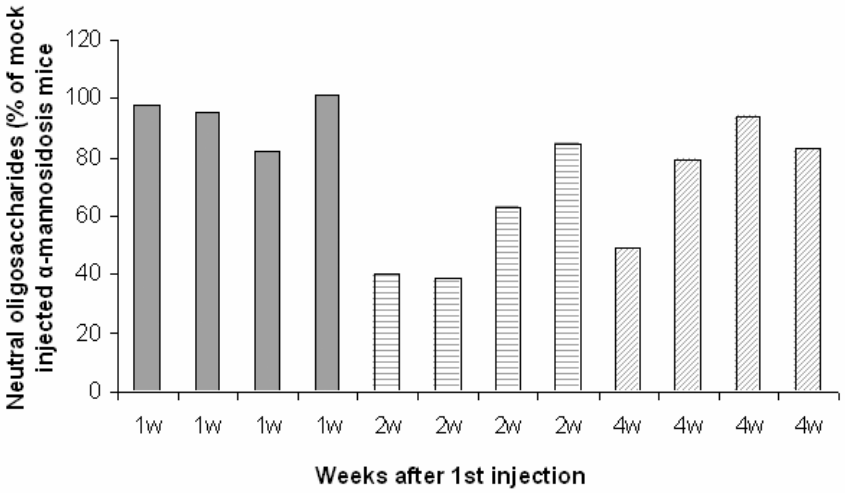


Fig. 3.24: Neutral oligosaccharides in brain extracts of α-mannosidosis mice after injecting twice a week 250 mU of human α-mannosidase per g body weight

The mice were killed 1, 2, and 4 weeks after injection. The neutral oligosaccharides in the tissue extracts of brain were separated by thin-layer chromatography and quantified as in Fig. 3.23. Individual mice values are shown.

3.9.3 Immune response

To asses the immune status of mice during these treatment with human LAMAN injections each 3.5 days, the general humoral immune response was determined measuring the IgG level with an ELISA (2.2.2.13), as described in section 3.8.3. Antibodies were again first observed after the 4th injection, reaching a maximum after 5-6 injections. High level of antibodies titers maintained until the end of the treatment. Antibody titers were observed in all treated mice (Fig 3.25)

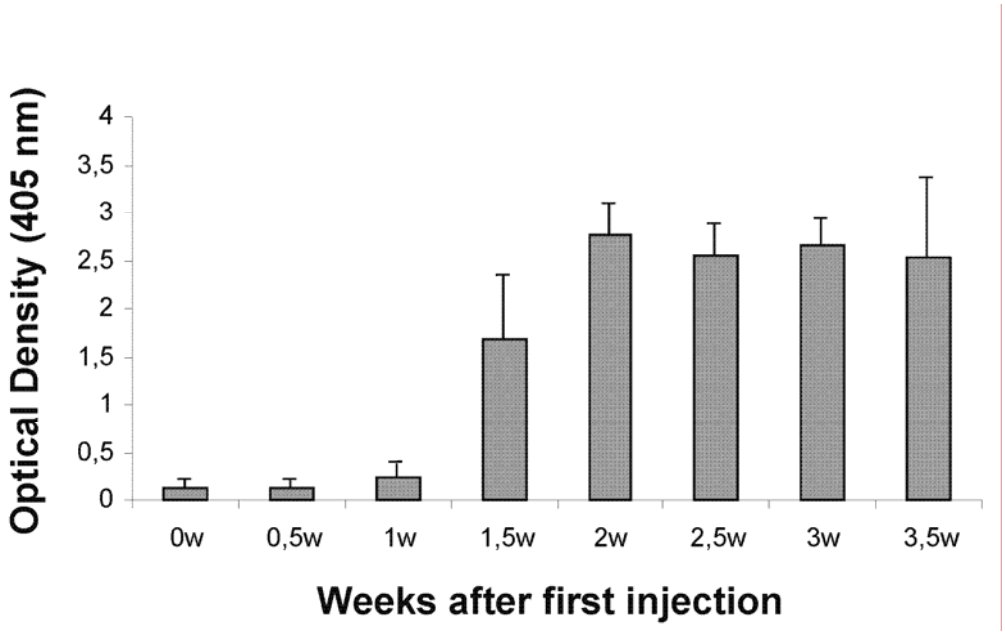


Fig 3.25: Development of antibodies (ELISA) with 2 weekly injections

The data show antibodies against IgG after each single injection. Data are double values from a single mouse during the 4 weeks of treatment.

Immunoprecipitation assays with Pansorbin (2.2.2.13) were performed with the mice serum of injected mice obtained 5 minutes after each single injection and no LAMAN activity was detectable in the pellet while the 91% of the LAMAN activity determined prior to IP was detectable in the supernatant indicating that

in no case the antibodies did precipitate human LAMAN. Therefore, the development of IgG antibody titers is not necessary related to human LAMAN.

To test if the IgG titers affect LAMAN activity towards a synthetic substrate (2.2.2.14), mice serum obtained 5 minutes after each single injection was incubated over night at 4 °C with human LAMAN. Activity was measured before and after incubation. LAMAN activity varied by less than 10% before and after incubation, therefore the IgG titers did not inactivate human LAMAN.

4. DISCUSSION

4.1 ERT in lysosomal storage diseases

Intravenous administration of the missing enzyme has been shown in several animal models of lysosomal storage disorders to effectively reduce the lysosomal storage in multiple visceral tissues (for review see Neufeld *et al.*, 2004). In the non-neuronopathic type I Gaucher disease which primarily affects cells of the monocyte/macrophage lineage, ERT with a modified form of the missing glucocerebrosidase that targets mannose receptors in monocytes/macrophage has become a powerful therapeutic option (Barton *et al.*, 1991). A major problem for ERT of most of the lysosomal storage disorders is the involvement of the central nervous system. Studies in a mouse model of MPS VII revealed that recombinant β-glucuronidase can reach the brain parenchyma when it is injected into newborns whose blood-brain barrier (BBB) is still leaky (O´Connor *et al.*, 1998). Two weeks later, however, the BBB is fully differentiated. The blood-brain barrier apparently prevents the crossing of lysosomal enzymes from the blood to the interstitial space surrounding neuronal and glial cells of the central nervous system (Dobrenis *et al.*, 2004). In spite of the inaccessibility of neural cells to intravenously administered lysosomal enzyme, ERT may have some indirect beneficial effects on brain function in lysosomal storage disorders. The latter may result from the clearance of storage material from brain endothelia and from the meninges which may improve the microcirculation and the flow of the cerebrospinal fluid (Kakkis *et al.*, 2001). The assessment of the efficacy of ERT depends on the availability of a suitable animal model and of sufficient amounts of the missing enzyme. The generation of a mouse model for α-mannosidosis and the production of recombinant LAMAN have made it possible to study the efficacy of ERT in this lysosomal storage disorder.

There are two major receptor systems responsible for the cellular uptake of circulating lysosomal enzymes. The Mr 300 kDa mannose 6-phosphate/ insulin like growth factor II receptors (MPR 300) which are expressed almost ubiquitously (Gosh *et al.*, 2003), and the mannose receptor, which is specific to cells of the monocyte/ macrophage linage (Pontow *et al.*, 1992). The asialoglycoprotein receptor present on hepatocytes and recognizing terminal galactose/N-acetylgalactosamine residues (Ashwell *et al.*, 1992) can also contribute to the clearance. This is explained by the predominance of high-mannose type oligosaccharides in lysosomal enzymes. The phosphorylation of mannose residues is an early event in the processing of N-linked oligosaccharides of lysosomal enzyme precursors and impairs their subsequent processing to sialylated complex type oligosaccharides (Kornfeld & Mellman, 1989; Sousa *et al.*, 1995). Lysosomal enzymes isolated from the secretions of cells are therefore enriched in precursor forms containing mannose 6-phosphate residues, while lysosomal enzymes isolated from tissues are enriched in forms that have been subjected to limited proteolysis and trimming of their oligosaccharides by phosphatases and glycosidases in lysosomes.

4.2 Comparison of the three LAMAN species

In the present study we have used LAMAN from three different species. LAMAN purified from bovine kidney consisted of mature polypeptides with a low mannose 6-phosphate content. The recombinant human LAMAN isolated from the secretion of CHO cells consisted mainly of the precursor form, but was also poorly phosphorylated. The low mannose 6-phosphate content of this preparation is likely to be due to dephosphorylation during isolation. The recombinant mouse LAMAN which was purified from the secretions of mouse embryonic fibroblasts lacking mannose 6-phosphate receptors, had a high content of mannose 6-phosphate and was represented by the precursor form. The clearance from circulation was faster for the two poorly phosphorylated

LAMAN preparations from bovine kidney and CHO cells than for the highly phosphorylated mouse LAMAN. The uptake of the different LAMAN preparations by organs was not directly compared. However, comparing the data of the corrective effect in different experiments suggests that of the poorly phosphorylated human and of the highly phosphorylated mouse LAMAN similar fractions are taken up by the liver, kidney and heart. Only in spleen was the uptake different and facilitated by higher phosphorylation. In a related study on the uptake of phosphorylated and non-phosphorylated ß-glucuronidase in mice no difference was observed for the uptake into liver and spleen, while uptake into kidney and heart was higher for the phosphorylated enzyme (Sands *et al.*, 2001). For α-galactosidase preparations that differed in glycosylation and phosphorylation a similar uptake by liver, spleen and kidney was observed (Ioannu et al., 2001). This indicates that the effect of glycosylation and phosphorylation on the uptake of lysosomal enzymes into different organs depends on the enzyme.

4.3 Effect of a single injection of LAMAN

A major difference between the LAMAN preparations from different sources was their stability. Assuming first order kinetics the activity of mouse LAMAN (see Table 3.3) decreased in liver, kidney and spleen with an apparent half life of 3 h or less, while the apparent half life of human LAMAN was more than 3 times longer. Bovine LAMAN activity decreased in a fashion similar to mouse LAMAN (H. Bustami, personal communication). This may explain the higher corrective efficacy of human LAMAN on the oligosaccharide storage in spleen, kidney and heart (see Fig. 3.6).

A single intravenous administration of LAMAN led to a rapid and pronounced decrease of the lysosomal storage of neutral oligosaccharides irrespective of the source of the enzyme (see sections 3.2 and 3.4). The corrective effect was

most pronounced in liver. This is also the tissue where the increase of LAMAN activity relative to that in wild type mice was highest. Histological examination revealed a correction of storage in both parenchymal and non-parenchymal liver cells. These was true for the highly phosphorylated mouse LAMAN and for the poorly phosphorylated human LAMAN (see Figs. 3.9). This was unexpected as an earlier study with ß-glucuronidase had shown that non-phosphorylated enzyme forms localized almost exclusively to non-parenchymal liver cells, while the phosphorylated forms localized to both parenchymal and non-parenchymal liver cells (Sands *et al.*, 2001). The corrective effect of LAMAN was transient in all tissues. The neutral oligosaccharides started to reaccumulate 2 to 6 days after the injection.

4.4 Effect of a double dose of human LAMAN

A remarkable correction of storage was observed when we injected of 250 mU human LAMAN per g body weight. That reduced storage in spleen and kidney by 80-90% was repeated after 3.5 days (see section 3.5). Within one week the storage disappeared not only in liver, but also in kidney and heart, two of the organs that presented more resistance to the treatment. More importantly, the concentration of neutral oligosaccharides also decreased in brain to about one fourth of that in mock-injected α-mannosidosis mice. The latter cannot be directly attributed to an uptake of LAMAN into neural cells. That was the first experiment in which an effect of the treatment on the storage of oligosaccharides was observed in brain, and further studies are needed to certificate this result.

4.5 Effect of multiple injections of human LAMAN

While almost a total correction of storage was observed when the mice received two times 250 mU human LAMAN per g body weight at an interval of 3.5 days

(section 3.5), in the long term studies based on multiple injections of 250 mU human LAMAN per g body weight (see section 3.8, once per week and section 3.9, twice a week), a full correction of the storage in peripheral organs was not observed. We hypothesized that an immune reaction against human LAMAN could interfere with the ERT and could explain the lower efficiency of the long term therapy with regard to storage material. Therefore, the general humoral immune response was determined in these long term studies, measuring the IgG level. After 4-5 injections, mice showed a high amount of IgG titers. Immunoprecipitation studies indicate that the antibodies did not precipitate LAMAN and did not affect LAMAN activity when incubated with synthetic substrate. Thus, the non-achievement of total correction could have to explanations: the immune response is unspecific, but could some how affect the uptake of LAMAN by the organs (see table 3.8) and the consequent correction of the oligosaccharides storage (see Figs. 3.20 and 3.23), or could be also due to differences on a quality of the LAMAN preparation (different polypeptide pattern, different phosphorylation). Further studies with new LAMAN preparations are needed to clarify these observations and to state the causes of the high immune response.

These long term studies based on repeated injections of 250 mU human LAMAN per g body weight (see section 3.8 and 3.9) resulted also in a decreased concentration of neutral oligosaccharides in the brain of some treated mice (see Figs. 3.20 and 3.24). To verify that the correction of the storage in brain was real, and not due to a loss of sugars during the preparation procedure, ^{3}H-labelled oligosaccharides were prepared and used as spike-in control for the isolation of oligosaccharides from brain (see section 3.6).
In mice the blood-brain barrier matures within the first two weeks of life, and lysosomal enzymes administered intravenously after this period do not cross the blood-brain barrier (Vogler *et al.*, 1999; Yu *et al.*, 2000; Miranda *et al.*, 2000; Du *et al.*, 2001). In α-mannosidosis mice, the integrity of the blood-brain barrier is preserved. Intravenously administered Evans blue did not cross the blood-brain barrier (section 3.7) and immunoglobulin G was excluded from

extravascular tissue. In the brain homogenates of treated mice we observed trace amounts of LAMAN (see Table 3.3 and 3.6). These activities are attributed to extraneural cells, such as endothelia, choroid plexus epithelium and incompletely removed meninges. If it is unlikely that the administered LAMAN gets access to oligosaccharides stored in neuronal and glial cells alternative mechanisms have to be considered. Clearance of oligosaccharides via the blood circulation or via an improved flow of the cerebrospinal fluid may contribute to the decrease of neutral oligosaccharide storage in brain. A careful histological and biochemical analysis of the brain of treated and non-treated α-mannosidosis mice will be required to clarify in which cell types and regions of the brain ERT decreases the storage of neutral oligosaccharides.

Experimental attempts to overcome the closed BBB comprise invasive strategies, e.g., intracerebroventricular infusion or temporary disruption of the tight junctions between cerebral endothelial cells by infusing hypertonic solutions (review by Scherrman, 2002). The associated risks of infection and neuropathological side effects directed attention to noninvasive delivery strategies based on conjugates between blood-brain shuttle vectors and therapeutic enzymes. Examples for shuttle vectors that have been investigated are certain antibodies which are transcytosed across the BBB and atoxic fragments of tetanus and cholera toxin which gain access to the CNS via retroaxonal transport (Dobrenis *et al.*, 1992; Alisky *et al.*, 2002)

In summary, this study yielded encouraging data on the efficacy of ERT in a mouse model of α-mannosidosis. A long term study based on repeated injection of LAMAN and complemented by ultrastructural analysis of multiple tissues including brain and the measurement of cognitive abilities and motor skills is needed to evaluate the full potential of ERT in mouse α-mannosidosis.

5 SUMMARY

A comparative study between the three different sources of LAMAN (purified from bovine kidney, recombinant mouse and recombinant human) led us to conclude that the corrective effect of the treatment on the storage of mannose oligosaccharides was highest for the recombinant human LAMAN. The corrective potential of human LAMAN was higher in kidney and heart, two organs which are more resistant to metabolic correction than liver and spleen. Human LAMAN was rapidly cleared from the circulation (half life of 8 min) and the stability of the enzyme internalized by liver, kidney and spleen was higher than that of the bovine and mouse LAMAN. Therefore, human LAMAN was used to prove the efficacy of ERT in α-mannosidosis mice.

After a single injection of LAMAN, a total correction of the storage in liver was observed 1 and 3 days after the injection, and the maximal correction in spleen and kidney was observed 3 and 6 days after the injection, respectively. Neutral mannose oligosaccharides started to reaccumulate again in all the organs 3 to 6 days after the injection. Afterwards the mice were injected twice with the same dose of human LAMAN (at day 0 and day 3.5) and 7 days after the first injection a total correction of the storage was observed in all the organs analyzed. Most notably, a correction of the storage was observed in the brain of treated α-mannosidosis mice. All this data demonstrate that the corrective effect of ERT on the storage of neutral oligosaccharides was time-, tissue- and dose-dependent.

To find the best therapeutic strategy of the ERT, long term experiments were performed, administrating human LAMAN at different intervals. Storage material remained at a residual level in all the studied organs throughout 16 weeks of treatment. A correction on the storage of the neutral mannose oligosaccharides was observed again in the brains of treated mice.

This data clearly underline the efficacy of ERT for the correction of storage in α-mannosidosis and suggests that ERT can substantially decrease storage also in the brain.

6. BIBLIOGRAPHY

Alisky J.M., van de Wetering C.I., Davidson B.L. (2002) Widespread dispersal of cholera toxin subunit b to brain and spinal cord neurons following systemic delivery. *Exp Neurol*., **178**(1):139-46.

Artelt, P., Morelle, C., Ausmeier, M., Fitzek, M., and Hauser, H. (1988). Vectors for efficient expression in mammalian fibroblastoid, myeloid and lymphoid cells via transfection or infection. *Gene,* **68,** 213-219.

Ashwell, G. and Harford, J. (1982) Carbohydrate-specific receptors of the liver. *Ann. Rev. Biochem.,* **51**, 531-554.

Barton, N.W., Brady, R.O., Dambrosia, J.M., Di Bisceeglie, A.M., Doppelt, S.H., Hill, S.C et al. (1991) Replacement therapy for inherited enzyme deficiency-macrophage-targeted glucocerebrosidase for Gaucher's disease. *N. Engl. J. Med.,* **324**, 1464-1470.

Beccari, T., Apolloni, M.G., Constanzi, E., Stinchi, S., Stirling, J.L., della Fazia, M.A., Servillo, G., Viola, M.P., and Orlacchio, A. (1997). Lysosomal alpha-mannosidases of mouse tissues: characteristics of the isoenzymes, and cloning and expression of a full-length cDNA. *Biochem J.,* **327**, 45-49.

Berg, T., Riise, H.M., Hanse, G.M., Malm, D., Tranebjarg, L., Tollersrud, O.K. and Nilssen, O. (1999) Spectrum of mutations in α-mannosidosis. *Am J. Hum. Genet.,* **64**, 77-88.

Beutler E, Grabowski G.A. Gaucher disease (2001). In Schriver C.R., Beudet A.L., Sly W.S., Valle D. Eds. Metabolic and molecular bases of inherited diseases, 3635-3668.

Bijsterbosch M.K., Donker W, van de Bilt H., van Weely S., van Berkel T.J., Aerts J.M. (1996) Quantitative analysis of the targeting of mannose-terminal

glucocerebrosidase. Predominant uptake by liver endothelial cells. *Eur J Biochem*., **237**(2):344-9.

Bijvoet A.G., Van Hirtum H., Kroos M.A., Van de Kamp E.H., Schoneveld O., Visser P. et al. (1999) Human acid alpha-glucosidase from rabbit milk has therapeutic effect in mice with glycogen storage disease type II. *Hum Mol Genet*., **8**(12):2145-53.

Burditt, L.J., Chtai, K., Hirani, S., Nugent, P.G., Winchester, B. and Blakmore, W.F. (1980) Biochemical studies on a case of feline mannosidosis. *Biochem. J.*, **189**, 467-473.

Byers S., Crawley A.C., Brumfield L.K., Nuttall J.D., Hopwood J.J. (2000) Enzyme replacement therapy in a feline model of MPS VI: modification of enzyme structure and dose frequency. *Pediatr Res*., **47**(6):743-9.

Cantor A.B., Baranski T.J., Kornfeld S. (1992) Lysosomal enzyme phosphorylation. II. Protein recognition determinants in either lobe of procathepsin D are sufficient for phosphorylation of both the amino and carboxyl lobe oligosaccharides. *J. Biol. Chem.* **267**:23349-56.

Crawley A.C., Brooks D.A., Muller V.J., Petersen B.A., Isaac E.L., Bielicki J., King B.M., Boulter C.D., Moore A.J., Fazzalari N.L., Anson D.S., Byers S., Hopwood J.J. (1996) Enzyme replacement therapy in a feline model of Maroteaux-Lamy syndrome. *J Clin Invest*., **97**(8):1864-73.

Danon, M.J., Oh, S.J., DiMauro, S., Manaligod, J.R., Eastwood, A., Naidu, S., and Schliselfeld, L.H. (1981). Lysosomal glycogen storage disease with normal acid maltase. *Neurology,* **31**, 51-57.

De Duve C., Pressman B.C., Gianetto R. et al. (1995) Tissue fraction studies. 6 : Intracellular distribution patterns of enzymes in rat liver tissue. *Biochem J* **60**, 604-617.

Dobrenis K., Joseph A., Rattazzi M.C. (1992) Neuronal lysosomal enzyme replacement using fragment C of tetanus toxin. *Proc Natl Acad Sci,* **89**(6):2297-301.

Dobrenis, K. (2004) Cell-mediated delivery systems. In: Platt, F.M., Walkley, S.V., (eds.) *Lysosomal disorders of the brain*, Oxford University Press, pp. 339-380

Du, H., Schiavi, S., Levine, M., Mishra, J., Heur, N., and Grabowski, G.A. (2001) Enzyme therapy for lysosomal acid lipasi deficiency in the mouse. *Hum. Mol. Genet.,* **10**, 1639-1648.

Eskelinen EL, Illert AL, Tanaka Y, Schwarzmann G, Blanz J, Von Figura K, Saftig P. (2002) Role of LAMP-2 in lysosome biogenesis and autophagy. *Mol Biol Cell.,***13**(9), 3355-68.

Eskelinen E.L., Tanaka Y., Saftig P. (2003) At the acidic edge: emerging functions for lysosomal membrane proteins. *Trends Cell Biol.*,**13**(3), 137-45. Review.
Eskelinen *et al.*, 2002 y 2003

Evers, M., Saftig, P., Schmidt, P., Hafner, A., McLoghlin, D., Schmahl, W., von Figura, K. and Peters, C. (1996) Targeted disruption of the arylsulfatase B gene results in mice resembling the phenotype of mucopolysaccharidosis VI. *Proc. Natl Acad. Sci. USA*, **93**, 8214-8219.

Fratantoni J.C, Hall C.W, Neufeld E.F. (1969) The defect in Hurler and Hunter syndromes. II. Deficiency of specific factors involved in mucopolysaccharides degradation. *Proc Natl Acad Sci* , **64**(1), 360-6.

Futerman A.H., van Meer G. (2004) The cell biology of lysosomal storage disorders. *Nature*, **5**, 554-565. Review.

Ghosh, P., Dahms, N.M. and Kornfeld, S. (2003) Mannose 6-phosphate receptors: new twists in the tale. *Nature reviews,* **4**, 202-212.

Gravel R.A., Kaback M.M., Proia R.L. *et al*. (2001). The GM2 gangliosidosis. In Schriver C.R., Beudet A.L., Sly W.S., Valle D. Eds. Metabolic and molecular bases of inherited diseases, 3827-3876.

Griffiths G., Gruenberg J. (1991) The arguments for pre-existing early and late endosomes. *Trends in Cell Biology,* Volume1, Issue1, 5-9 .

Harmatz P, Whitley CB, Waber L, Pais R, Steiner R, Plecko B, Kaplan P, Simon J, Butensky E, Hopwood JJ. (2004) Enzyme replacement therapy in mucopolysaccharidosis VI (Maroteaux-Lamy syndrome).*J Pediatr*., **144**(5):574-80.

Hasilik A., Waheed A., von Figura K. (1981) Enzymatic phosphorylation of lysosomal enzymes in the presence of UDP-N-acetylglucosamine. Absence of the activity in I-cell fibroblasts. *Biochem Biophys Res Commun*., **98**(3):761-7.

Haskins, M.E., Jezyk, P.F., Desnick, R.J., McDonough, S.K. and Patterson, D.F. (1979) Alpha-L-iduronidase deficiency in a cat: a model of mucopolysaccharidosis I. *Pediatr. Res.*, **13**, 1294-1297.

Hess, B., Saftig, P., Hartmann, D., Coenen, R., Lüllmann-Rauch, R., Goebel, H., Evers, M., von Figura, K., D'Hooge, R., Nagels, G., De Deyn, P., Peters, C. and Gieselmann, V. (1996) Phenotype of arylsulfatase A-deficient mice: relationship to human metachromatic leukodystrophy. *Proc. Natl Acad. Sci. USA*, **93**, 14821-14826.

Hirsch, C., Blom, D. and Ploegh, H.L. (2003) A role for N-glycanase in the cytosolic turnover of glycoproteins. *EMBO J.*, **22**, 1036-1046.

Hirschhorn R., Reuser A. (2001) Glycogen storage disease type II: acid alpha-glucosidase (acid maltase) deficiency. In Schriver C.R., Beudet A.L., Sly W.S., Valle D. Eds. Metabolic and molecular bases of inherited diseases, 3389-3420.

Hocking, J.D., Jolly, R.D, Blatt R.D. (1972) Deficiency of α-mannosidase in Angus cattle. An inherited lysosomal storage disease. *Biochem. J.*, **128**, 69-78.

Hoflack B., Kornfeld S. (1985) Purification and characterization of a cation-dependent mannose 6-phosphate receptor from murine P388D1 macrophages and bovine liver. *J Biol Chem*., **260**(22):12008-14.

Hopwood J.J, Bunge S., Morris C.P., Wilson P.J., Steglich C., Beck M., Schwinger E., Gal A. (1993) Molecular Basis of mucopolysacharidosis type II. *Hum Mutat*., **2**(6):435-42. Review.

Jeyakumar M., Thomas R., Elliot-Smith E., Smith D.A., van der Spoel A.C., d'Azzo A., Perry V.H., Butters T.D., Dwek R.A., Platt F.M. (2003) Central nervous system inflammation is a hallmark of pathogenesis in mouse models of GM1 and GM2 gangliosidosis. *Brain*, **126**, 974-987.

Ioannu, Y.A., Zeidner, K.M., Gordon, R.E. and Desnick, R.J. (2001) Fabry disease: preclinical studies demonstrated the effectivness of α-galacosidase replacement in enzyme-deficient mice. *Am. J. Hum. Genet.,* **68**, 14-25.

Johnson K.F., Kornfeld S. (1992) The cytoplasmic tail of the mannose 6-phosphate/insulin-like growth factor-II receptor has two signals for lysosomal enzyme sorting in the Golgi. *J Cell Biol.*, **119**(2):249-57.

Kakkis, E.D., Schuchman, E., He X *et al*. (2004). Enzyme replacement therapy in feline mucopulosaccharidosis. *I Mol Genet Metab*., **72**, 199208.

Kakkis, E.D., Muenzer, J., and Tilller, G.E. (2001) Enzyme replacement therapy in mucopolysaccharidosis. *N. Engl. J. Med.,* **344**, 182-188

Kasper D, Dittmer F, von Figura K, Pohlmann R. (1996) Neither type of mannose 6-phosphate receptor is sufficient for targeting of lysosomal enzymes along intracellular routes. *J Cell Biol*., **134**(3):615-23.

Kornfeld R., Kornfeld S. (1985) Assembly of asparagine-linked oligosaccharides. *Ann Rev. Biochem* **54**, 631-64.

Kornfeld S, Mellman I. (1989) The biogenesis of lysosomes. *Annu Rev Cell Biol*., **5**, 483-525. Review.

Kornfeld S. (1987) Trafficking of lysosomal enzymes. *FASEB J.* **1**, 462

Kornfeld S. (1992) Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors*. Annu. Rev. Biochem.* **61**, 307-30.

Lachmann R.H. (2004) Alpha-glucosidase (CHO) (Genzyme). *Curr Opin Investig Drugs*., **5**(10):1101-10. Review.

Lang L, Reitman M, Tang J, Roberts RM, Kornfeld S. (1984) Lysosomal enzyme phosphorylation. Recognition of a protein-dependent determinant allows specific phosphorylation of oligosaccharides present on lysosomal enzymes. *J Biol Chem*., **259**(23),14663-71.

Lazzarino D.A., Gabel C.A. (1988) Biosynthesis of the mannose 6-phosphate recognition marker in transport-impaired mouse lymphoma cells. Demonstration of a two-step phosphorylation 2 steps phosphoriation. *J Biol Chem*., **263**(21), 10118-26.

Lundberg P, Langel U. (2003) A brief introduction to cell-penetrating peptides. *J Mol Recognit*., **16**(5):227-33. Review

Malm, D. (2004) Prologue. The doctor as parent. In: Platt, F.M., Walkley, S.V., (eds.) *Lysosomal disorders of the brain*, Oxford University Press, pp. XXI-XXVII

Mancini et al, 2005, Lysosomal transport disorders.

Masson C., Cisson I., Simon V., Insalaco P, Audran M. (2004). Fabry disease : A review. *Joint Bone Spine*, **71** (5), 381-3.

Mignani R., Cagnoli L. (2004) Enzyme replacement therapy in Fabry's disease: recent advances and clinical applications. *J Nephrol.* May-Jun;17(3):354-63. Review.

Miranda, S.R., He, X., Simonara, C.M., Gatt, S, Desnick, R.J., and Schuchman, E.H. (2000) Infusion of recombinant human acid sphingomyelinase into Niemann-Pick disease mice leads to visceral, but not neurological correction of the pathophysiology. *FASEB J.,* **14**, 1988-1995.

Muenzer J., Lamsa J.C., Garcia A., Dacosta J., Garcia J., Treco D.A. (2002) Enzyme replacement therapy in mucopolysaccharidosis type II (Hunter syndrome): a preliminary report. *Acta Paediatr Suppl*., **91**(439), 98-9. Review.

Munier-Lehmann H, Mauxion F., Bauer U., Lobel P., Hoflack B. (1996) Re-expression of the mannose 6-phosphate receptors in receptor-deficient fibroblasts. Complementary function of the two mannose 6-phosphate receptors in lysosomal enzyme targeting. *Biol Chem.,* **271**(25):15166-74.

Neufeld, E.F. (2004) Enzyme replacement therapy. In: Platt, F.M., Walkley, S.V., (eds.) *Lysosomal disorders of the brain*, Oxford University Press, pp. 327-338.

Nishino I., Fu J., Tanji K., Yamada T., Shimojo S., Koori T., Mora M., Riggs J.E., Oh S.J., Koga Y., Sue C.M. et al. (2004) Primary LAMP-2 deficiency causes X-linked vacuolar cardiomyopathy and myopathy (Danon disease). *Nature*, **406**(6798), 906-10.

O'Connor L.H., Erway L.C., Vogler C.A., Sly W.S., Nicholes A., Grubb J., Holmberg S.W., Levy B., Sands M.S. (1998) Enzyme replacement therapy for murine mucopolysaccharidosis type VII leads to improvements in behavior and auditory function. *J Clin Invest*., **101**(7), 1394-400.

Öckerman, P. A. (1967) A generalized storage disorder resembling Hurler's syndrome. *Lancet,* **2**, 239-241.

Phaneuf, D., Wakamatsu, N., Huang, J.Q., Borowski, A., Peterson, A.C., Fortunato, Pontow, S.E., Kery, V., and Stahl, P.D. (1992) Mannose receptor. *Int. Rev. Cytol.,* **137B**, 221-244.

Phaneuf D., Wakamatsu N., Huang J.Q., Borowski A., Peterson A.C., Fortunato S.R., Ritter G., Igdoura S.A., Morales C.R. et al. (1996) Dramatically different phenotypes in mouse models of human Tay-Sachs and Sandhoff diseases. *Hum Mol Genet*., **5**(1):1-14.

Pohlmann R., Boeker M.W., von Figura K. (1995) The two mannose 6-phosphate receptors transport distinct complements of lysosomal proteins. *J Biol Chem*., **270**(45), 27311-8.

Raas-Rothschild A., Cormier-Daire V., Bao M., Genin E., Salomon R., Brewer K., Zeigler M., Mandel H., Toth S., Roe B., Munnich A., Canfield W.M. (2000) Molecular basis of variant pseudo-hurler polydystrophy (mucolipidosis IIIC) J Clin Invest.,**105**(5), 673-81.

Reitman M.L., Kornfeld S. (1981) Lysosomal enzyme targeting. N-Acetylglucosaminylphosphotransferase selectively phosphorylates native lysosomal enzymes. *J Biol Chem.*, **256**(23), 11977-80.

Sachs and Sandhoff diseases. *Hum. Mol. Genet.*, **5**, 1-14.

Sands, M.S., Vogler, C.A., Ohlemiller, K.K., Roberts, M.S., Grubb, J.H., Levy, B. and Sly, W.S. (2001) Biodistribution, kinetics and efficacy of highly phosphorylated and non-phosphorylated ß-glucuronidase in the murine model of mucopolysaccharidosis VII. *J. Biol. Chem.,* **276**, 41160-43165.

Saint-Pol, A., Cordogno, P. and Moore, S.E.H. (1999) Cytosol to lysosome transport of free polymannose-type oligosaccharides. *J. Biol. Chem.,* **274**, 13547-13555.

Scherrmann J.M. (2002) Drug delivery to brain via the blood-brain barrier. *Vascul Pharmacol*., **38**(6), 349-54. Review.

Sousa M, Parodi A. J. (1995) The molecular basis for the recognition of misfolded glycoproteins by the UDP-Glc:glycoprotein glucosyltransferase *EMBO J.* **14(17)** :4196-223.

Stinchi, S., Lüllmann-Rauch, R., Hartmann, D., Coenen, R., Beccari, T., Orlaccio, A., von Figura, K., and Saftig, P. (1999) Targeted disruption of the lysosomal α-mannosidase gene results in mice resembling a mild form of human α-mannosidosis. *Hum. Mol. Gen.,* **8**, 1365-1372.

Thomas, G.H. (2001) Disorders of glycoprotein degradation: α-mannosidosis, ß-mannosidosis, fucosidosis and sialidosis. In: Scriver, C., Beaudet, A., Sly, W., Valle, D., Childs, B., Kinzler, K., Vogelstein, B. (eds.) *The Metabolic and Molecular Bases of Inherited Disease*, McGraw-Hill, New York, 8th ed., pp. 3507-3533

Tollersrud, O.K., Berg, T., Healy, P., Evjen, G., Ramachandran, U. and Nilssen, O. (1997) Purification of bovine lysosomal α-mannosidase, characterization of its gene and determination of two mutations that cause α-mannosidosis. *Eur. J. Biochem.,* **246**, 410-419

Van der Hout J.M.., Kamphoven J.H., Winkel L.P. *et al*.(2004). Long-term intravenous treatment of Pompe disease with recombinant human alpha-glucosidase from milk. *Pediatrics*, 113 (5), e448-57.

Vogler, C., Levy, B., Galvin, N.J., Thorpe, C., Sands, M.S., Barker, J.E., Barty, J., Birkenmeier, E.H., and Sly, W.S. (1999) Enzyme replacement therapy in murine mucopolysaccharidosis type VII: neuronal and glial response to ß-glucuronidase requires early initiation of enzyme replacement therapy. *Pediatr. Res.,* **45**, 838-844.

Walkely, S.U., Thuall, M.A., Dobrenis, K., Huang, M., March, .A., Siegel, D.A. and Wurzelmann, S. (1994) Bone marrow transplantation corrects the enzyme defect in neurons of the central nervous system in a lysosomal storage disease. *Proc. Natl. Acad. Sci. U.S.A.,* **91**, 2970-2974.

Wall, D.A., Grange, D.K., Goulding, P., Danies, M., Luisiri, A. and Kotagal, S. (1998) Bone marrow transplantation for the treatment of α-mannosidase. *J. Pediatr.,* **133**, 282-285.

Will, A., Cooper, A., Hatton, C., Sardhawalla, I.B., Evans, D.I.K. and Stevens, R.F. (1987) Bone marrow transplantation in the treatment of α-mannosidosis. *Arch. Dis. Childhood,* **62**, 1044-1049

Wraith J.E., Clarke L.A., Beck M., Kolodny E.H., Pastores G.M., Muenzer J., Rapoport D.M., Berger K.I., Swiedler S.J., Kakkis E.D., Braakman T., Chadbourne E., Walton-Bowen K,. Cox G.F. (2004) Enzyme replacement

therapy for mucopolysaccharidosis I: a randomized, double-blinded, placebo-controlled, multinational study of recombinant human alpha-L-iduronidase (laronidase). *J Pediatr*., **144**(5), 581-8.

Yamanaka, S., Johnson, M.D., Grinberg, A., Westphal, H., Crawley, J.N., Taniike, M., Suzuki, K. and Proia, R.L. (1994) Targeted disruption of the *Hexa* gene results in mice with biochemical and pathologic features of Tay-Sachs disease. *Proc. Natl Acad. Sci. USA*, **91**, 9975-9979.

Yu, W.H., Zhao, K.W., Ryanzantsev, S., Rozengurt, N., and Neufeld, E.F. (2000) Short term enzyme replacement in the murine model of Sanfilippo syndrome type B. *Mol. Genet. Metab.,* **71**, 753-780

Zhu Y., Li X., Kyazike J., Zhou Q., Thurberg B.L., Raben N., Mattaliano R.J., Cheng S.H. (2004) Conjugation of mannose 6-phosphate-containing oligosaccharides to acid alpha-glucosidase improves the clearance of glycogen in pompe mice. *J Biol Chem*., **279**(48):50336-41.

APPENDIX

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Lebenslauf - CURRICULUM VITAE

• PERSÖNLICHE ANGABEN

Name und Vorname :	Prieto Roces, Diego
Geb. am:	15.07.78 in Avilés, Asturias, Spanien
Adresse :	Annastr. 17 App. 4, 37075 Göttingen, Deutschland
Telefonnummer:	(+49)5513891739 / (+49)1798276169
E-mail:	diego.prieto@gmx.net

• HOCHSCHULSTUDIUM

2002- **Dissertation:** „Enzyme replacement theraphy mit knock-out alpha-mannosidosis Mäuse“
Institüt Biochemie 2, Georg-August Universität, Göttingen. Arbeitsgruppe von Prof. Dr. K.von Figura.
EU Framework programme EURAMAN (in Kollaboration mit dem Firma Zymanex A/S).

2000-2002 **Diplom in Biochemie** an der Medizinische Fakultät der Universität von Oviedo, Asturias, Spanien.

1996-2001 **Diplom in Chemie** (Schwerpunkt: Organische Chemie) an der Chemische Fakultät der Universität von Oviedo, Asturias, Spanien.

• WEITERE QUALIFIKATIONEN

1998 Kurs über „**Chemische Verfahrensweisen zur Beseitigung industrieller Abfälle**” („Ciencia Tecnología y Cultura de las Residuo Auditorias Ambientales”), an der Bergbau Fakultät der Universität von Oviedo, Asturias.

1997 Kurs über „**Chemische Technologien im Umweltschutz**” (“Ciencia Tecnología y Cultura de los Residuos Medioambientales”), an der Bergbau Fakultät der Universität von Oviedo, Asturias.

• PRAKTIKA

März-August 2002 **Forschungsprojekt** über die **Hormonelle regulation des Glucose-6-Phosphat-Transporters auf DNA-Ebene**, am Institut für Medizinische Biochemie und Molekularbiologie an der Ernst-Moritz-Arndt-Universität in Greifswald, Deutschland. (ERASMUS-STIPENDIUM).

2000 **Forschungsprojekt** über "**Fischer's Carbene Synthese**" unter Leitung von Dr. Francisco J. González Fernández an der Chemischen Fakultät der Universität von Oviedo, Asturias (2 Monate)

- **PUBLIKATIONEN**

2004 "Efficacy of enzyme replacement therapy in α-mannosidosis mice: a preclinical animal study". Human Molecular Genetics, 2004, **13**, No. 18, 1979-1888.
Als "Research Highlights" in Nature, Vol 431, 16 September 2004

- **SPRACHKENNTNISSE UND INFORMATIK**

Englisch: Gute Kenntnisse in Wort und Schrift. **First Certificate of English.**

Deutsch: Gute Kenntnisse in Wort und Schrift. Sprachkurs an der **Bauhaus Universität** in Weimar, Deutschland (100 Stunde)

Informatik: Kenntnisse in Windows XP, Office XP (Word, Excel, Access, Power Point), Internet Explorer, Netscape und Outlook.