# Enzyme Therapy in Infantile Pompe's Disease

A clinical study on the effect of recombinant human alpha-glucosidase produced in the milk of transgenic rabbits

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Enzym Therapie voor de infantiele vorm van de ziekte van Pompe

Een klinische studie naar het effect van recombinant humaan alpha-glucosidase
geproduceerd in de melk van transgene konijnen

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Aan mijn ouders
Voor Harald, Suzanne en Iris

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# *CHAPTER 1*

## Introduction

## Introduction

Pompe's disease is a progressive and lethal, metabolic disorder. No therapeutic option was available until January 1999 when a clinical study was started aiming to test the safety and efficacy of enzyme replacement therapy with recombinant alpha-glucosidase from rabbit milk. In this thesis I will describe the outcome of this study and compare the results with the natural course.

The introducing paragraphs below provide information about the clinical, diagnostic and elementary aspects of Pompe's disease and depict the road towards therapy.

## 1.1 Pompe's Disease in a Historic Perspective

### *1.1.1 Pompe's Disease*

On December 27th, 1930, the Dutch pathologist, J.C. Pompe performed a post-mortem examination on a 7-months old infant with idiopathic cardiac hypertrophy. Pompe combined the clinical and histopathological features and was the first to describe these in 1932 as a generalised glycogen storage disease[1].

In earlier case reports both the cardiomegaly and the vacuolar histopathology had been described, but the glycogen storage had not been identified. In the absence of a reliable method to stain glycogen, the idiopathic hypertrophy of the heart was thought to be the result of a diffuse benign tumour (rhabdomyomatosis)[2, 3]. In 1900 Best described a staining method to visualise glycogen with light microscopy[4]. Soon afterwards Abricosov observed the presence of glycogen in a case of rhabdomyoma[5]. However, it took a few more years before Pompe, shortly afterwards followed by Putschar[6], postulated that the glycogen storage in Pompe's disease was part of a metabolic disorder. The metabolic defect was elucidated by Hers and de Barsy in 1963 by demonstrating the deficiency of acid alpha-glucosidase as the cause of the disease[7]. In the same year Lejeune et al.[8] established the lysosomal localisation of the enzyme. This discovery made Pompe's disease the first proven lysosomal storage disorder.

### *1.1.2 Clinical features and diversity*

Although the disease was first described in infants, Gunther[9] was the first who noticed that vacuolar glycogen storage did also occur in older children. In 1968 Engel and Dale, later followed by Hudgson et al., described muscular glycogen storage in an adult[10, 11]. With these observations it was recognised that there are several variants of Pompe's disease.

The infantile form presents shortly after birth as a rapidly progressive myopathy. Infants suffer from muscular weakness, poor motor development and failure to thrive and are reported to die within the first[12] to first two years[13] of life. The heart is characteristically involved[14]. Therefore this subtype of the disease is also called the cardiac subtype. More detailed information on the infantile subtype is given in chapter 2 of this thesis.

The late onset phenotype has a more heterogeneous course. The disease may present at any age, which has led to further sub-typing in adult, juvenile or childhood and non-classical

infantile forms of Pompe's disease or Glycogen Storage Disease type II (GSD II). In the adult phenotype only the skeletal muscles are involved. The lower limbs are more severely affected than the upper limbs. Hepatomegaly and cardiomegaly are absent. The presentation of the adult subtype varies. The disease may present with a slowly progressive proximal myopathy, but also with acute respiratory failure caused by weakness of the respiratory muscles[13]. Start of symptoms may be delayed until the second to sixth decade of life. In virtually all cases respiratory support is ultimately needed, and respiratory failure is the main cause of death[13].
The juvenile or childhood phenotype is mainly distinguished from the adult form of GSD II by age of onset and progression of the disease. First symptoms present within the first decade of life. Skeletal muscle weakness is the major problem, cardiac involvement generally does not occur. Pulmonary infections and wasting of the respiratory muscles mostly become fatal before the third decade[12].
Recently, Slonim and co-workers proposed another intermediate phenotype[12]; the non-classical infantile form of Pompe's disease. They reported that patients with the non-classical infantile subtype present with predominant involvement of skeletal muscles in the first year of life. Left ventricular hypertrophy of the heart occurs, but cardiac hypertrophy is reported to be less significant than in the classical infantile form and outflow tract obstruction was not noticed. Patients are reported to survive beyond the first year of life. Respiratory insufficiency may require start of artificial ventilation early in life. They reported that, in contrast to patients with the classical infantile form, non-classical infantile patients do not die from cardiac failure. The clinical picture of the non-classical infantile form may show overlap with the juvenile form of the disease.
Overall the appearance of Pompe's disease is a spectrum with a more defined, infantile, rapidly progressive phenotype with cardiac involvement at one end and the heterogeneous late onset phenotype at the other.

*1.1.3 Lysosomal alpha-glucosidase deficiency as primary defect*

The common cause in all forms of Pompe's disease is the deficiency of lysosomal, acid alpha-glucosidase. This enzyme hydrolyses the glycogen captured in the lysosome. More specifically it releases the alpha-1,4 and alpha-1,6 glucosidic linkages at an optimal pH of 4.0 to 5.0. Deficiency of alpha-glucosidase leads to accumulation of lysosomal glycogen, causing swelling of the lysosome, displacement and compression of cellular organelles and ultimately muscle damage. The histopathologic mechanism, which leads to cellular injury, is not fully understood.
Pompe's disease is an autosomal recessive inherited disease, which occurs with an estimated frequency of 1: 40.000[15-23]. The human acid alpha-glucosidase gene is localised on chromosome 17 in the region q25.2-q25.3[24]. Mutations in the gene cause alpha-glucosidase deficiency. Hoefsloot et al[25, 26] in our centre and Martiniuk et al[27-30] found that the gene is approximately 20 kb long and contains 20 exons[25, 26, 28-30]. The cDNA encodes for a protein of 952 amino acids[27, 28, 31]. The primary translation product, the acid alpha-glucosidase precursor has a molecular mass of 110 kDa. It has a signal sequence[31], which guides the precursor to the lumen of the endoplasmatic reticulum where N-linked glycosylation occurs at all seven available positions. The precursor is processed at both the amino- and carboxy-terminal ends

to form a 95 kDa intermediate and a 76 and 70 kDa mature alpha-glucosidase. Phosphorylation occurs in the Golgi complex at two of the seven sites minimally[32]. During the maturation process the specific activity for the natural substrate glycogen increases[33, 34]. This is possibly the result of a conformational change that allows for more efficient access of the large substrate glycogen to an additional substrate-binding site and/or catalytic site[35]. A fraction of the 110 kDa precursor pool is secreted and can for instance be isolated from urine[33, 34]. Mature acid alpha-glucosidase can be purified from human placenta ( 76 and 70 kDa form), bovine testis (70 kDa) and several other tissues[36]. The enzyme derived from human urine and bovine testis is phosphorylated[36-40] while the 76 and 70 kDa placental forms are not[36, 41]. This may be the result of rapid dephosphorylation of the mature enzyme within lysosomes as seen with other lysosomal enzymes.

After elucidating the cause of Pompe's disease an explanation was sought for the clinical heterogeneity. It was speculated that the level of neutral alpha-glucosidases played a role in the clinical variation. However, Reuser et al. and Van der Ploeg[42] et al. from our group, as well as Henken et al[41] rejected this hypothesis when no significant difference was found between the activity of neutral alpha-glucosidase in the different phenotypes of Pompe's disease[40, 42]. More recently research has shown that the clinical phenotype is mainly determined by the level of residual alpha-glucosidase activity[12, 35, 38, 40, 43-47]. Classical infantile patients have no residual activity, whereas it varies between 3 and 25% of the mean normal value in adult patients[12].

*1.1.4 Genetic heterogeneity*

Generally, the residual enzyme activity and clinical phenotype correlate with different genotypes. Several polymorphism's and over 100 mutations have been reported in the acid alpha-glucosidase gene (http://www.Pompecenter.nl). The mutations are spread throughout the gene and vary from missense, nonsense and splice site mutations to smaller and larger deletions. In classical infantile Pompe's disease the mutations frequently result in lack of transcription or unstable acid alpha-glucosidase mRNA or protein. Some, infantile, mutations are reported to occur more frequently in certain populations. For instance the Asp645Glu mutation occurs especially in Taiwanese patients (allele frequency 0.8)[48]. In African and African American patients the Arg854X nonsense mutation is found quite often (allele frequency 0.5)[49], while del525T and del exon18 are frequent mutations in Dutch patients[13, 50]. In the late onset phenotype these deleterious mutations are found as well, but then combined with mutations that lead to a partial loss of function or to decreased synthesis of otherwise normal acid alpha-glucosidase. The latter mutations may also occur in homozygous form. Adult-onset mutations that were reported more than once include Pro545Leu, Arg672Trp, Arg672Gln, Arg725Trp and IVS6 (-22 →G). The mutation occurring most frequently in Caucasian adult-onset patients is the leaky splice-site mutation IVS1 (-13 T→G) (allele frequency of 0.67 to 0.46)[21, 51, 52]. This mutation is not found in homozygous form in patients, probably due to a sufficient level of residual activity.

Variation in onset of symptoms and progression of disease for "adult genotypes" is not fully explained by different levels of acid alpha-glucosidase activity. The underlying factors may be either environmental or genetic like differences in genetic background, somatic mosaicism, presence of a second mutation or polymorphism in the same allele, and differential 'leakage' of splice site mutation. In addition diet and exercise could play a role in phenotypic expression[12, 53, 54].

*1.1.5 Diagnostic procedures*

Acid alpha-glucosidase is present in all tissues and cells. Its activity can be determined using the natural substrate glycogen or the artificial substrate 4-methylumbelliferyl-alpha-D-glucopyranoside (4-MU). For diagnostic purposes the enzyme activity is usually measured in fibroblasts, leucocytes, lymphocytes, EBV-transformed B-cell lines and muscle. In leukocytes neutral alpha-glucosidase can influence the outcome of the measurement and therefore the natural substrate glycogen has to be used instead of the artificial 4-MU substrate. The diagnosis of Pompe's disease is most reliable performed in an assay on fibroblasts. Therefore, the invasive procedure of a muscle biopsy is not a pre-requisite for diagnosis. Prenatally amniotic cells and chorionic villi can be used.

The determination of residual enzyme activity allows for diagnostic differentiation between the clinical phenotypes. Generally infantile patients have an enzyme activity of less than 1 to 2% of normal, while adult patients have values up to 30% and juvenile patients have intermediate values[12]. However, overlap is seen in the enzyme activity of juvenile and adult patients [45, 55]. Moreover, the normal range is broad and the enzyme activity of carriers overlaps with the activity of both late-onset patients and healthy individuals. Therefore for carrier detection DNA analysis is required. This is also the preferable method for the prenatal diagnosis of late-onset patients, while infantile patients can reliably be diagnosed prenatally by enzyme analysis of uncultured chorionic villi or amniotic cells[56-58].

Infrequently, cases have been reported of a hypertrophic cardiomyopathy and vacuolar glycogen storage with normal alpha-glucosidase activity[59, 60]. Recently, it was demonstrated that the deficiency of a lysosomal membrane protein, LAMP2, is responsible for the disease in these patients (Danon's Disease)[61].

*1.1.6 Ancillary studies and pathology*

In all forms of Pompe's disease increased values of ASAT, ALAT, LDH and CK are commonly found. Ausems et al. proposed to use CK as a first screening parameter for adult Pompe's disease[62]. However, since CK levels can also be normal in Pompe patients caution has to be taken. Another common biochemical abnormality in Pompe's disease is the presence of an abnormal tetrasaccharide band in urine on thin layer chromatograpy[13]. The origin of this tetrasaccharide band is unknown. Sjöblad[63] suggested that undegraded glycogen is released into the circulation and degraded by serum amylase to the oligosaccharide present in urine.

The characteristic cardiomegaly in the infantile Pompe patients can be visualised by chest X-ray and echocardiography. On the electrocardiogram large QRS complexes, repolarisation disturbances and a borderline shortened PQ-interval are seen. It has been suggested by

Gilette and co-workers that glycogen lowers the conduction time and thereby the PQ-interval[14, 64, 65 and this thesis]. Echocardiography reveals an increased thickness of the left ventricular posterior wall and inter-ventricular septum, which may lead to outflow tract obstruction[14, 64, 66, 67].

In all phenotypes the electromyogram (EMG) shows myopathic patterns. At rest, fibrillation potentials and positive denervation potentials can be observed. Other EMG abnormalities are myotonic discharges without clinical myotonia (pseudomyotonic discharges) or an excessive irritability of the muscle fibres, for example at insertion of the needle. Motor unit potentials are of low amplitude with normal or short conduction times and often have a complex form during voluntary contraction. In rare cases, especially in the late onset phenotype, the EMG can be completely normal[13].

Muscle biopsies mostly reveal vacuolar glycogen storage (a vacuolar myopathy) on light microscopy. The glycogen contained in the vacuoles can be visualised with Periodic Acid Schiff (PAS) staining. The vacuoles also show an increased staining for acid phosphatase, indicating that the vacuoles are lysosomes. Excessive glycogen storage may lead to damage of muscle fibers. Ultimately this may lead to replacement of muscle by adipose and/or fibrous tissue. Preferential involvement of type I fibers is reported by some authors, but has not been confirmed by others[68-71]

In the infantile or generalised form vacuolisation eventually is present in all muscles, giving them the classical appearance of a lace-work pattern. In the late onset phenotype muscles are involved more heterogenously. Both healthy and severely affected muscle can be found in the same patient. Thus, normal histopathology does not exclude Pompe's disease [72]. In general, muscle weakness correlates with the histopathological changes observed under the microscope[71, 73-75].

Electron microscopy shows that glycogen accumulates in freely dispersed state as well as in vacuoles. The intracellular accumulation of glycogen causes displacement or compression of normal cellular organelles[76].

At autopsy of infantile patients with GSD II, glycogen accumulation is observed in liver, heart, skeletal muscle, smooth muscle, kidney, the eye, skin, endothelial cells, lymphocytes and in the peripheral and central nervous system[7, 13, 77-83]. In nervous tissue, glycogen accumulation is prominent in Schwann cells, spinal neurones (including anterior horn cells and motor nuclei of the brain and spinal ganglia), myenteric plexus, astrocytes, oligodendroglia, endothelial cells and pericytes.

In late-onset patients glycogen accumulation is less generalised. Storage is mainly found in skeletal muscle, but also in the smooth muscle of blood vessel walls. Notably, glycogen storage is reported in the blood vessel wall of the basilar artery[71, 77, 82-85]. Fusiform dilatations and aneurysms of the basilar artery were described by others[86-89], which suggests that glycogen storage in smooth muscle cells may lead to aneurysms[88]. Infrequently glycogen deposition has been reported in glia and neuronal cells in specific regions of the brain and anterior horn cells in the spinal cord of late infantile or juvenile patients[69, 90].

## 1.2 Enzyme Replacement Therapy

*1.2.1 Lysosomes and storage diseases*

The discovery of the lysosome[91], and the elucidation of its function, were important milestones for the identification of lysosomal storage diseases and the development of enzyme replacement therapy. The lysosome degrades cellular and extra-cellular (macro-) molecules resulting in products that can be re-utilised for metabolic functions and cell renewal[7, 91, 92]. Thus, the cell can digest and renew its own interior. In some cells the lysosomes have specialised functions. In polymorphonuclear granulocytes and macrophages they have a function in the destruction of micro-organisms. In hepatocytes lysosomes can release cholesterol from low-density lipoproteins. In osteoclasts they allow the remodelling of bone during growth and in the thyroid gland they converse thyreoglobuline into thyroxine and tri-iodothyronine[7, 93, 94]. The accessibility of the lysosome for exogenous macromolecules, makes lysosomal enzyme deficiencies amenable for enzyme replacement therapy[95].

*1.2.2. Historical perspective*

Pompe's disease was the first lysosomal storage disorder in which enzyme replacement was ever attempted. In 1964 Baudhuin et al[96] administered a crude extract of acid alpha-glucosidase Aspergillus niger to a 5-months old infant. The extract was injected intra-muscular 3 times per day for 3 days without any effect. Lauer et al[97] similarly attempted intra-muscular enzyme replacement therapy with a preparation from Aspergillus Niger. Alpha-glucosidase activity in the liver increased, but glycogen concentration remained elevated. The first clear effect was noticed by Hug and Schubert after long term intravenous administration of a comparable extract[79]. After 18 days of daily therapy in an infantile patient they reported normalisation of alpha-glucosidase activity and glycogen content in the liver, slight improvements in the electro-microscopic defects and EKG abnormalities and amelioration of the condition of the patient. However, after 4 months the therapy was complicated by a hypo-complementic immune nephritis. The administrations were discontinued and the patient died 6 days later. In order to avoid immune reactions De Barsy et al[98, 99] attempted treatment with human alpha-glucosidase purified from human placenta. They administered a single intravenous gift to one patient and repeated infusions to another. Although alpha-glucosidase activity increased in the liver, it did not in muscle. The glycogen content did not diminish either. Despite the utilisation of an enzyme from a human source the patient developed hyperthermia. The last and longest trial with enzyme replacement therapy from Aspergillus foetidus was reported by Gillette et al[65]. They administered the enzyme intravenously to a patient during 3 courses of 5 days with a monthly interval. Glycogen concentration in the anterior thigh muscle decreased after the first course. The EKG improved as the cardiac hypertrophy diminished, and the PR interval increased. After discontinuation of the therapy glycogen concentration in the anterior thigh muscle increased and the EKG deteriorated. During the second series of infusions the patient developed an anaphylactic reaction to the enzyme, manifested by urticaria and wheezing. The effect of the enzyme replacement diminished during the last two courses and the patient died of progressive cardiorespiratory failure after the third course at the age of 19 months.

In retrospect the failure of early attempts of enzyme replacement therapy was due to the administration of low doses of impure, heterologous and immunogenic enzyme preparations. Moreover, no special attention was given to the administration of high uptake forms of alpha-glucosidase. In the lysosomal uptake of enzymes the mannose 6-phosphate receptor plays an important role. Kaplan et al[100] and Sando and Neufeld[101] were the first to recognise the importance of the mannose 6-phosphate receptor in the process of endocytosis of lysosomal enzymes. It is now known that the receptor has a function in the sorting of lysosomal enzymes[36, 102-106], and in the recapture of lysosomal enzymes after secretion[107].

The mannose 6-phosphate receptor is essential for the uptake of enzyme in vitro. Reuser et al. [36] demonstrated that alpha-glucosidase derived from human placenta, which lacks mannose 6-phosphate groups, is not taken up by fibroblasts and cultured skeletal muscle. In contrast mannose 6-phosphate containing alpha-glucosidase from bovine testis or human urine efficiently corrects the enzyme deficiency in cultured fibroblasts and muscle cells of patients with Pompe's disease and results in degradation of lysosomal glycogen[26, 36, 38-40, 108, 109]. The uptake is inhibited by free mannose 6-phosphate. Thus, the presence of mannose 6-phosphate groups on alpha-glucosidase is crucial for in vitro correction of muscle cells. However, the in vitro situation does not mimic the in vivo situation in every detail. To reach the target tissue in vivo (muscle) the enzyme has to cross the endothelial barrier of the capillaries and the interstitial connective tissue. Van der Ploeg et al.[110] demonstrated, by using an isolated rat heart as a model, that perfusion with mannose 6-phosphate containing alpha-glucosidase results in lysosomal uptake and correction of enzyme activity in the heart, proving that the enzyme can indeed cross the endothelial barrier and the interstitial connective tissue of the heart. They further showed[110] that both mannose 6-phosphate and non-mannose 6-phosphate containing enzyme species are taken up by muscle cells of healthy mice after intravenous administration of alpha-glucosidases from human placenta and bovine testis. The uptake of mannose 6-phosphate containing enzyme was at most 2 to 3 times better, whereas mannose 6-phosphorylation makes a hundredfold difference for uptake in vitro[110].

Based on the outcome of these experiments it was decided to pursue further attempts on enzyme replacement therapy in humans, and to invest in the development of human alpha-glucosidase production methods.

*1.2.3. Production of alpha-glucosidase*

Large-scale production of lysosomal enzymes is difficult since natural sources are insufficient. Urine can serve as a natural source, but the enzyme concentration is low and the collection is practically impossible. The use of enzyme from non-mammalian sources like yeast and fungi[65, 79, 96, 97] introduces the almost unavoidable risk of an immunological response.

Cloning of the human alpha-glucosidase gene[25, 27] made biotechnological production a feasible alternative. Several systems were explored for the production of recombinant human alpha-glucosidase. The choice is limited due to differences in post-translational modification between species. Bacteria are unable to perform complicated glycosylation and essential post-

translational modifications. Production was attempted but the derived alpha-glucosidase appeared inactive[111]. Production in insect cells with the help of baculoviruses results in non-phosphorylated alpha-glucosidase species[112].
Two production systems have proven their value for the production of both catalytically active as well as properly glycosylated alpha-glucosidase; i.e. Chinese Hamster Ovarian Cells (CHO-cells)[113, 114] and transgenic animals[115-117].

*1.2.4. Production of recombinant human alpha-glucosidase in CHO-cells*

Two CHO-cell lines were developed, one in a joint project of our centre and the Women's and Children's Hospital, North Adelaide, Australia[113] and another with our cDNA at Duke University Medical Centre[114]. Both lines were transfected with the same acid alpha-glucosidase cDNA, but with a different transcriptional control. In one system the human polypeptide elongation factor 1 alpha gene promotor[113] was used. The other system uses the cytomegalovirus promotor in combination with the dihydrofolate reductase gene[114].
Both systems produce a recombinant human phosphorylated precursor of alpha-glucosidase, which can be purified from the medium.
The alpha-glucosidase derived from CHO-cells was pre-clinically tested. A twenty-four hours administration of 1290 nmol/h/ml CHO-enzyme to cultured fibroblasts and muscle cells of Pompe patients showed enzyme uptake in a mannose 6-phoshate dependent manner and lysosomal conversion of the administrated 110 kDa form to the 76 kDa mature form[113, 114, 118]. The enzyme deficiency was corrected. Normalisation of lysosomal glycogen was obtained after a twenty-four hours administration of 2000 nmol/h/ml CHO enzyme. Correction of enzyme activity and glycogen concentration lasted for 7 days, suggesting that one infusion per week is sufficient for treatment.
The effect of recombinant human enzyme derived from the medium of CHO cells was also tested in a healthy Guinea pig[114]. A single intravenous administration of 32.4 mg/g resulted in the elevation of enzyme activity.
The alpha-glucosidase knock-out mouse developed at our centre[119, 120] and the naturally occurring alpha-glucosidase deficient quail were used to prove the efficiency of the recombinant enzymes in alpha-glucosidase deficient animals. The knock-out mouse has the advantage of being evolutionary closer to humans than the quail. It mimics the infantile phenotype as to residual enzyme activity and histopathology. However, the clinical course differs as the knock-out mouse reaches adulthood as opposed to the infantile patients who die in the first year of life. The alpha-glucosidase deficient quail does not resemble the infantile but the juvenile phenotype. It has a partial enzyme deficiency, late onset of symptoms and a milder histopathology. The quails show a progressive myopathy in that they cannot fly, nor flap their wings, nor elevate from supine position from 4 weeks after hedging.
Both quails[121] and mice[116] received a single intravenous injection of 100 µg of recombinant human alpha-glucosidase from CHO-cells. In both species there was significant uptake of enzyme in muscle tissue, and lysosomal targeting was proven by conversion of the 110 kDa precursor to 76 kDa mature alpha-glucosidase.

Subsequently a longer experiment was performed. Two quails were treated every 2 to 3 days for a total period of 18 days with 14 mg/kg recombinant human alpha-glucosidase from CHO cells. This resulted in normalisation of alpha-glucosidase activity in heart and skeletal muscle[121]. The quails learned to flap their wings, elevate from supine position and one quail was reported to fly more than 1 meter high.

*1.2.5. Production of human proteins in milk of transgenic animals*

Another source of human recombinant enzyme was developed over the last two decades. Milk of transgenic animals offers an ideal source of therapeutic proteins. The mammary gland of transgenic animals appears to carry out proper post-translational modification of human proteins required for full biological activity[122]. In comparison with mammalian cell bioreactors, transgenic livestock with mammary gland targeted expression is able to produce valuable human therapeutic proteins in very high concentrations. The field of transgenic bioreactors has rapidly developed over the last decade. After the production of alpha-1-antitrypsin in the milk of transgenic sheep in 1991 an ever-increasing number of proteins was produced in several animal species. The proteins range from small unstable peptides (salmon calcitonin peptide[123]) to large, complex proteins needing post-translational modification (factor VIII[124, 125]). Two recombinant human proteins from milk were tested in clinical trials: anti-thrombin III from transgenic goats and alpha-1-antitrypsin from sheep[126, 127].

*1.2.6. Production of recombinant human proteins in transgenic mice and rabbits*

We focussed on the production of recombinant human alpha-glucosidase in transgenic animals. Bijvoet et al[115, 116] first investigated the feasibility of transgenic production in mice. Two constructs were tested, one consisting of cDNA, the other using the total genomic sequence, both under control of the bovine alpha-$S_1$-casein gene promotor[115-117]. The constructs were injected into the pronucleus of fertilised oocytes. Embryos were implanted in foster mothers and a line of transgenic animals was obtained by germ line transmission. The transgenic female expressed human alpha-glucosidase in her milk. The genomic construct (producing 2 mg alpha-glucosidase per ml milk) turned out to be more suitable for the production than the cDNA construct with a production of 1.5 microgram alpha-glucosidase per ml milk. The purified recombinant enzyme from the mice-milk resembles the natural alpha-glucosidase precursor from human urine and the recombinant precursor secreted by genetically engineered CHO-cells. The milk enzyme also is a 110 kDa precursor and is similarly taken up by cultured fibroblasts and muscle cells of Pompe patients in a mannose 6-phosphate dependent manner. Uptake leads to normalisation of alpha-glucosidase activity[115, 116].

Based on the successful outcome of in vitro experiments, the milk enzyme was tested in vivo. One single intravenous dose of 100 microgram corrects the alpha-glucosidase deficiency of knock-out mice[116]. The 110 kDa precursor is converted into the 76 kDa mature form in heart and skeletal muscle, as a sign of uptake in the lysosomal system.

Stimulated by these positive results it was decided together with Pharming B.V. Leiden, to further explore the large-scale production of recombinant human alpha-glucosidase for clinical application in animals. To enhance the process rabbits were chosen since these have a short duration of pregnancy and large nests, they reach sexual maturity at a few months of age, and they produce 100-200 ml of milk per day with a protein concentration which is even higher than in cow milk.

The gene construct used for this purpose encompasses the entire human alpha-glucosidase gene with all exons and introns, including the untranslated exon 1 (28.5 kb) and 9 kb of the 3'-UTR. High-level, cell type-specific, expression was obtained by fusing this gene at the 5' side to 6.3 kb of the bovine alpha-S1-casein gene promotor. The construct was injected into fertilised rabbit oocytes as described earlier. Newborns carrying the transgene were selected by Southern blot analysis. The ones who transmitted the transgene in a Mendelian fashion were used for breeding. Expression of the transgene was investigated in following generations by Northern blot analysis and by measuring the acid alpha-glucosidase activity in milk samples. As a result of testing and selective breeding a line of rabbits was obtained producing recombinant human alpha-glucosidase during lactation in a concentration of 2 to 8 mg/ml[117].

The end products from CHO-cells and milk are very similar in molecular mass (110 kDa), and kinetic properties, but the mannose 6-phosphate content is different[113, 114, 116]. The 110 kDa precursors from human urine, cell culture media and mouse milk are taken up by fibroblasts and muscle cells via mannose 6-phosphate receptor-mediated endocytosis, but the uptake of the rabbit milk precursor via this receptor is poor.

In the knock-out mouse model, however, the uptake of the various enzymes by target tissues is not consistently different (A.J.J. Reuser unpublished results[128]). A single intravenous administration of 17 mg/kg alpha-glucosidase derived from rabbit milk resulted in normalisation of alpha-glucosidase activity in all tissues except brain[117]. Long term treatment of knock-out mice also led to positive results. Three knock-out mice received four injections of 40-68 mg/kg alpha-glucosidase from rabbit milk with a 6-day interval. Lysosomal processing was demonstrated. Alpha-glucosidase activity in liver increased up to 60 times normal and in heart and skeletal muscles up to 3 times normal. Glycogen content of liver and heart decreased, but remained the same in skeletal muscles. Encouraged by these results a 25 weeks experiment with long-term administration of alpha-glucosidase to knock-out mice was started. Sixteen mice were used for this experiment. Eight received the recombinant enzyme from rabbit milk, and the other eight a placebo. The first dose was 68 mg/kg. This was followed one week later by 17 mg/kg, which was continued weekly. After 13 doses the alpha-glucosidase activity in the liver had increased to 120 times normal and the glycogen content in the liver had normalised. However, only one of the three mice showed an increase of alpha-glucosidase activity in heart and skeletal muscle, without a concurrent decrease of glycogen. As it appeared, the mice had developed a high antibody titre. Mice with a low antibody titre were kept on treatment for an additional 12 weeks and received an increased dose of 68 mg/kg. After the total treatment period of half a year, the alpha-glucosidase activity in all tissues except kidney and brain, was above normal and the glycogen content was either normal or significantly lower than in the same tissues of placebo treated mice. A significant improvement in histopathology was seen[117].

The promising effect of enzyme replacement therapy in preclinical studies led us to explore the effect of human recombinant alpha-glucosidase from rabbit milk in humans. In a phase 1 study with recombinant human alpha-glucosidase from rabbit milk no major side effects were found in healthy volunteers (Pharming). Thus a long line of research performed at the Erasmus Medical Rotterdam, finally opened the way to clinical trials.

### 1.3 Aims of the study

This study aims to evaluate the safety and efficacy of enzyme replacement therapy as a treatment for Pompe's disease, a fatal and otherwise incurable metabolic disorder. The study consists of three elements:

1. Delineation of the natural course of Pompe's disease in infants in order to establish in- and exclusion criteria and clinical endpoints
2. Design of a study protocol with the established in- and exclusion criteria and clinical endpoints
3. Experimental treatment of four infants with Pompe's disease over a 3 ½ -year period and evaluation of the results.

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# CHAPTER 2

# The natural course of infantile Pompe's disease; 20 original cases compared with 133 cases from the literature

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## Abstract

*Objective*: Infantile Pompe's disease is a lethal cardiac and muscular disorder. Current developments towards enzyme replacement therapy are promising. The aim of our study is to delineate the natural course of the disease in order to verify endpoints of clinical studies.

*Methods:* 20 infantile patients diagnosed by the collaborative Dutch Centres and 133 cases reported in literature were included in the study. Information on clinical history, physical examination and diagnostic parameters was collected.

*Results:* The course of Pompe's disease is essentially the same in the Dutch and the general patient population. Symptoms start at a median age of 1.6 months in both groups. The median age of death is 7.7 and 6 months, respectively. Five percent of the Dutch patients and 8% of all reported patients survive beyond one year of age. Only 2 patients from literature became older than 18 months.

A progressive cardiac hypertrophy is characteristic for infantile Pompe's disease. The diastolic thickness of the left ventricular posterior wall and cardiac weight at autopsy increase significantly with age.

Motor development is severely delayed and major developmental milestones are generally not achieved. For the Dutch patient group, growth deviates significantly from normal despite start of nasogastric tube feeding. Levels of ASAT, ALAT, LDH, CK or CK-MB are typically elevated, while ASAT, ALAT and LDH increase significantly with age. The patients have fully deleterious mutations. Acid alpha-glucosidase activity is severely deficient.

*Conclusions:* Survival, decrease of the diastolic thickness of the left ventricular posterior wall and achievement of major motor milestones are valid endpoints for therapeutic studies for infantile Pompe's disease. Mutation analysis and measurement of the alpha-glucosidase activity should be part of the enrolment program.

## Introduction

Pompe's disease or Glycogen storage disease type II (GSDII) is a lysosomal storage disorder in which deficiency of acid alpha-glucosidase leads to accumulation of glycogen and finally to destruction of muscle tissue. Complete deficiency of alpha-glucosidase causes a progressive lethal cardiac and skeletal muscle disorder known as infantile Pompe's disease[1]. Partial deficiency leads to a milder late onset phenotype. The latter condition may present at any age and is subdivided in non-classical infantile, childhood, juvenile and adult Pompe's disease[2-4]. The estimated combined incidence is 1:40.000 births[5, 6]. There is as yet no registered therapy for Pompe's disease, but the first clinical studies on enzyme replacement therapy with recombinant human alpha-glucosidase from rabbit milk and CHO cells have started. The preliminary data of phase II studies in infantile patients are promising[7-9]. However, to fully appreciate the effect of enzyme therapy precise knowledge is needed on the natural course of infantile Pompe's disease. This information is currently lacking.

Only two reviews on infantile Pompe's disease were published over the last 50 years. One review dates from 1950 and describes 14 patients[10]. The other review is from 1962 and describes 40 additional cases[11]. Notably these reviews date from the time that the primary enzyme defect was still unknown[12]

This review depicts the natural course of classical infantile Pompe's disease based on original data from 20 patients who where seen by the Dutch collaborative centres between 1980 and 1998 and a collection of 133 cases from the literature.

## Methods

We analysed the clinical and laboratory data of the patients who were biochemically or genetically diagnosed with infantile Pompe's disease at the department of Clinical Genetics of the Erasmus University Rotterdam between 1980 and 1998. These data were compared with those obtained from the literature. We included all publications identified via Pubmed by a search for "infantile Pompe's disease", "infantile acid maltase deficiency", "infantile glycogenosis type 2", "infantile alpha-glucosidase deficiency" and "glycogenosis type 2a", written in English, German, French, Dutch, or Italian. Case reports cited in the collected articles and additional ones cited by Hirschhorn and Reuser[1] were added. We excluded publications lacking clinical information and cases of normal alpha-glucosidase activity (Danon's disease), prenatal death and experimental treatment.

Information on clinical history, physical examination and diagnostic data were collected. Data were often incomplete. Symptoms or findings of physical examination that were not reported were scored as negative.

*Biochemical-genetic studies*

Fibroblasts were homogenised in water, and 2000 x g supernatants were used to determine alpha-glucosidase activity[13, 14]. Mutation analysis was performed using genomic and cDNA, as described earlier[15]. The functional effect of mutations was studied by assay of alpha-

glucosidase synthesis and activity in transiently transfected COS cells or in cultured fibroblasts of patients[15, 16].

*Growth*

Data on growth were collected and expressed in the standard deviation score for weight ([weight of the patient – weight according to the P50 for height] / standard deviation).

*Clinical chemistry*

Aspartate aminotransferase (ASAT), Alanine aminotransferase (ALAT) and Creatine Kinase (CK) were measured according to the guidelines of the International Federation of Clinical Chemistry. Lactate dehydrogenase (LDH) was measured according to the guidelines of the Dutch Association of Clinical Chemistry. CK-MB was measured by immune inhibition. All measurements were performed at an assay-temperature of 37°C.

*Cardiology*

The thickness of the left ventricular posterior wall was measured at the diastole (LVPWd) by M-mode echocardiography in compliance with the guidelines of the American Society of echocardiography[17].

*Statistics*

Characteristics for which repeated measurements within patients were available (weight, length, enzyme activities, LVPWd) were analysed using mixed model ANOVA (random coefficients model for SAS PROC MIXED). Correlation coefficients given are Spearman's $r_s$. Data given are medians, or indicated otherwise. Two sided p-values $\leq 0.05$ were considered significant.

## Results

*Population*

Twenty infantile Pompe patients were diagnosed between 1980 and 1998; 14 patients were from Dutch, 4 from Turkish, 1 from Italian and 1 from Taiwanese ancestry. The patients were admitted to 5 academic hospitals (Beatrix Clinic Groningen, Wilhelmina Children's Hospital Utrecht, University Medical Center Nijmegen, Emma Children's Hospital Amsterdam and the Sophia Children's Hospital Rotterdam). Thirty percent of the patients was female, 70 % male. In literature we located 83 publications describing 133 patients, 42% female, 50% male [2, 10, 11, 18-97]. Sex was not reported in 8% of the cases.

*Molecular characterisation*

All patients identified via the Department of Clinical Genetics Rotterdam had a severe alpha-glucosidase deficiency (Table 1). The median activity in fibroblast was 0.52 nmol MU/h/mg protein (range 0.18 to 1.15 nmol/h/mg protein, normal value 36-166 nmol MU/h/mg protein, median 84 nmol MU/h/mg, n=254). In 15 patients we identified the mutations in both alpha-glucosidase alleles. In two more patients one mutation was detected (Table 1). In total 9 different mutations were found. The effect of 7 mutations was known: delT525[98], del exon18[23], Asp645His[58, 99], insC2741/insG2743[15], Pro768Arg[15], del Lys903[100], and Gly309Arg[101]. All these mutations are fully deleterious and result in severe deficiency of alpha-glucosidase activity. Two new mutations were detected: Ala880Asp and InsA1827 resulting in Tyr609Ter. The latter mutation will certainly lead to loss of function. The effect of Ala880Asp was studied in transiently transfected COS cells, and also leads to complete loss of alpha-glucosidase activity. The delT525 and del exon18 mutations were found most frequently, ten times each. In all but one case the patient was from Dutch ancestry. One patient from Italy was homozygous for the exon18 deletion. Her parents came from the Italian region of Catania where this mutation prevails as in the Dutch population[102]. Of all mutations found in the Dutch patient group the delT525 mutation, the insC2741/insG2743 mutation, and the Tyr609Ter mutation result in absence of cross reactive immunological material (CRIM-negative). The delT525 mutation leads to frame shift and formation of unstable messenger. Two patients are homozygous for this mutation, and therefore completely CRIM negative. Two of the four patients from Turkish ancestry are homozygous for the insC2741/insG2743 mutation and therefore also CRIM negative. Both these patients were offspring of a consanguineous marriage. Another Turkish patient was homozygous for the Pro768Arg mutation, while in the fourth Turkish patient the mutation is as yet unknown. One patient from Taiwan appeared homozygous for the G1933C mutation known to occur in infantile patients from Taiwan and certain areas of China[58, 99]. The great majority of case reports from literature is incomplete as to investigating the patients genotype. Related literature data are therefore not included in this review.

*Course of Disease*

First symptoms were noted at a median age of 1.6 months in both the Dutch patient group and in literature[2, 10, 11, 18, 20, 21, 23, 25, 27-36, 40-46, 48-60, 62, 63, 65-76, 78-88, 91-96, 99] (Table 2).

Patients were hospitalised at median ages of 2.8 months in the Dutch patient group and 4.0 months in literature[10, 11, 18-21, 24, 26, 27, 29, 30, 33, 34, 36-38, 40-46, 48, 51-53, 55, 61-63, 65, 68, 70, 72, 74, 78, 80, 82, 84, 87, 89, 90, 92, 95-97, 103], and diagnosed at respective ages of 5.3 and 4.5 months[2, 11, 18, 22, 25, 27, 28, 32, 41, 44, 46, 47, 58, 61, 66, 73, 80, 83, 85, 91, 93, 99]. The median time from diagnosis to death was 2 months in both groups[2, 11, 18, 22, 25, 27, 28, 32, 41, 44, 46, 61, 73, 80, 83, 85, 91, 93]. In the Dutch patient group death occurred at a median age of 7.7 months. Patients described in literature died at a median age of 6.0 months[2, 10, 11, 18-21, 23-34, 36-44, 46, 48-57, 59-65, 67-75, 78-97].

**Table 1:** *Alpha-glucosidase activity, mutations in the lysosomal alpha-glucosidase gene and the resulting aminoacid changes per patient*

| Patient | Alpha-glucosidase activity | Nucleotide change | AA alteration | CRIM | Nucleotide change | AA alteration | CRIM* |
|---|---|---|---|---|---|---|---|
| 1 | 1.15 | DelT525[110] | Thr175→shift | - | Unknown | | |
| 2 | 0.59 | DelT525[110] | Thr175→shift | - | Unknown | | |
| 3 | 0.43 | DelT525[110] | Thr175→shift | - | DelT525[110] | Thr175→shift | - |
| 4 | 0.29/0.42 | DelT525[110] | Thr175→shift | - | DelT525[110] | Thr175→shift | - |
| 5 | 0.38 | DelT525[110] | Thr175→shift | - | InsA1827 | Tyr609Ter | - |
| 6 | 0.33 | DelT525[110] | Thr175→shift | - | Del exon18[111] | Del 55aa 828→ | + |
| 7 | 1 | DelT525[110] | Thr175→shift | - | Del exon18[111] | Del 55aa 828→ | + |
| 8 | Undetectable | DelT525[110] | Thr175→shift | - | Del exon18[111] | Del 55aa 828→ | + |
| 9 | 0.18 | Del exon18[111] | Del 55aa 828→ | + | Del exon18[111] | Del 55aa 828→ | + |
| 10 | Not available | Del exon18[111] | Del 55aa 828→ | + | Del exon18[111] | Del 55aa 828→ | + |
| 11 | 0.44 | Del exon18[111] | Del 55aa 828→ | + | 2639C→A | Ala880Asp | + |
| 12 | Undetectable | Del exon18[111] | Del 55aa 828→ | + | 3 nucleotide deletion | Del Lys903[100] | + |
| 13 | 0.6 | Del exon18[111] | Del 55aa 828→ | + | 925G→A[101] | Gly309Arg | + |
| 14 | 0.73 | 1933G→C[58] | Asp645His | + | 1933G→C[58] | Asp645His | + |
| 15 | 0.7 | 2741AG→CAGG[15] | Frameshift | - | 2741AG→CAGG[15] | frameshift | - |
| 16 | 1.1 | 2741AG→CAGG[15] | Frameshift | - | 2741AG→CAGG[15] | frameshift | - |
| 17 | 0.52 | 2303C→G[15] | Pro768Arg | + | 2303C→G[15] | Pro768Arg | + |
| 18 | 1.1 | Unknown | | | Unknown | | |
| 19 | 0.2 | Unknown | | | Unknown | | |
| 20 | 0.23 | Unknown | | | Unknown | | |

**Presence (CRIM+) or absence (CRIM-) of immunologically detectable alpha-glucosidase*

**Table 2:** *Course of disease of the Dutch population and of the cases collected from literature*

| | The Netherlands | | | | Literature | | | |
|---|---|---|---|---|---|---|---|---|
| | Median* | Mean* | Range* | N | Median* | Mean* | Range* | N |
| | | | | 20 | | | | 133 |
| First Symptoms | 1.6 | 1.9 | 0-6.8 | 20 | 1.6 | 2.1 | 0-11 | 107 |
| Hospitalisation | 2.8 | 3.4 | 0-7.3 | 20 | 4.0 | 4.0 | 0-14 | 77 |
| Diagnosis | 5.3 | 4.8 | 0.6-9.3 | 20 | 4.5 | 5.4 | 0-23 | 22 |
| Death | 7.7 | 7.6 | 3.8-12.4 | 20 | 6.0 | 6.3 | 0-34.5 | 119 |
| Time from diagnosis to death | 2.0 | 2.8 | 0.1-6.4 | 20 | 2.0 | 2.7 | 0-10 | 18 |

**The age of the patients is given in months*

The survival curve of both groups is presented in figure 1. Only one Dutch infant survived beyond one year of age. This baby was born prematurely at a gestational age of 32 weeks. The age corrected for the duration of gestation was 10.6 months. The patients described in literature died in majority within the first year of life (109 of 119 patients). Ten patients survived beyond 1 year of age[2, 19, 29, 38, 56, 61, 85, 91, 97]. Only 2 patients were reported with ages of death above 1.5 year (29 and 34.5 months)[38,97].

In the Dutch patient group, the clinical course of the CRIM-negative patients did not differ from the CRIM positive patients. The five CRIM negative patients died between 6.6-8.6 months of age.

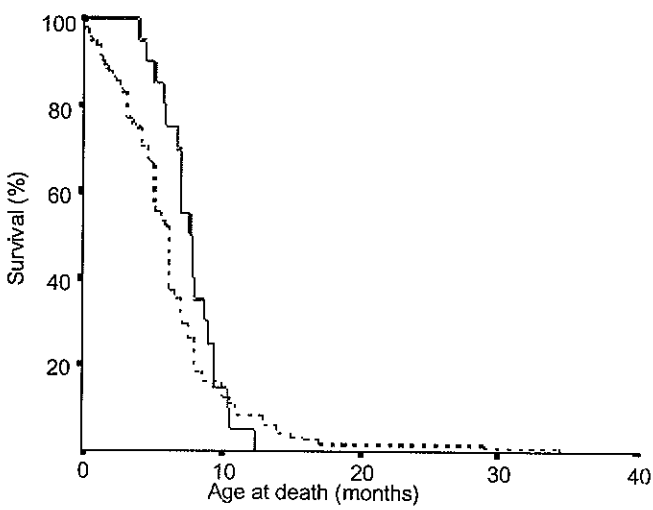


**Figure 1:** *Survival curve of patients with infantile Pompe's disease. The Dutch patient population (—) and the literature cases (----) are illustrated separately*

*Clinical History; first symptoms*

We kept record of the first symptoms of all Dutch patients. Information on an additional 66 cases was available from the literature[2, 10, 11, 18-20, 22-24, 26-32, 34-38, 41, 42, 44, 45, 48-52, 55-58, 60-76, 78, 79, 81, 82, 84, 85, 87, 88, 92, 95-97, 99, 104-106]. Feeding problems and/ or failure to thrive were the first symptoms in 55% of the Dutch patients and in 44% of the cases reported in literature. Motor problems, like muscular weakness, motor retardation and paucity of movements, were the first symptom in 40% of the Dutch patient group and in 20% of the cases described in literature. Respiratory problems (like air way infections and respiratory difficulty) were the first symptom in 40 and 27% of the cases. Cardiac problems (like cardiac failure and rhythm disturbances) were noticed as first symptom in 15% of the Dutch patients and in 23% of the cases from literature (The total percentage exceeds 100% due to the fact that patients may present with combinations of first symptoms).

Other less frequently reported symptoms were discomfort (like malaise, sweatiness, fatigue, irritability and a weak cry) and gastrointestinal complaints (like constipation, vomiting and regurgitation). Neurological symptoms like spasm, and tremor were noticed in 2 Dutch cases. In three cases from literature mental retardation was suspected[27, 38, 75]. Reasons for hospitalisation were respiratory problems, cardiac problems, feeding problems, and growth retardation in decreasing order.

*Physical examination*

The 20 Dutch patients were physically examined. An additional 103 physical examination reports were available from literature[2, 10, 11, 18-23, 26-42, 44-46, 48-52, 55-57, 59-65, 67-74, 76-79, 81, 82, 84, 85, 87, 88, 92, 95-97]. At first sight, patients were tachy- or dyspnoeic (75% of the Dutch patients and 41% of the cases from literature), pale (40% of the Dutch patients, 20% of the cases described in literature) and /or cyanotic (30% and 23% respectively). An enlarged tongue was noticed in 45% of the Dutch patients and 29% of the cases from literature. The Dutch patients typically were hypotonic (95%). Hypotonocity was reported to a lesser extent in literature (52%). In 75 % of the Dutch patients and in 46% of the cases from literature a heart murmur was present. A gallop rhythm was reported in 17% of the cases from the literature. Auscultation of the lungs was abnormal in 55% of the Dutch patient group and in 47% of the cases reported in literature. At palpation of the abdomen a moderate hepatomegaly was found in 90% of the Dutch patients and reported in 29% of the cases from literature (median size 3 and 3.2 cm below the costal margin, respectively). A moderate splenomegaly was found in 15 and 6% of the patients, respectively (median size 2 cm below the costal margin in both populations). Absent deep tendon reflexes (35% of the Dutch patients, 33% of the cases from literature) was the main abnormality observed during neurological examination. Incidentally tongue fibrillations (3%),

absence of tongue movements (2%) and spasms of the legs (2%) were reported in literature. In the Dutch patient group these were not noticed.

*Motor development*

A complete set of data on motor development was available for 16 Dutch patients. None of these patients ever learned to turn, sit or stand. Of the 133 patients reported in literature only 2 patients were reported to turn from supine to prone position [18, 63]. The age at which they achieved this motor milestone was not mentioned, but they lost this ability at the age of 4 and 7 months, respectively. Three patients were reported to sit. One achieved this motor milestone at the age of 6 months and subsequently lost it at the age of 7 months[63]. The other patients were reported "no longer to sit without support" and "no longer to support themselves in a sitting position" at ages of 7.5 months[72] and 10.5 months[33], respectively.

*Growth*

Longitudinal data on weight, length and head circumference were available for 18 of the Dutch patients (mean duration of follow up 4.3 months). The standard deviation score for weight decreased significantly with age (p=0.001, Fig 2), showing a deviation from the normal weight curve, despite the start of nasogastric tube feeding in 14 patients. Increase of length and head circumference with age was normal.

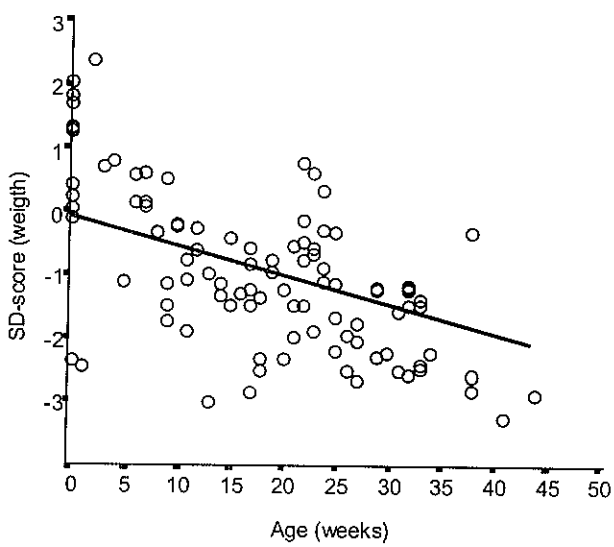


**Figure 2:** *Standard Deviation score of weight versus age in weeks (p=0.001) for the Dutch patient population. SD-score =[weight of the patient-weight according to the P50 for weight]/ standard deviation of weight.*

*Clinical Chemistry*

Levels of CK, CK-MB, LDH, ASAT and ALAT generally appeared to be increased in the Dutch patients (n=19). The median value of CK was 690 IU (range 175 to 2307 IU, upper limit of normal 295 IU ($P_{95}$), number of measurements 40). A normal CK value was measured 5 times. The median value of CK-MB was 29 (range 14 to 64 IU, upper limit of normal 18 IU, number of measurements 20). CK-MB was normal in 3 measurements. The median value of LDH was 1956 IU (range 801 to 5714 IU, upper limit of normal 1097 IU, number of measurements 35). LDH was normal in 4 measurements. The median value of ASAT was 321 IU (range 115-859 IU, upper limit of normal 89 IU, number of measurements 51) and of ALAT 184 (range 9 to 397 IU, upper limit of normal 60 IU). ASAT and ALAT were one time normal in one patient, but elevated in later measurements. In none of the cases normal values for CK, CK-MB, LDH, ASAT and ALAT were found at the same time. There was a significant increase of LDH, ALAT and ASAT with age ($p<0.001$, Fig 3 a, b and c). Data from literature were too scarce to draw conclusions. Control ranges and assay temperatures were often lacking.

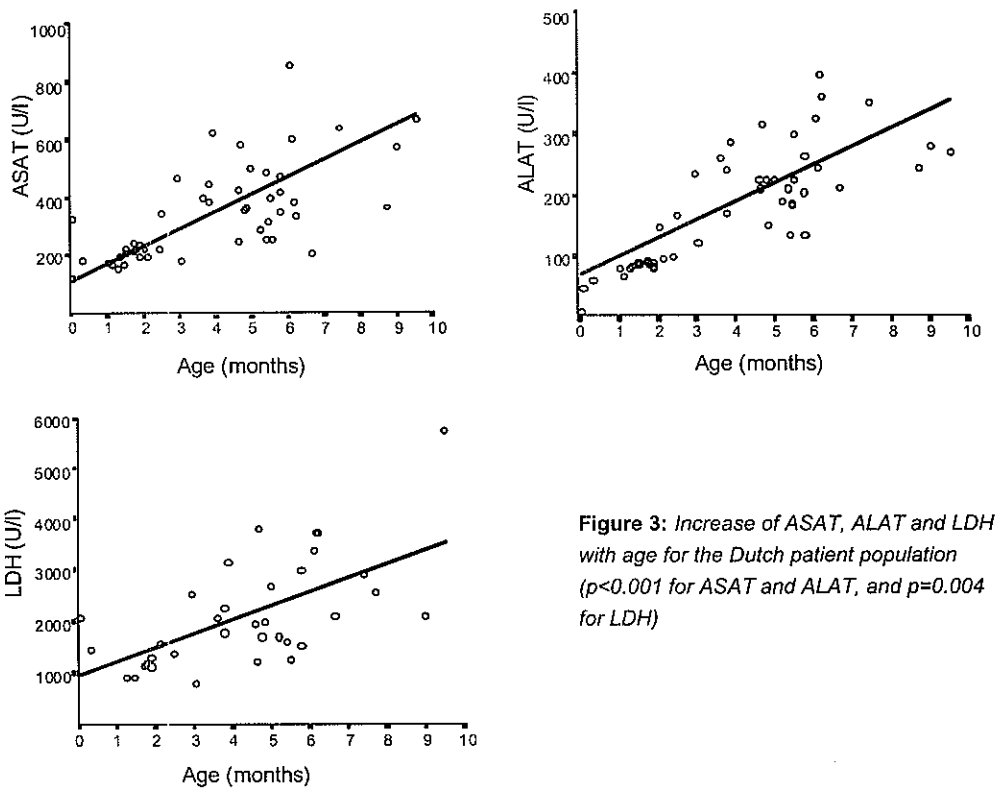


**Figure 3:** *Increase of ASAT, ALAT and LDH with age for the Dutch patient population ($p<0.001$ for ASAT and ALAT, and $p=0.004$ for LDH)*

*Cardiology*

All available chest roentgenograms of the Dutch patients (n=19) showed a cardiomegaly at ages ranging from 0 to 7.2 months. In literature an increased heart size was reported in 99% of the cases described (n=82[10, 11, 18-21, 26-42, 44, 45, 48-52, 55, 56, 60, 61, 63-68, 70-72, 74, 76-79, 81-83, 85, 87, 88, 95, 97, 107]).

On the electrocardiograms (EKG) a left ventricular hypertrophy was noticed in all Dutch patients (n=19). A biventricular hypertrophy was seen in 84%, and an atrium hypertrophy in 11% of the patients. In literature left ventricular hypertrophy was reported in 60 (83%) of 72 cases[2, 10, 11, 18, 19, 22, 24, 26, 27, 29, 30, 32, 34-41, 44, 45, 49, 50, 52, 55, 56, 60, 61, 63-68, 70, 72, 76-79, 81, 83, 85, 87, 88, 95, 97].

In 10 cases the cardiomegaly was noticed on the chest roentgenogram, but not on the EKG. In only two patients the EKG was totally normal[2, 88]. Atrium hypertrophy was seen in 4% of the cases described in literature.

Another EKG abnormality reported is a borderline to shortened PQ-interval. This was present in 58% of the Dutch patient group (median 0.08 sec.) and in 51% of the cases described in literature (median 0.08 sec.). Repolarisation disturbances like T-inversion and ST-depression were present in 84% of the Dutch and 56% of the literature cases.

Echocardiographic data of 18 Dutch patients were available (Fig 4). All patients had a cardiac hypertrophy at ages varying from 0.1 to 8.8 months, as shown by the increased thickness of the left ventricular posterior wall and the intra-ventricular septum. The thickness of the left ventricular posterior wall increased significantly with age (p=0.01, Fig 4). In literature the echocardiographic data of 23 patients were presented[18, 19, 26, 28, 32, 35, 37, 39, 49, 56, 60, 66, 67, 76, 77, 81, 83, 108]. All patients had a cardiac hypertrophy as illustrated by the thickening of the Left Ventricular Posterior Wall and/or Intra-Ventricular Septum.

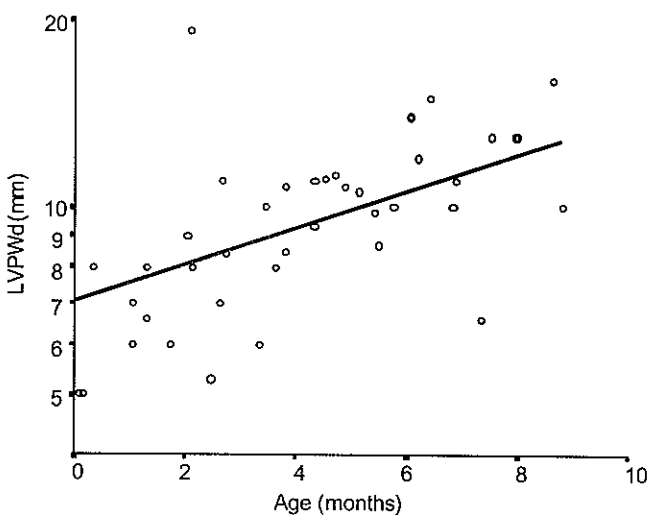


**Figure 4:** *Increase of the diastolic thickness of the left ventricular posterior wall (LVPWd) with age (p=0.01)*

In 67 cases heart-weight at obduction was reported[10, 11, 18-21, 24, 26, 27, 29, 30, 33, 34, 36-38, 40, 41, 44-46, 48, 51-53, 55, 61-65, 68, 70, 72, 74, 78, 80, 82, 84, 87, 89, 92, 95-97]. The heart weight shows a significant increase with age ($r_s$=0.57, Fig 5) in accordance with the cardiac ultrasound data of the Dutch patients. Outflow tract obstruction was described in 8 cases from literature[11, 32, 39, 45, 49, 76, 83, 109], as observed either by echocardiography, catheterisation or autopsy (6%).
When all cardiac data from the literature are compiled there is information available for 109 patients; 107 of them have a cardiomegaly and 2 do not[2, 88].

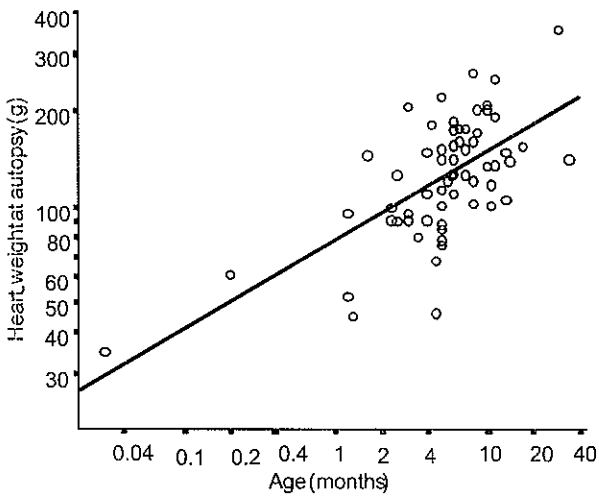


**Figure 5:** *Increase of heart weight with age ($r_s$ =0.57)*

*Neurological diagnostics*
Data on brain imaging were available for 8 of the 20 Dutch patients. An ultrasound of the brain was performed in 6 patients, a Magnetic Resonance Imaging (MRI) in 2, and a Computed Tomography (CT-scan) in 2 patients. The ultrasound was normal in all 6 patients. A MRI performed in one of these patients was also normal. CT-scans made in 2 additional patients showed some widening of the anterior horn of the left ventricle in one patient and a possible widening of the peripheral liquor spaces in the other patient. The MRI in the latter patient revealed central and cortical atrophy.

## Discussion

With the phase II clinical studies on the effect of enzyme replacement therapy in Pompe's Disease underway it becomes increasingly important to achieve accurate knowledge on the natural course of the disease in order to verify endpoints. The first results of enzyme replacement therapy in infants are promising. Patients seem to survive longer and an effect on cardiac hypertrophy is observed. However, systematic surveys of larger groups of untreated patients, needed to fully appreciate the effect, are lacking. Therefore, we followed the natural course of infantile Pompe's disease in 20 Dutch patients and studied the published case reports of an additional 133 patients. The Dutch patients were identified via the enzyme diagnostic laboratory of the department of Clinical Genetics, Erasmus University, Rotterdam, thereby precluding selection based on pre-set clinical criteria.

The literature data show that Pompe's disease occurs world-wide. The course of the disease is essentially the same in the Dutch and the general patient population. Symptoms start in both groups shortly after birth (median age 1.6 months). Age at hospitalisation (2.8 and 4.0 months), diagnosis (5.3 and 4.5 months) and death (7.7 and 6.0 months) also compare well.

All Dutch patients have a severe alpha-glucosidase deficiency of less than 1.5% of the median control value (median 0.6 % of control value) in fibroblasts. Patients from Taiwanese, Turkish, Dutch and Italian ancestry are included. Mutations were discovered in 32 out of 40 mutant alleles. All revealed fully deleterious mutations. The frequently occurring IVS1 (-13T→G) mutation, typically associated with late onset Pompe's disease was not present in the infantile patient group. This finding is consistent with the prediction that only the combination of two fully deleterious mutations leads to the classical early-onset phenotype. Patients from different ethnic origin had different mutations. For instance, delT525 and del exon18 are common among the Dutch patients. We detected two new severe mutations: Ala880Asp and InsA1827 (leading to Tyr609Ter). Their effect was demonstrated by expression studies. Currently, more than 80 fully deleterious mutations are known
(www.pompecenter.nl).

In both patient groups feeding problems, failure to gain weight, muscular weakness, motor retardation, cardiac problems, respiratory difficulty and airway infections were frequently reported as first symptom of the disease. Once referred to the hospital, symptoms of respiratory and/or cardiac failure were evident. The children mostly were tachy- and/or dyspnoeic and often pale or cyanotic. On clinical examination, a cardiac murmur was frequently present. The moderate enlargement of the liver is assumed to result from glycogen storage, but may also result from cardiac decompensation. Hypotonicity was noted as a prominent feature in the Dutch patients, but is less reported in the literature. We consider it unlikely that the Dutch and general patient group differ in this aspect and attribute the apparent difference to incompleteness of the case reports. Absence of deep tendon reflexes was the most common abnormality on neurological examination. Enlargement of the tongue was reported in less than half of the patients (45% of the Dutch cases and 29% of the cases from literature).

The following findings are key elements for the definition of the classical infantile subtype of Pompe's disease. First, infantile Pompe patients typically die in the first year of life. Death occurs at a median age of 7.7 months in the Dutch patient group and 6.0 months in literature.

Similar survival data were reported by Amalfitano et al.[7-9] (mean age of death 8.6 months) and Slonim et al.[2-4] (calculated median age of death 8.0 months). There is no significant difference between the age of death of CRIM negative and CRIM positive patients in the Dutch patient group. A minority of patients became older than one year (8%). Ninety-eight percent of all patients died before the age of 1 ½ years. None of the patients grew older than 2.9 years of age. Based on n=133, statistical calculation shows that the upper limit of 95% confidence interval for 3 years survival equals 3%.

Second, infants with classical Pompe's disease have a rapidly progressive cardiac hypertrophy. The diastolic thickness of the left ventricular posterior wall appears to be a good measure for follow-up. The left ventricular mass (LVM) or left ventricular mass index (LVMI) might be used as follow-up on cardiac weight. In the Dutch patients a cardiomegaly was found at any timepoint in life, even when the chest roentgenogram, the electrocardiogram or echocardiography was performed at birth. This indicates that cardiac hypertrophy already develops during gestation. In literature 107 out of the 109 patients appeared to have a cardiomegaly during cardiac evaluation. From the two patients from literature who were reported to have a normal heart on EKG and chest roentgenogram, one had an unusual high level of residual alpha-glucosidase activity (36% of normal in fibroblasts, and 46% of normal in muscle), which does not combine with infantile Pompe's disease[88]. The other patient had two brothers who died at relatively advanced age (3 and 4 years of age) as compared to all other cases in this study[2]. From the combined data we conclude that the absence of cardiomegaly is atypical for classical[2-4] infantile Pompe's disease.

Three other key elements of infantile Pompe's disease are motor development, growth and laboratory findings. Motor development is severely delayed. Important milestones are generally not achieved or lost shortly after acquisition. None of the Dutch patients ever learned to turn, sit or stand. In literature only 3 of the 133 patients were reported to turn or sit, but subsequently lost this ability quickly.

Weight gain appears to be significantly reduced in infantile patients, despite the start of nasogastric tube feeding.

The levels of ASAT, ALAT, LDH, CK and CK-MB are typically elevated in infantile Pompe's disease. None of the Dutch patients has normal values for ASAT, ALAT, LDH, CK and CK-MB at the same time. ASAT, ALAT and LDH increase significantly with age and appear an even better marker of disease progression than CK. It is most likely that the enzymes originate from muscle. However, it is not excluded that the liver contributes in part.

Based on the data obtained in our study on the natural course of infantile Pompe's disease it is concluded that survival is a good endpoint for enzyme replacement therapy studies. In an ongoing open label enzyme replacement therapy study we currently treat four infantile Pompe patients with recombinant human alpha-glucosidase from rabbit milk[7-9]. All four patients are still

alive at the age of three years. Based on this result statistical calculations show that the 95% confidence interval for the 3 years survival percentage ranges from 40 up to 100% in a group of infantile patients receiving enzyme therapy. This means that if the enzyme therapy study with the same therapeutic regimen would be repeated with a larger number of infants, the 3-years survival percentage is expected to be 40% at least. Comparing this figure with the upper limit of the 95% confidence interval for 3 years survival of untreated children (this manuscript), which is 3%, there is a strong indication that enzyme therapy has an effect on survival.

However, since optimisation of care (like application of artificial ventilation, treatment of infections and other supportive measures) also may prolong survival of untreated children, the results obtained with enzyme therapy should ideally be compared with results obtained in a matched group of children, who receive exactly the same supportive care but no enzyme therapy.

Based on the assumption that one-year survival increases from 10% in the "placebo" group to at least 75% in the enzyme replacement therapy group, it is calculated that in a placebo controlled enzyme therapy study 11 patients have to be included in both the treatment and the placebo group to obtain 80 percent power at alpha=0.05 (Fisher's exact test) for the comparison of survival.

A decrease of cardiac hypertrophy, evidenced by a decrease in the diastolic thickness of the left ventricular posterior wall or left ventricular mass index can provide additional proof for efficacy of therapy and serve as secondary endpoint. Improvement of clinical condition should accompany the latter finding to demonstrate the clinical benefit for the patients. Achievement of major milestones provides evidence for the efficacy of enzyme therapy on motor function.

If for ethical reasons a non-placebo controlled study is performed and historical data are used as control, it is of utmost importance that no selection bias is introduced. To our opinion molecular genetic delineation of the patients should always be part of the enrolment program, besides characteristic clinical and cardiac features, in order to identify patients with non-infantile phenotypes, who have a prolonged natural course.

**Acknowledgment**

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# CHAPTER 3

# Recombinant human alpha-glucosidase from rabbit milk in Pompe patients

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## Abstract

Pompe's disease is a fatal muscular disorder caused by lysosomal alpha-glucosidase deficiency. In an open-label study, four babies with characteristic cardiomyopathy were treated with recombinant human alpha-glucosidase (rhAGLU) from rabbit milk at starting doses of 15 mg/kg or 20 mg/kg, and later 40 mg/kg. The enzyme was generally well tolerated. Activity of alpha-glucosidase normalised in muscle. Tissue morphology and motor and cardiac function improved. The left-ventricular-mass index decreased significantly. We recommend early treatment. Long-term effects are being studied.

Infantile Pompe's disease is a metabolic myopathy with a rapidly progressive course, and is commonly fatal in the first year of life. The disease presents in the first few months after birth with respiratory and feeding difficulties and hypotonia, and hypertrophic cardiomyopathy is characteristic. Major developmental milestones, such as rolling over, sitting, and standing are not achieved[1]. The late-onset form presents as a slowly progressive proximal myopathy. The disease is caused by lysosomal alpha-glucosidase deficiency and concomitant storage of lysosomal glycogen[1].

In the development of enzyme therapy for Pompe's disease, production of rhAGLU was tested in genetically modified Chinese hamster ovary cells[2, 3] and milk of transgenic animals[4]. The two sources seemed suitable, but the high yield in milk and efficacy of the enzyme seen in mice led to large-scale production of rhAGLU* in transgenic rabbits being chosen[4]. A phase I study of healthy volunteers showed no major side-effects. We report on the first 36 weeks of treatment in patients, during which safety and efficacy data were gathered.

We did a single-centre, open-label pilot study, approved by the institutional review board. Four patients were included with typical symptoms of infantile Pompe's disease (table 1) and virtual absence of alpha-glucosidase. We obtained written informed consent from the parents.

**Table 1:** *Patients' characteristics*

| Patient | Onset of symptoms | Head lag/ axial hypotonia* | Cardiac hypertrophy/ ECG abnormality* | Oxygen need* | Age at diagnosis | Age at inclusion |
|---|---|---|---|---|---|---|
| 1 | At birth | + | + | - | 1 month | 3 months |
| 2 | 3 months | + | + | + | 4 months | 7 months |
| 3 | At birth | + | + | - | 14 days | 2.5 months |
| 4 | 3 months | + | + | + | 6 months | 8 months |

**During inclusion period*

RhAGLU was administered intravenously once weekly, at starting doses of 20 mg/kg in babies lighter than 6.5 kg (patients 3 and 4) and 15 mg/kg in babies weighing 6.5 kg or more (patients 1 and 2). Doses were increased to 40 mg/kg for all patients. These doses are generally well tolerated without premedication. Adverse events reported were fever, malaise, erythematous rash, sweating, hypoxia, flushing, and tachycardia. The role of IgE-type antibodies in these responses was not evident, but IgG-type antibodies may be relevant. Adverse events were transient and manageable by adaptation of the infusion rate.

Alpha-glucosidase activity in muscle on the starting doses showed a ten-fold increase at 12 weeks of treatment (from 0.15-0.37 nmol/mg per h to 2.1-4.9 nmol/mg per h), but was still lower than normal (8-40 nmol/mg per h). 12 weeks later, with 40 mg/kg rhAGLU, alpha-glucosidase activity was in the normal range for all four patients. On histological assessment, lysosomal

* In the original article recombinant human alpha-glucosidase was referred to as rhGAA.

glycogen storage was lowered and tissue morphology improved. The total tissue glycogen content did not change significantly.

Skeletal muscle function and strength improved in all patients, most significantly for patient 1, who had the least severe disease at start of treatment. This infant reached milestones that are beyond realistic expectations for a patient with the disease. At 12 months, he could crawl in a four-point position and stand with the support of one arm. Patient 3, who had more severe disease, learned to touch her feet in play. Her improvement has continued, despite producing no endogenous acid alpha-glucosidase (cross-reactive-immunological-material [CRIM] negative). Patients 2 and 4 also gained strength, most notably in the arms. At start of treatment these two patients (ages 7 months and 8 months) had end-stage disease and muscle function was almost lost. Patient 2 became dependent on a respirator during the inclusion period, as did patient 4, after 10 weeks of treatment, during a bout of pneumonia. The two patients, included before age 3 months, developed normal respiration and became outpatients. All patients showed progress in mental development.

The most prominent effect was on the heart. Left-ventricular posterior-wall thickness and left-ventricular-mass index (figure 1) decreased in all patients from the start of treatment. Patient 4 responded best. Her left-ventricular-mass index at 36 weeks of treatment was less than 30% of baseline.

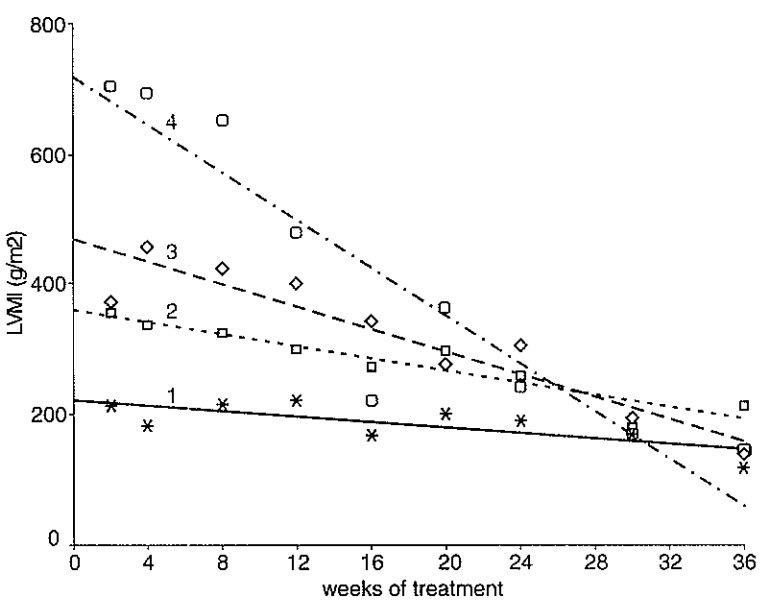


**Figure 1:** *Linear regression analyses of left ventricular-mass index. R=-0.58, r=-0.58, r=-0.82 and r=-0.91 for patients 1, 2, 3 and 4 respectively; each p<0.05*

Symptoms of cardiac instability disappeared in all patients, which was life-saving for patient 4. All patients passed the critical age of 1 year.

RhAGLU resulted in uptake of alpha-glucosidase in skeletal muscle, improved tissue morphology, and stimulated muscle function. The treatment reduced cardiac size, improved cardiac function and clinical condition, and seemed to prolong life. We recommend that treatment be started early. Confirmatory studies in a larger population and long-term follow-up are needed to assess final outcome and quality of life. The milk of transgenic animals seems to be a safe source and opens the way for further exploration of this production method.

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# CHAPTER 4

# Enzyme therapy for Pompe's disease with recombinant human alpha-glucosidase from rabbit milk

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**Abstract**

Pompe's disease is a metabolic myopathy caused by deficiency of lysosomal acid alpha-glucosidase. In this report we review the first 36 weeks of a clinical study on the safety and efficacy of enzyme therapy aimed to correct the deficiency. Four patients with infantile Pompe's disease were enrolled. They received recombinant human alpha-glucosidase from transgenic rabbit milk. The product is generally well tolerated and reaches the primary target tissues. Normalisation of alpha-glucosidase activity in skeletal muscle is obtained and degradation of PAS positive material is seen in tissue sections. The clinical condition of all patients improved. The effect on the heart was most significant with an impressive reduction of the LVMI. Motor function improved. The positive preliminary results stimulate continuation and extension of efforts towards the realisation of enzyme therapy for Pompe's disease.

## Introduction

Pompe's disease is a lysosomal storage disorder involving heart and skeletal muscles[1]. The most serious form of the disease is seen in babies with the infantile form of the disease. Feeding difficulties, respiratory infections, and motor delay are the presenting symptoms in the first months of life. A characteristic increased heart size, due to the presence of a hypertrophic cardiomyopathy, is seen on chest X-rays in all patients, and often raises suspicion for the diagnosis. Both cardiac failure and respiratory insufficiency add to the cause of death. Patients usually do not survive beyond the age of one year.

A milder course is seen in patients with late onset forms of the disease. Symptoms may start at any age and are related to progressive dysfunction of skeletal muscles. The heart is mostly not involved. When the disease proceeds, patients become wheelchair bound and dependent on artificial ventilation. A scoliosis may develop. Respiratory failure is the main cause of death. The age of death highly depends on the rate of progression of the disease and degree of involvement of respiratory muscles and varies from early childhood to late adulthood.

The disease is caused by a deficiency of acid alpha-glucosidase needed for degradation of lysosomal glycogen[2]. The correlation between the level of residual enzyme activity and the severity of disease is rather strict[3]. Alpha-glucosidase activity is virtually absent in the severe infantile cases, whereas residual activities up to 20% of normal are seen in late onset patients.

The rational of enzyme therapy for treatment of lysosomal storage disorders is based on the natural process whereby extracellular compounds gain access to the lysosomal system via endocytosis. The first and following attempts at enzyme therapy for Pompe's disease started from 1964, but failed[4-6]. Alpha-glucosidases from non-human sources appeared antigenic and low doses were administered compared to the dose we presently think is required to correct the deficiency in skeletal muscles. Developments in DNA technology have given enzyme therapy for lysosomal storage diseases a new chance by allowing large-scale production of recombinant human enzymes[7, 8]. The present report covers the first 36 weeks of a study on enzyme therapy in patients with infantile Pompe's disease with recombinant human alpha-glucosidase produced in the milk of transgenic rabbits.

## Methods

*Study design*

The clinical study was performed at the Sophia Children's Hospital, Rotterdam, The Netherlands, and was a single-centre, open-label pilot study. The study was approved by the Institutional Review Board. Written Informed Consent was obtained from the parents. All assessments were performed at baseline and on regular basis thereafter. Four patients with infantile Pompe's disease were included.

Muscle biopsies were scheduled at baseline and subsequently at 12-week intervals to assess uptake of alpha-glucosidase and changes in histopathology. Biopsies were taken from the quadriceps muscle via an open muscle biopsy, one day after infusion of recombinant human alpha-glucosidase (rabbit milk rhAGLU).

Neuro-motor and mental development were assessed with the Alberta Infant Motor Scale (AIMS) every 4-6 weeks[9], the Bayley Scales of Infant Development (BSIDII) every 12 weeks[10], and regular standardised neurological examinations.

*Biochemistry*

Tissue specimens for measurement of alpha-glucosidase activities were immediately frozen in liquid nitrogen and stored at -80°C until use. The tissue was homogenised in water. Alpha-glucosidase activity was determined using the artificial substrate 4-methyl-umbelliferyl (MU) - alpha -D-glucopyranoside at pH 4.0[11]. Protein concentration of the supernatant was determined using the bicinchoninic acid (BCA) protein assay as described by the manufacturer (Pierce, USA).

*Histology*

For histology purposes, tissue specimens were fixed in 4% glutaraldehyde and embedded in glycol-methacrylate (GMA). Tissue sections (4 μm) were stained with Periodic Acid Schiff (PAS). Slides prepared at different time points were stained in one session.

*Enzyme production*

rhAGLU production in transgenic rabbits was achieved essentially as described before[12]. An overview of the procedure is given in the results section. The milk is defatted, caseins are removed by filtration, and rhAGLU is purified by multi-step column chromatography. Additional measures are taken to remove hazardous contaminants, for instance viruses that can potentially be present. Both breeding of rabbits and down-stream processing of rabbit milk rhAGLU were performed by Pharming N.V., Leiden, The Netherlands.

## Results

*Production of recombinant human alpha-glucosidase (rhAGLU)*

The recombinant alpha-glucosidase that was used in the study is produced in transgenic rabbits by the epithelial cells of the mammary gland. The gene construct used for this purpose encompasses the human alpha-glucosidase gene with all exons and introns including the untranslated exon 1 (28.5 kb) and 9 kb of 3'-UTR. High level, cell type specific expression is obtained by fusing this gene at the 5' side to 6.3 kb of the bovine $\alpha_{S1}$-casein promoter. Fig. 1 depicts the transgene and illustrates the further procedure. The transgene is microinjected into the pronucleus of fertilised rabbit oocytes. These are implanted in foster mothers. Newborns carrying the transgene are selected by Southern blot analyses. Those who transmit the transgene in a Mendelian fashion are used for breeding. Expression of the transgene is investigated in following generations by Northern blot analysis and by measuring the acid alpha-glucosidase activity in milk samples. As a result of testing and selective breeding, a line of rabbits was obtained producing recombinant human alpha-glucosidase (rhAGLU) during lactation.

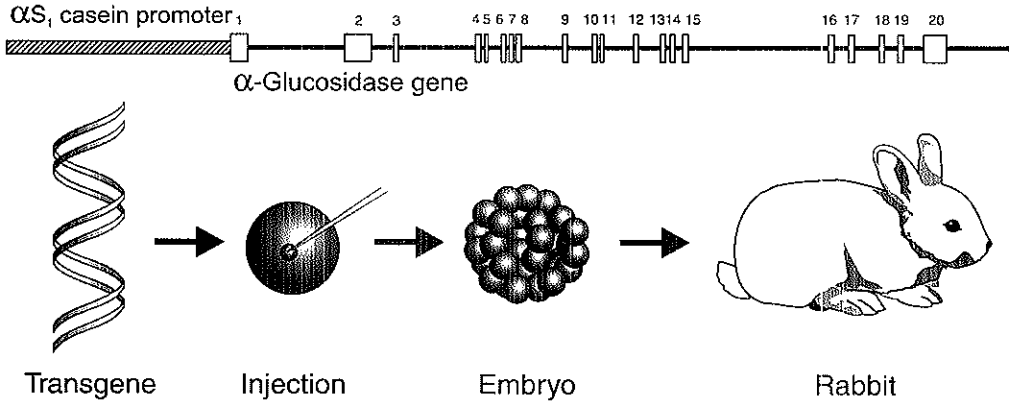


**Figure 1:** *Schematic drawing of the gene construct used to produce recombinant human alpha-glucosidase in rabbit milk by transgenesis*

RhAGLU is extracted from the milk by multi-step column chromatography and mixed with infusion fluid for intravenous administration. Safety, tolerance and pharmacokinetic studies were completed in rats and healthy volunteers (Phase I) before start of the Phase II clinical study in patients with Pompe's disease.

*Inclusion and exclusion criteria*

The clinical study started with patients having the most severe infantile form of the disease.

The in- and exclusion criteria are listed in Table 1. The clinical presentation of the patient had to be consistent with the most severe infantile form of Pompe's disease, including presence of a hypertrophic cardiomyopathy. Cardiac size was determined by ultrasound. A left ventricular mass index over 68.7 g/m$^2$ ($P_{97.5}$) was considered to be abnormal[13]. The acid alpha-glucosidase activity in fibroblasts and skeletal muscle had to be deficient, and there had to be evidence of lysosomal glycogen storage in muscle upon histological examination. The upper age limit for inclusion of the patients was set at 10 months.

Ventilator dependency was an exclusion criteria, as was allergy to food and medication, and congenital abnormalities not consistent with Pompe's disease.

**Table 1:** *Inclusion/Exclusion criteria*

| Inclusion Criteria | Exclusion Criteria |
|---|---|
| Symptoms of Infantile Pompe's disease | Congenital abnormalities |
| Hypertrophic cardiomyopathy | Allergy to food, proteins |
| Severe alpha-glucosidase deficiency | Ventilator dependency |
| Diagnosis confirmed by muscle biopsy | |
| Age less than 10 months | |

**Table 2:** *Patient characteristics*

| Patients | Onset of symptoms | Age at diagnosis | Oxygen need* |
|---|---|---|---|
| Patient 1 | At birth | 1 month | - |
| Patient 2 | 3 months | 4 months | + |
| Patient 3 | At birth | 14 days | - |
| Patient 4 | 3 months | 6 months | + |

* = *during inclusion period*

Four patients were enrolled (Table 2). They all presented with symptoms before 3 months of age. The final diagnosis was made before 6 months of age, in all cases (14 days, 1 month, 3 months and 6 months respectively). Two patients were included at the relatively young age of 2.5 and 3 months (patient 1 and 3 in Table 2). They had the best clinical condition at inclusion. A cardiomegaly at birth led to the diagnosis, indicating that the hypertrophic cardiomyopathy already develops during gestation. The youngest of the two was most severely affected. She had signs of cardiac failure and respiratory distress and was dependent on nasogastric tube feeding since birth. Axial hypotonia, a head lag and slipping through were seen in both cases.
The other two patients were included relatively late (7 and 8 months, respectively) in an end stage of the disease. Both patients were oxygen dependent and close to respiratory failure at the time of inclusion. There were signs of cardiac instability. They were hardly able to move arms, and legs lay flat on the surface in a frog like position. Both these patients became respirator dependent: one immediately after inclusion, even before the first alpha-glucosidase infusion was given; the other 10 weeks after start of treatment during a bout of pneumonia. The latter patient had a complete atelectasis of the left lung due to extensive cardiomegaly.
Endpoints of the study were exploratory and comprised gathering of safety and efficacy data. Survival was taken as the primary endpoint.

*Safety*

This report covers the first 36 weeks of treatment with recombinant human alpha-glucosidase from rabbit milk (144 infusions in total). Patients over 6.5 kg with 15 mg/kg (patients 1 and 2, in Table 2) and patients under 6.5 kg started with a dose of 20 mg/kg (patient 3 and 4, in Table 2). The dose was increased later to 40 mg/kg for all patients. The enzyme was administered intravenously via a central venous catheter and has been generally well tolerated. Infusion reactions were seen in all four patients, but were well manageable by adaptation of the infusion rate. Pre-medication with antihistamines or corticosteroids is not needed. Changes in blood pressure were never seen. Two patients are treated as outpatients, the other two in hospital.

*Uptake of alpha-glucosidase by muscle*

Alpha-glucosidase activity was assessed in muscle tissue obtained by open biopsy from the quadriceps muscle. All patients had a severe deficiency of alpha-glucosidase activity in muscle at inclusion (Table 3) ranging from 1-2 % of the normal value.

**Table 3:** *alpha-glucosidase levels (nmol/mg/h) skeletal muscle*

| Patient | Muscle t=0 | Muscle t=1 | Muscle t=2 |
|---|---|---|---|
| Patient 1 | 0.15 | 4.9 | 27 |
| Patient 2 | 0.27 | 2.7 | 8 |
| Patient 3 | 0.20 | 2.1 | 13 |
| Patient 4 | 0.37 | 2.7 | 16 |

*t=0: baseline; t=1: 12 weeks after start of treatment with 15 or 20 mg/kg. t=2: after 12 more weeks of treatment with 40 mg/kg*

Twelve weeks after treatment (t=1) with weekly dose of 15 or 20 mg/kg, a second biopsy was taken. A 7-30 fold increase of alpha-glucosidase activity in muscle was seen, demonstrating that the target tissue was reached.

However, the values obtained were still below normal, and the condition of patient 4 became very critical. Therefore, it was decided to increase the dose of all patients to 40 mg/kg weekly. Twelve weeks there after a new biopsy was taken. All patients had now acquired normal alpha-glucosidase activities (Table 3).

*Histopathology*

At baseline, all patients showed an impressive storage of lysosomal glycogen, as judged by the presence of PAS positive concentrates in muscle tissue sections (Fig. 2A, page 120, patient 1). The extent of pathological findings was related to the age of the patient and the severity of symptoms at the time of biopsy. Twelve weeks after dose increase a significant reduction of PAS positive material was observed (Fig. 2B, page 120, patient 1), but the total tissue glycogen content had not changed significantly.

*Clinical findings*

Normalisation of alpha-glucosidase activity and improvement of tissue morphology was reflected by improvement of clinical condition. The late included patients had the poorest motor condition at start of treatment. They were able to lift their arms only briefly while their legs lay flat on the surface in a frog-like position without any movement. During the 36 weeks of treatment, the patients gained strength in their arms. They learned to play with toys above their head and to transfer objects. Head balance improved slightly. The best effect was seen in the two patients who were included early. At 36 weeks of treatment, the younger of the two can lift her legs freely from the surface and has learned to touch her feet in play. She turns her upper body completely, but is not able to roll over. Her condition is still showing progress. Patient 1 who had the best motor condition at start of treatment has shown the most remarkable progress. At 9.5 months of age he learned to sit independently without arm support and at 10 months he started to crawl. At 11 months he pulled to a standing position and cruised along furniture. At 12 months, he learned to creep in a four-point position and to stand with support of one arm.

All patients survived beyond the age of one year, which exceeds the mean age of survival of untreated infantile Pompe patients[14, 15]. The later included patients are still dependent on

artificial ventilation. The young included patients, however, did not become dependent on artificial ventilation or oxygen, indicating that the rhAGLU infusions support respiratory muscle function when started early.

The most significant effect was seen on the heart. All patients had a cardiomegaly at start of treatment, characteristic for infantile Pompe's disease. The left ventricular mass index (LVMI) exceeded 170 grams/$m^2$ in all, which is high above normal (68.7 grams/$m^2$ , $P_{97.5}$). The cardiac enlargement already develops during gestation. The two best performing patients in the study both had a cardiomegaly at birth. The left ventricular mass index and left ventricular posterior wall thickness decreased significantly after start of treatment with rhAGLU[16]. The largest reduction was seen in patient 4. At 36 weeks of treatment the LVMI had decreased to 30 % of the base line value. Both the atelectasis of the left lung and the signs of cardiac instability had disappeared. The combined effects of rhAGLU were life saving for this patient.

## Discussion

This report reviews the first 36 weeks of a study on the safety and efficacy of recombinant human alpha-glucosidase from mouse milk in four patients with infantile Pompe's disease. The period of treatment is too short to draw final conclusions, but important lessons have been learned. First of all, evidence was obtained that human medicines can be produced in the milk of transgenic animals. rhAGLU is one of the very first products in this line that are currently being investigated in Phase II and III trials. The product is well tolerated and does not evoke major side effects. Further, there are several lines of evidence that rhAGLU treatment serves its purpose.

Intravenous administration of rhAGLU leads over the 36 weeks period to an increase of alpha-glucosidase activity in the target tissues. The lysosomal storage of glycogen in muscle seems to decrease, at least when judged by the less intense PAS staining after 36 weeks. However, we did not yet register a significant decrease in the overall glycogen content of the muscle at this time point. An explanation is difficult to give, as the contribution of lysosomal versus cytoplasmic glycogen to the total glycogen content of the muscle is unknown. Importantly, we did see improved muscle structure in the best performing patient (patient 1). Efficacy of enzyme therapy with rabbit milk rhAGLU is most notable by decline of LVMI seen in all four patients, along with improved cardiac function. Stabilisation of the clinical condition, and for some patients progressive developmental milestones, also point to therapeutic effect. The natural course is one of decline. Patients with severe infantile Pompe's disease do not reach major milestones like sitting and rolling and usually die around one year of age.

Long term follow up of these children under continuous treatment is necessary to evaluate the true prospects of enzyme therapy for infantile Pompe's disease.

Limited as our results are in this early stage, they do show the benefits of enzyme replacement therapy for patients with Pompe's disease. It is time to scale up the efforts and make enzyme therapy for Pompe's disease a real industrial enterprise. Phase III trials are required and patients with late onset forms of disease need to be included. These are the challenges for the near future.

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# CHAPTER 5

# Long term IV treatment of Pompe's disease with recombinant human alpha-glucosidase from milk

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*Submitted*

## Abstract

*Objective:* Recent reports warn that the worldwide cell culture capacity is insufficient to fulfil the increasing demand for human protein drugs. Production in milk of transgenic animals is an attractive alternative. We tested the long term safety and efficacy of recombinant human alpha-glucosidase from rabbit milk for the treatment of a lysosomal storage disorder (Pompe's disease).

*Methods:* In the beginning of 1999 four critically ill patients with infantile Pompe's disease (2.5-8 months old) were enrolled in a single Centre Open Label Study and treated intravenously with 15-40 mg/kg/week recombinant human alpha-glucosidase.

*Results:* The genotypes of the patients were consistent with the most severe form of Pompe's disease. Transgenic alpha-glucosidase was tolerated well during more than three years of treatment. Clinical effects were significant. All patients survived beyond the age of four years, whereas untreated patients succumb at a median age of 6-8 months. Alpha-glucosidase activity normalised. Muscle morphology improved markedly in one and cardiac hypertrophy in all patients. Two infants achieved motor milestones that are unmet in infantile Pompe's disease, but only one patient learned to walk.

*Conclusion:* Our study shows that a safe and effective medicine can be produced in milk of mammals and encourages further development of enzyme replacement therapy for the several forms of Pompe's disease. The therapeutic effect depends on enzyme dose and residual muscle function at start of treatment. We advise to evaluate the condition of the patients critically before and during treatment.

## Introduction

Production of therapeutic proteins in milk of transgenic animals is a challenging new technology in light of the rapidly expanding market for protein drugs and the ever-increasing costs of healthcare[1]. As the transgenic protein concentration in milk is very high, kilogram quantities of product per year can be obtained at relatively low costs, even in small animals like rabbits[2].
Since the first report in 1988 on alpha-1-antitrypsin production in sheep milk[3] a variety of transgenic proteins has been produced. These range from small unstable peptides (salmon calcitonin peptide[4]) to large proteins with complex post-translational modifications (factor VIII[5, 6] and lactoferrin[7]). But, despite the great promises of this novel production platform there is as yet not a single transgenic medicine on the market. Three recombinant proteins from milk are being tested in clinical trials: antithrombin III from goats, alpha-1-antitrypsin from sheep and alpha-glucosidase from rabbits[8-10].
We have focussed on the production of recombinant human acid alpha-glucosidase for the treatment of Pompe's disease, an autosomal recessive muscle disorder. The classic infantile form of the disease leads to death at a median age of 6-8 months[11] and is diagnosed by absence of alpha-glucosidase activity and presence of fully deleterious mutations in the alpha-glucosidase gene[12]. Cardiac hypertrophy is characteristically present[11]. Loss of muscle strength prevents infants from achieving developmental milestones like sitting, standing and walking[11-13]. Milder forms of the disease caused by less severe mutations and partial deficiency of alpha-glucosidase are also known. Pompe's disease is a lysosomal glycogen storage disorder with an estimated frequency of 1:40,000 births[14, 15] and is thereby an orphan disease.
We have successfully explored the feasibility of enzyme therapy for Pompe's disease in preclinical studies[16-19]. The principle of treatment is based on the capacity of lysosomes to engulf exogenous proteins via endocytosis[20]. After cloning the human alpha-glucosidase gene we have focussed on production of the enzyme in CHO cells and milk of transgenic animals[18, 19, 21-25]. The high yield of 2 grams per litre and the short reproduction time of rabbits has led to the pharmaceutical production of recombinant human alpha-glucosidase (rhAGLU) in milk of transgenic rabbits. The product's safety and efficacy were tested in phase I and phase II clinical studies[10]. Here we report how four patients with infantile Pompe's disease responded to more than 3 years of IV treatment.

## Methods

*Enzyme purification and characterisation*

A line of transgenic rabbits producing rhAGLU was obtained[19]. Rabbit milk was collected and stored at –20 °C until use. An alpha-glucosidase containing whey fraction was prepared from skimmed milk by tangential flow filtration using a Biomax 1000 membrane cassette (Millipore, Bedford, MA) and subsequently concentrated by ultrafiltration using a Biomax 30 membrane (Millipore, Bedford, MA). Following a virus inactivation step with Tween-80 in 1% and tri-n-butylphosphate in 0.3% concentration for 6 hours at 25 °C, the alpha-glucosidase was subsequently captured by Q Sepharose Fast Flow chromatography (Amersham Pharmacia

Biotech, Uppsala, Sweden). After intermediate purification on a Phenyl Sepharose HP column (Amersham Pharmacia Biotech, Uppsala, Sweden) alpha-glucosidase was polished by Source Phenyl 15 chromatography (Amersham Pharmacia Biotech, Uppsala, Sweden). Following a second viral removal step by nanofiltration, purified alpha-glucosidase was concentrated by ultrafiltration (Biomax 30 membrane; Amersham Pharmacia Biotech, Uppsala, Sweden) and sterilised by microfiltration (0.2 µm dead-end filter). The enzyme has a specific activity of more than 250 µmoles/mg/hr for 4-methylumbelliferyl-alpha-D-glucopyranoside and is >95% pure. Toxicity studies were performed in mice, rats and dogs in doses up to 100 mg/kg. A phase I study was successfully performed in humans. All information is contained in Investigator Brochures.

*Biochemical-genetic studies*

Fibroblasts and muscle tissue were homogenised in water, and 2000 x g supernatants were used to determine alpha-glucosidase activity, glycogen content and protein concentration[19, 26]. Mutation analysis was performed on genomic and cDNA, as described earlier[27]. The functional effect of mutations was studied by assay of alpha-glucosidase synthesis and activity in transiently transfected COS cells[27, 28].

*Study design*

The study was a single-centre, open-label phase II study. The Institutional Review Board approved the study, and written Informed Consent was obtained from the parents of all patients. The objective of the study was to evaluate the safety and efficacy of rhAGLU.

*Inclusion criteria*

Patients qualified for inclusion if they had symptoms characteristic of the infantile form of Pompe's disease, including a hypertrophic cardiomyopathy. The upper age limit was 10 months. Confirmation of the diagnosis was required by an open biopsy from the quadriceps muscle, revealing a virtual absence of alpha-glucosidase and the presence of lysosomal glycogen storage.

*Clinical studies*

RhAGLU was administered intravenously as a 1-2 mg/ml solution in saline with 5% glucose and 0.1% human serum albumin, in single starting doses of 20 mg/kg weekly in patients under 6.5 kg and 15 mg/kg in patients over 6.5 kg. Later, the dose was increased to 40 mg/kg weekly in all infants. During infusions, heart rate, temperature, transcutaneous oxygen saturation and blood pressure were continually recorded.

Before the start of rhAGLU treatment, there was a period of up to 2 weeks in which baseline assessments were performed. Thereafter patients were assessed at regular intervals. Muscle biopsies were taken from the quadriceps muscle via an open muscle biopsy one day after rhAGLU infusion. Tissue specimens for measurement of alpha-glucosidase were immediately

frozen in liquid nitrogen and stored at –80 °C until use. For histology purposes, tissue specimens were fixed in 4% glutaraldehyde and embedded in glycolmethacrylate (GMA). Tissue sections (4 µm) were stained with Periodic Acid Schiff (PAS). Slides prepared at different time points were stained in one session.

IgE and IgG antibody titres were detected using a standard ELISA assay in which the plates were coated with antigen (1 µg/ml) and the samples were diluted 5 fold for IgE and 100 fold for IgG. Samples from healthy volunteers served as negative controls.

Left ventricular dimensions were determined by M-mode echocardiography, in compliance with the guidelines of the American Society of Echocardiography, using a Hewlett Packard Sonos 5500[29]. The LVPWd and the calculated LVMI were used as measures of hypertrophic cardiomyopathy[30]. Psychomotor development was assessed using the Alberta Infant Motor Scale (AIMS)[31], the Bayley Scales of Infant Development (BSIDII)[32] and regular standardised neurological examinations.

## Results

*RhAGLU from rabbit milk*

In order to achieve high level expression of recombinant human acid alpha-glucosidase (rhAGLU) in the mammary gland, we placed the entire alpha-glucosidase gene under control of the bovine $alpha_{S1}$-casein gene promoter. The gene construct was injected into fertilised rabbit oöcytes and these were implanted in foster mothers. Thus, we obtained a line of transgenic rabbits with high level expression of rhAGLU during lactation[18]. The production line yields on average 2 g of crude rhAGLU per litre of milk during the first 3 weeks of lactation. The molecular mass of alpha-glucosidase from milk is 110 kDa (Fig. 1A), similar to the mass of the acid alpha-glucosidase precursor produced in genetically engineered CHO cells[18, 24, 33]. Rabbit milk acid alpha-glucosidase contains uncleaved N- and C-terminal pro-peptides.

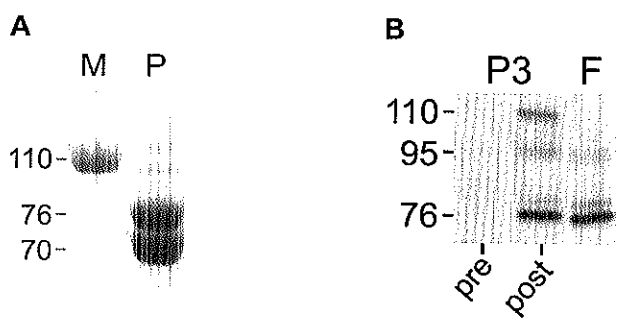


**Figure 1:** *Characterisation of rhAGLU from rabbit milk. A. SDS-PAGE (8%) analysis under reducing conditions. The gel was loaded with 50 µg of protein per lane and stained with Coomassie brilliant blue. The 110 kDa precursor from rabbit milk (M) is compared with the 76 and 70 kDa mature forms of acid alpha-glucosidase from human placenta. B. Western blot analysis of alpha-glucosidase derived from muscle of patient 3 (P3) pre- (t=0) and post (t=3) treatment. Alpha-glucosidase from fibroblasts of a healthy individual is used to mark and identify the various molecular forms.*

**Table 1:** *Clinical characteristics of the patients at baseline*

| Patient no. | Sex/age at start of treatment | Clinical appearance |
|---|---|---|
| 1 | M/3 months | Cardiac hypertrophy, head-lag, slipping through, axial hypotonia |
| 2 | F/7 months | Cardiac hypertrophy, heart failure, hypercapnia, oxygen need, head-lag, slipping through, axial hypotonia, poor head balance, paresis arms, paralysis legs, nasogastric tube feeding |
| 3 | F/2.5 months | Cardiac hypertrophy, heart failure, tachypnoea, borderline oxygen saturations, extreme perspiration, head-lag, slipping through, axial hypotonia, poor head balance, nasogastric tube feeding |
| 4 | F/8 months | Cardiac hypertrophy, heart failure, oxygen need, atelectasis left lung, head-lag, slipping through, axial hypotonia, poor head balance, paresis arms, paralysis legs, nasogastric tube feeding, growth retardation, intractable fever |

*Clinical condition and molecular delineation of patients*

RhAGLU from rabbit milk was tested for its therapeutic effect in an open label study in four patients with the most severe infantile form of Pompe's disease fulfilling the inclusion criteria as described in the methods. The clinical status of the four patients at baseline is summarised in Table 1. Thorough molecular characterisation of the patients with regard to the degree of acid alpha-glucosidase deficiency, pattern of alpha-glucosidase synthesis, and type of alpha-glucosidase gene mutations was performed to sustain the clinical diagnosis of classic infantile Pompe's disease and to investigate whether patients were naïve (CRIM negative) or non naïve (CRIM positive) for alpha-glucosidase. The alpha-glucosidase activity in skeletal muscle and fibroblasts of all four patients was below the lower limit of detection (<2% of normal) (Table 2).

**Table 2:** *Biochemical-genetic delineation of the patients*

| Patient | α-glucosidase [a] activity | Genotype | Amino Acid Substitution |
|---|---|---|---|
| 1 | 0.6 | G1799A/del exon 18 | R600H/del55AA |
| 2 | 0.5 | A1115T/delT525 | H372L/ - [b] |
| 3 | 0.1 | delT525/delT525 | - / - [b] |
| 4 | 0.7 | G1913T/silent | G638V/ - [c] |
| Control | 40-160 | | |

*a. The activities are expressed as nmole 4-Methylumbelliferon (MU) formed per hour per mg cellular protein.*

*b. The delT525 is not expressed at the protein level.*

*c. The G1913T mutation was heterozygous in genomic DNA and homozygous in cDNA obtained by RT-PCR indicating that only the G1913T allele is expressed.*

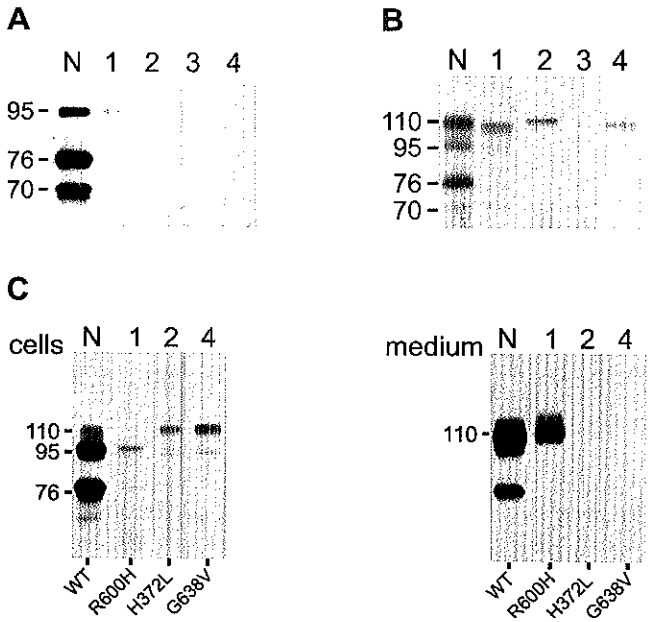


**Figure 2:** *Molecular forms of alpha-glucosidase.* ***A****, Western blot analysis. Alpha-glucosidase was immunoprecipitated from fibroblast lysates with rabbit anti-human-alpha-glucosidase antibodies and subjected to SDS-PAGE. Alpha-glucosidase on the Western blots was visualised with mouse anti-human-alpha-glucosidase antibodies and chemoluminescence. The numbers above the lanes correspond to the patient numbers. N are cells from a healthy individual. Each lane shows the relative amount and molecular mass of the alpha-glucosidase species present in an aliquot of lysate containing 1mg of total cellular protein.* ***B****, Synthesis and post-translational modification of alpha-glucosidase. Fibroblasts were incubated for 8 hours in medium containing $^{35}S$ methionine and then harvested. Alpha-glucosidase was immunoprecipitated from cell lysates with rabbit anti-human alpha-glucosidase antibodies and subjected to SDS-PAGE. The autoradiogram shows the processing forms of alpha-glucosidase. The numbers above the lanes correspond to the patient numbers. N are cells from a healthy individual. Equal numbers of cells were labelled per lane.* ***C****, Functional effects of novel mutations. Mutations were introduced in wild type cDNA by site-directed mutagenesis and transiently expressed in COS cells. Alpha-glucosidase was immunoprecipitated from cells and media at 72 hours after transfection and analysed by Western blotting, as described above. The numbers above the lanes correspond to the patient numbers. N is the wild type construct. Equal numbers of cells and equal volumes of media were used per lane.*

Three of the four patients lacked all biosynthetic forms of alpha-glucosidase, when investigated by Western blot analysis (molecular species of 110, 95, 76, and 70 kDa) (Fig. 2A).
A trace amount of the naturally occurring 95 kDa biosynthetic intermediate was seen in cultured fibroblasts from the fourth patient (patient 1).When we used a more sensitive detection method, $^{35}S$ methionine incorporation, we could detect low level synthesis of alpha-glucosidase in three of the four patients (patient 1, 2, and 4) and some post-translation modification from 110 kDa to

95 kDa in one of them (patient 1) (Fig. 2B). Patient 3 remained totally deficient with both detection methods and was therefore declared CRIM-negative. As third mode of molecular delineation we analysed the genotype of the patients (Table 2). The CRIM-negative patient (patient 3) is homozygous for delT525, a mutation known to lead to unstable mRNA, frame shift and absence of cross reactive immunologic material[34]. Both her parents are carriers of this mutation. Each of the other three patients has at least one alpha-glucosidase allele with a mutation permitting low level synthesis of the 110 kDa precursor when overexpressed in COS-cells (Fig. 2C). In two cases (patient 2 and 4) the precursor is degraded, but in one (patient 1) the Arg600His substitution is compatible with the formation of some 95 kDa intermediate. Importantly, none of the mutant alleles generates catalytically active alpha-glucosidase, despite over-expression. Thus, the genotype of all patients enrolled in the study is consistent with the infantile phenotype.

*RhAGLU tolerability*

RhAGLU was administered intravenously, in an initial dose of 15 to 20 mg/kg per week. The dose was later increased to 40 mg/kg per week. The duration of the infusions ranged from 4 to 6 hours. Infusion associated reactions were observed in the initial phase of treatment (starting at week 5-7) as reported earlier. They comprised fever, malaise, erythematous rash, sweating, hypoxia, flushing and tachycardia[10]. Corticosteroids and antihistamines were given in this period, but did not have a significant effect and were discontinued. The events appeared transient and stopped to occur since low infusion rates were applied (2 to 10 ml/h) for the first 2 hours. Events did not reoccur when the dose was doubled. In the later phase of treatment infusion reactions only occurred sporadically in the form of low-grade fever and rash. They were transient and mild. None of the patients receives at present antihistamines or corticosteroids. The infusions are easily manageable on an outpatient basis. One patient receives the infusions already for more than two and a half years at home.

The IgE titre did not rise above background levels during the study. Anti-rhAGLU IgG titres increased to levels between 5 and 13 times baseline values during the first 20 to 48 weeks and declined to 1 and 8 times baseline values during the following infusions. IgG titres against rabbit whey also increased (4-12 times baseline values) during the first 24 weeks and then stabilised between 3 and 9 times baseline values. We did not observe a consistent difference in antibody formation in the CRIM-positive versus the CRIM-negative patient. Notably, we found the highest IgG titre in a 34-year-old patient with 10-20 % of normal CRIM who was treated in a separate late onset patient study with the same enzyme preparation.

*Alpha-glucosidase activity in tissues*

During the first 12 weeks of treatment, muscle alpha-glucosidase activity increased from <2% to 10-20% of normal in all patients (Table 3A). In order to optimise the therapeutic effect, we increased the rhAGLU dose in all infants to 40 mg/kg and this resulted, 12 weeks later, in normal alpha-glucosidase activity levels.

**Table 3:** *Uptake of alpha-glucosidase in muscle and correction of glycogen storage*

**A**

| Patient | Alpha-glucosidase activity* | | | |
|---|---|---|---|---|
| | T=0 | T=1 | T=2 | T=3 |
| 1 | 0.2/0.4 | 4.9/3.7 | 27.4/29.3 | 27.1/25.8 |
| 2 | 0.3/0.3 | 2.7/3.1 | 8.0/9.5 | 28.9/12.1 |
| 3 | 0.2/0.7 | 2.1/3.8 | 13.0/7.4 | 8.2/8.9 |
| 4 | 0.4/0.2 | 2.7/1.8 | 16.2/11.3 | 5.8/4.4 |
| Control | 8-40 | | | |

**B**

| Patient | Glycogen-concentration* | | | |
|---|---|---|---|---|
| | T=0 | T=1 | T=2 | T=3 |
| 1 | 747/1022 | 755/1351 | 892/1440 | 86/503 |
| 2 | 2810/2543 | 3270/3199 | 2450/3190 | 2460/3020 |
| 3 | 1650/1397 | 2330/1847 | 2020/2440 | 2300/2550 |
| 4 | 3690/2720 | 4630/3700 | 3060/2720 | 2130/2310 |
| Control | 30-180 | | | |

**Multiple measurements of alpha-glucosidase activity (nmol/mg/h) (A) and glycogen concentration (microgram/mg) (B) in muscle at baseline (t=0) and after 12 weeks of treatment with 15 to 20 mg/kg (t=1), 12 more weeks of treatment with 40 mg/kg (t=2) and 72 weeks of treatment (t=3).*

These were maintained until the last measurement in week 72. Importantly, all four patients, including the patient without any endogenous alpha-glucosidase (CRIM-negative), revealed mature 76 and 70 kDa forms of alpha-glucosidase on Western blot after treatment with the 110 kDa (precursor) rhAGLU (Fig.1A and B). As the final steps of enzyme maturation occur in lysosomes, this observation provides evidence that rhAGLU reaches its target.

*Muscle morphology*

At baseline, all patients had severe glycogen storage in the quadriceps muscle as revealed by intense PAS-positive staining and lacework patterns in HE-stained tissue sections. The two older patients had more severely affected muscle at inclusion than the two younger ones. The muscle pathology correlated at each time point with the severity of signs. After 12 weeks of treatment with 15 or 20 mg/kg rhAGLU, the PAS intensity had diminished, while the number of vacuoles had increased. Twelve weeks after dose elevation we observed signs of muscle regeneration in three of the four patients. Further improvement of muscular architecture was only seen in the patient who learned to walk (Fig. 3, page 121). The glycogen concentration in muscle stabilised in patient 2, 3 and 4, and decreased in patient 1 during the course of treatment (Table 3B).

*Survival*

All patients in the study are currently alive at an age of 4 years, whereas the life expectancy of untreated patients with the classic infantile form of Pompe's disease is typically less than 1 year[10-13].

*Cardiac morphology and function*

All four patients had the characteristic cardiac hypertrophy at start of treatment, with a left ventricular mass index (LVMI) exceeding 170 g/m$^2$ ($P_{97.5}$ 86.6 g/m$^2$ [35]). During 84 weeks of treatment, the LVMI decreased from 171, 203, 308 and 599 g/m$^2$ at baseline to 70, 160, 104 and 115 g/m$^2$ for patients 1, 2, 3, and 4, respectively. The decrease of the LVMI along time appeared linear ($r$ = -0.86, -0.91, -0.89 and -0.80 for the four respective patients; all $P$ <0.01). The diastolic thickness of the left ventricular posterior wall (LVPWd) was increased before start of rhAGLU treatment. We found a significant change of slope for LVPWd against time at t=0 for each separate patient ($P$ <0.01) (Fig. 4). The decrease in LVPWd after the start of treatment levelled out in two patients (patients 3 and 4) when the $P_{95}$ of normal was approached, as evidenced by a second significant change of slope using the "broken stick" method ($P$ <0.05). Systolic function normalised and diastolic function improved in all patients.

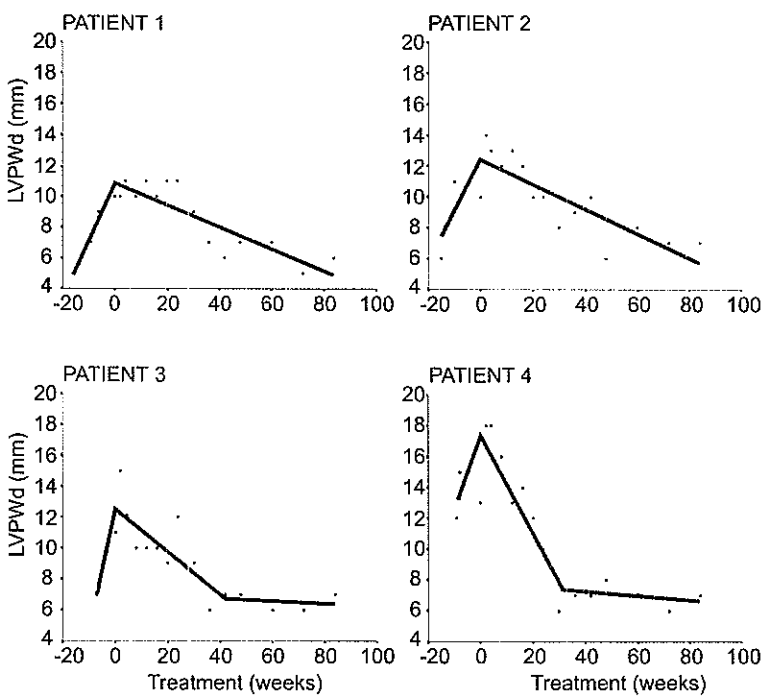


**Figure 4:** *Changes in LVPWd during treatment for patients 1-4. Piece-wise linear regression ("broken-stick" method) is used to illustrate the statistically significant change in slope after start of treatment.*

*Respiratory condition*

During the first 2 years of life, the two younger patients (patient 1 and 3) remarkably showed no significant respiratory problems. Patient 3 recovered from a life-threatening bronchiolitis at the age of 1 year without sequelae, despite borderline oxygen saturation at inclusion. At the age of two she became ventilator-dependent after surgical removal of an infected Port-A-Cath. The respiratory course of patient 1, now four years old, remained uneventful. Patients 2 and 4, who were older and most severely affected at inclusion, had a marginal respiratory condition from the start of treatment, and both required oxygen. Patient 2 ($pCO_2$ 10.6 kPa equivalent to 80 mm Hg at start; normal range 4.5-6.8 kPa) became ventilator-dependent before the first rhAGLU infusion and remained fully ventilated. Patient 4 (pCO2 9.8 kPa equivalent to 75 mm Hg at start) became ventilator-dependent during a bout of pneumonia after 10 weeks of low-dose treatment. She could be gradually weaned from the ventilator after one year of high dose treatment and was eventually completely ventilator free for 5 days, but this situation could not be maintained. At the age of 4 her ventilator needs are 22-23 hours per day.

*Motor development*

The most remarkable progress in motor development was seen in the younger patients (patients 1 and 3, Fig. 4, page 121). Patient 1 learned to crawl (12 months), walk (16 months), squat (18 months) and climb stairs (22 months). Patient 3 learned to sit unsupported (Fig 5), and her condition further improved until the age of 2, when she became ventilator dependent, and her condition declined. At baseline, the older patients (patients 2 and 4) could hardly lift their arms while in a supine position, and their legs lay immobile on the surface.

During treatment, the muscle function of the arms improved significantly. Patient 2, however, lost her regained muscle strength after a series of airway infections. In her case, restoration of function appears to be a difficult process. Patient 4 maintained the strength in her arms. When she was 24 months old she could roll over onto her side and play for longer periods while seated in a wheel chair. Her condition has not improved since then.

*Mental development*

As rhAGLU is unlikely to pass the blood-brain barrier, we had concerns about long-term mental development and neurological performance. Neurological examinations performed from the start of treatment at pre-set time points showed no signs of CNS involvement so far. The patients seem to have normal mental development and attend school at the age of four. As unexpected finding we measured in all patients an elevated response threshold of brainstem auditory evoked potentials (BAEP) at inclusion. This did not change during treatment.

## Discussion

Our study demonstrates that a safe and effective medicine for IV treatment of human disease can be produced in the milk of transgenic animals; in this case rhAGLU from rabbit milk for patients with Pompe's disease. The safety of the product is proven by the fact that more than 700 infusions were well tolerated by critically ill patients. The efficacy of the therapy is evident

from several observations. First, all four patients are presently more than 4 years old, whereas the average life span in untreated infantile Pompe's disease is less than one year[10-13].
Second, the cardiac hypertrophy diminished and cardiac function improved, whereas cardiac failure is a major cause of death in infantile Pompe's disease. Third, patients gained muscle strength, while normally there is a progressive loss of muscle function. Two patients achieved developmental milestones that are unmet in untreated patients. Notably, the motor score on the Bayley Scale of Infant Development of one patient normalised at the age of two years.
The efficacy of treatment is further supported by biochemical findings. Correction of alpha-glucosidase deficiency occurred in skeletal muscle of all four patients. After three months of single weekly doses of 15 to 20 mg/kg the correction was partial. A 100% correction was achieved after three additional months on a weekly dose of 40 mg/kg. Conversion of the 110 kDa precursor from milk to mature 76/70 kDa alpha-glucosidase provides evidence that the enzyme is targeted to lysosomes, where this proteolytic processing occurs[36]. Moreover, we see a decrease of the PAS-staining intensity and the appearance of empty vacuoles in quadriceps muscle tissue sections of all patients, suggestive for an effect on the lysosomal glycogen pool. In the best performing patient this results in greatly improved muscle morphology and glycogen content[37].
It is evident, that the four patients respond differently to the treatment and we have tried to find an explanation. All patients appeared to have molecular-genetic defects and clinical characteristics that are consistent with classic infantile Pompe's disease[11-13]. Notably, the best performing patient had the most severe hearing deficit (80dB). This makes it very unlikely that the difference in response is explained by inclusion of patients with milder non-classic infantile phenotypes.
We find it equally unlikely that an inhibitory immune response, as described in a study by Amalfitano et al. with alpha-glucosidase from CHO-cells[38], explains the difference in efficacy. The latter study claims, that neutralizing antibodies caused the loss of treatment benefit at the age of 8 months in two CRIM-negative patients. This counter-effect did not occur in our study. On the contrary, our truly CRIM–negative patient was the second best responder. Enzyme therapy studies for Gaucher's disease, Fabry's disease and MPS I do not indicate either that a CRIM-negative status per se or antibody formation interferes with the efficacy of enzyme replacement therapy[39-43].
In our view, the degree of impairment of skeletal muscle function and the age of the patient at start of treatment play the decisive roles in the outcome. The two patients, included at 2.5 and 3 months of age, had milder symptoms at inclusion and responded clearly better than the 7 and 8 months old patients who were in an end stage of the disease at the time of inclusion.
We have learned that active muscle movement is important and probably required to restore muscle function. In Pompe's disease proximal muscles are more affected than distal muscles and the legs more than the arms. In this respect the quadriceps muscle (from which the muscle biopsies were taken) is expected to reflect the most severe muscle pathology. Muscle pathology and muscle function appeared to be closely related and explains to our opinion why

muscle morphology and total glycogen content only normalised in the patient, who learned to walk[37].

Despite the significant effects of our therapy we warn that infantile patients are at risk to develop residual disease with contractures, scoliosis or respiratory insufficiency if treatment is started too late or with a too low dose. The therapeutic window in infantile patients is small. We may have lost precious time by treating infantile patients for three to six months with a possibly sub-optimal dose of 15-20 mg/kg/week instead of 40 mg/kg/week.

The results of our study are encouraging for the further exploration of enzyme replacement therapy. This holds for infantile patients, but even more so for the patients with milder forms of Pompe's disease. In their case the therapeutic window is larger as we observe in our pilot study with 3 late onset patients aged 12-33 years. One of them, who was wheelchair-bound for four years, started to walk again after 2 years of treatment with 20 mg/kg/week of recombinant human alpha-glucosidase[44].

The effective dose of rhAGLU is high compared with the 1 mg/kg dose of glucocerebrosidase used for the treatment of Gaucher's disease type I and the 0.2-1 mg/kg dose of alpha-galactosidase used for treatment of Fabry's disease[39-41, 43, 45]. These differences are explainable by the different target tissues. In Pompe's disease, the target tissue (skeletal muscle) is shielded from the enzyme by the capillary endothelium and the interstitial tissue. In contrast, the targets in Gaucher's disease (liver and spleen macrophages) and Fabry's disease (endothelial cells) are directly exposed to circulating enzyme. Notably, in Fabry's disease 3 mg/kg α-galactosidase is sufficient to reach the endocard but insufficient to reach the cardiomyocytes[40]. The higher dose required to correct enzyme activity in skeletal muscle is supported by studies in animal models[19, 33, 46, 47], and was demonstrated for various enzyme species, among which recombinant human alpha-glucosidase from both transgenic mammals as well as genetically engineered CHO cells.

## Conclusion

The results of our study demonstrate both the safety and efficacy of enzyme replacement therapy in Pompe's disease as well as the feasibility of producing medicines in the milk of transgenic animals. This novel production platform can potentially reduce the costs of therapeutics, but scale-up persists as major challenge in the rhAGLU production process. The currently used transgenic rabbit production line supplies approximately 10 gram of rhAGLU per animal per year. At a dose of 10-40 mg/kg per patient per week the production of rhAGLU in rabbit milk is feasible in the initial phase of product development, but falls short to later supply the world-wide group of Pompe patients. The more conventional production system in genetically modified CHO cells can be used as alternative, but also has a capacity limitation[1]. Thus, timely investments need to be made in the development of alternative production platforms. Our studies show, that production in milk of sheep, goat or cow is the option of choice.

## Epilogue

When this manuscript was ready for submission, Patient 3 developed a high fever with an intractable course. She went into coma and died one week later. We have devoted an epilogue to her case at the end of this thesis

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# CHAPTER 6

# Cardiac response to enzyme replacement therapy in Pompe's disease

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*Submitted*

## Abstract

*Background:* Cardiac hypertrophy is a characteristic feature of various lysosomal storage disorders like Pompe's disease and (Anderson) Fabry's disease. We have studied the effects of enzyme therapy in Pompe's disease for more than 3.5 years.

*Methods and results:* In the beginning of 1999 four patients with the cardiac or infantile form of Pompe's disease were enrolled in an open label study at ages of 2.5-8 months. Recombinant human alpha-glucosidase was produced in milk of transgenic rabbits and administered intravenously in a dose of 15-40 mg/kg/week. Before start of treatment patients had a progressive concentric cardiac hypertrophy evidenced by progressive thickening of the left ventricular posterior wall (LVPWd) and highly elevated left ventricular mass indexes (LVMI) up to 599 g/m$^2$. After start of IV treatment a significant decrease of both LVPWd and LVMI was found, with a decrease of the LVMI to 16, 35, 50 and 75% of pre-treatment levels ($p<0.05$). The effect levelled out when the LVMI approached the upper limit of normal. LVMI became normal in three of the four patients. Concentric remodelling was observed. On EKG the amplitude of the QRS complexes decreased; and the borderline abbreviated PR interval increased in 3 of the four patients. Shortening fraction normalised. Clinical condition ameliorated. Cardiac disease was no longer life threatening as in untreated Pompe's disease. Diastolic function remained mildly disturbed.

*Conclusions:* The early and good response to therapy shows that the heart in Pompe's disease is amenable to enzyme replacement therapy.

## Introduction

Infantile Pompe's disease is an autosomal recessive inborn error of metabolism caused by complete deficiency of alpha-glucosidase[1]. Glycogen accumulation occurs in lysosomes of various cell types and tissues such as endothelial cells, smooth muscle, cardiomyocytes, myotubes, neuronal cells and liver parenchymal cells. Progressive storage eventually leads to impairment of muscle function and the occurrence of a hypertrophic cardiomyopathy, the most characteristic feature of the infantile or cardiac form of the disease[2-7]. Cardiorespiratory failure is the major cause of death. In a recent survey of 20 Dutch patients and 133 cases from literature we found a median age of survival of 7.7 months in the Dutch patients compared to 6 months in the world-wide population[7].

Milder forms of Pompe's disease also occur. In these patients skeletal muscle weakness is the main symptom and cardiac hypertrophy is not a prominent feature. Alpha-glucosidase is partial deficient.

Cardiac hypertrophy is not only seen in Pompe's disease but may also emerge in other lysosomal storage disorders such as Fabry's disease and MPS I.

Enzyme replacement therapy has recently been registered for the latter disorders. Effects on the heart are still largely unknown. We are involved in the development of enzyme replacement therapy for Pompe's disease[8-11]. After cloning of the alpha-glucosidase gene we have explored methods to produce recombinant human alpha-glucosidase (rhAGLU) in CHO cells[12] and transgenic animals[13-15]. The high production level in milk (2 g/litre) has led to further exploration of production in transgenic rabbits. Rabbits were chosen for their short reproduction time.

In the beginning of 1999 we started to treat 4 patients with the recombinant enzyme[16, 17]. Here we report on the cardiac response to enzyme therapy over a treatment period of 3.5 years.

## Methods

*Study Design*

The clinical study was performed at the Erasmus Medical Centre Rotterdam, the Netherlands as a single-centre, open-label pilot study. The Institutional Review Board approved the study. Written informed consent was obtained from the parents.

*Study Population*

Patients qualified for inclusion if they had symptoms characteristic of the infantile form of Pompe's disease, including a hypertrophic cardiomyopathy. The upper age limit was 10 months. The diagnosis of Pompe's disease was confirmed by the demonstration of alpha-glucosidase deficiency in muscle tissue and by mutation analysis.

*Treatment*

Recombinant human alpha-glucosidase was produced in transgenic rabbits, as described earlier[13-17]. Patients under 6.5 kg (patient 3 and 4) received a weekly dose of 20 mg/kg rhAGLU and patients over 6.5 kg (patient 1 and 2) received 15 mg/kg. After 12-23 weeks of treatment the dose was increased to 40 mg/kg in all.

*Clinical Evaluation*

Patients were evaluated by a clinical examination, complete laboratory testing, electro-cardiography and echocardiography at start of treatment and preset time-points thereafter. The patients were followed for a period of 3 and a half year.

*Electrocardiography.*

Twelve-lead standard EKG's were recorded using a portable PC-based acquisition system (Cardio Control Delft, The Netherlands). The following leads were taken: I, II, III, V1, V2, V3R, V4, V6 en V7.

The accumulated voltages of the R in lead V6 and the S in V1 were used as measure for left ventricular hypertrophy. The voltages of R in V1 and the S in V6 were accumulated as measure for right ventricular hypertrophy. One senior cardiologist assessed all EKG's throughout the study.

*Echocardiography*

The patients underwent detailed echocardiographic examination with a Sonos 5500 ultrasound system (Philips Medical Systems). All examinations were performed by the same operator.

The following left ventricular parameters were measured from 2D-guided M-mode tracings according to recommendation of the American Society of Echocardiography: end-diastolic left ventricular internal cavity dimension (LVIDd), inter-ventricular septum thickness (IVSd) and left ventricular posterior wall thickness (LVPWd). Left ventricular mass was calculated by the Devereux modified cube formula and indexed by body surface area (left ventricular mass index (LVMI)). Left ventricular hypertrophy (LVH) was considered to be present if the left ventricular mass index was above 86.6 g/m$^2$ ($P_{97.5}$ for infants)[18]. Relative wall thickness (RWT) was calculated as [(IVSd +LVPWd)/LVIDd]. Left ventricular geometry was classified as normal if both LVMI and RWT were normal, as concentric hypertrophy if both LVMI and RWT were increased and as concentric remodelling if LVMI was normal and the RWT increased. RWT was defined as increased if > 0.45.

The following indices of overall systolic function were calculated: shortening fraction (SF), the ratio of the non-corrected left ventricular pre-ejection period to ejection time (LVPEP/LVET) and the ratio of the non-corrected right ventricular pre-ejection period to ejection time (RVPEP/RVET).

Diastolic filling was assessed by measuring trans-mitral (M) and tricuspid (T) E and A wave peak flow velocities and E/A ratio.

*Statistical analyses*

Non-linear, least squares regression, repeated measurement, ANOVA allowing for between and within patient variations, was used to compare the longitudinal pre-treatment measurements of LVPWd with untreated controls. After visual inspection curves were fitted for LVMI and LVPWd for each patient separately, using non-linear least squares regression.

## Results

Between January and March 1999 four patients with infantile Pompe's disease were enrolled in an open label study at ages of 2.5, 3, 7 and 8 months. They were treated with an intravenous dose of 15 or 20 mg/kg/week recombinant human alpha-glucosidase from rabbit milk for the first 12-23 weeks and thereafter with 40 mg/kg/week.

The enzyme activity in skeletal muscle was partially corrected at the lower dose and increased to normal values at the higher dose[16].

*Clinical condition*

Before start of treatment the cardiac condition was severely compromised in three of the four patients (patient 2, 3 and 4). They had a marginal peripheral circulation, limited exercise tolerance and showed extreme perspiration. Three patients received anti-diuretics; one patient in combination with digoxin and an ACE inhibitor. The liver size varied from 3 to 5 cm below the costal margin. The cardiac condition of patient 4 became extremely critical at the age of 11 months. This led to the decision to increase the weekly dose of alpha-glucosidase for all patients.

During enzyme therapy the clinical condition of the patients ameliorated gradually. The exercise tolerance improved and the peripheral circulation normalised. Diuretics could be reduced. Digoxin and the ACE inhibitor were successfully discontinued. Liver size became normal in all four patients.

*Radiography*

All patients had an increased Cor-Thorax (C-T) ratio on chest X-rays at start of treatment. In patient 4 cardiac enlargement led to a total atelectasis of the left lung. During treatment the atelectasis of the left lung disappeared. The C-T ratio's decreased.

*Electrocardiography*

At baseline PR-times were at the lower limit of normal (0.08 sec.). All patients had signs of a pronounced bi-ventricular hypertrophy. In patient 2, 3 and 4, T-inversion was noticed in leads I, II, III, aVR, aVF, V3R, V1, V4, V6 and V7 reflecting a disturbance of repolarisation.

During therapy the absolute PR-time normalised in 3 of the 4 patients (0.09 sec in patient 1 and 0.10 sec. in patients 2 and 4). Repolarisation disturbances disappeared in patient 4. A decrease in left ventricular hypertrophy was seen in all. Signs of right ventricular hypertrophy disappeared in patients 1, 2 and 4. In patient 1 also the left ventricular hypertrophy disappeared. The EKG of the latter patient became normal.

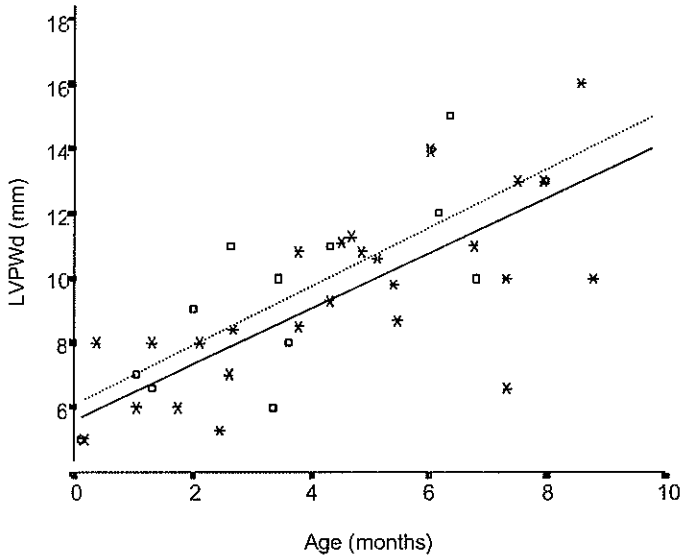


**Figure 1** : *LVPWd against age. Longitudinal pre-treatment values of patients (squares, —) in the trial compared with values obtained from other untreated patients with infantile Pompe's disease (*, ------).*

*Left Ventricular geometry*

Infantile patients have a progressive cardiac hypertrophy as shown by the significant increase of the LVPWd with age in untreated patients (Fig. 1).

Before start of enzyme therapy the increase of the LVPWd of the patients in study did not differ significantly from untreated patients, illustrating that the four patients in the trial were a representative sample (Fig. 1). At start of therapy the LVPWd, IVSd and LVMI were highly elevated (171, 203, 308 and 599 g/m$^2$ in patients 1, 2, 3 and 4, respectively, Table 1). Cardiac hypertrophy was concentric (RWT >0.45).

After start of enzyme therapy an exponential decrease of the LVMI and LVPWd was observed in all patients ($p<0.05$, Fig. 2 and 3). The most dramatic effect was seen in patient 4. In this patient the LVMI decreased to 16% of the value before start of treatment. In the other patients the LVMI decreased to 35, 50 and 75% of the onset value. In 3 of the 4 patients (patient 1, 3 and 4) the LVMI became within normal limits (below $P_{97.5}$). Concentric remodelling had occurred since the RWT was still > 0.45. After 3.5 years of treatment the LVPWd has normalised in one patient (patient 1). Outflow tract obstruction of the left ventricle, initially present in this patient, disappeared after 16 weeks of therapy.

**Table 1:** *echocardiographic parameters before therapy and after 3 ½ years of treatment*

| Patient | | Normal | 1 | | 2 | | 3 | | 4 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | before | after | before | after | before | after | before | after (w96) |
| Weight | kg | | 7.8 | 17,8 | 8.3 | 19.7 | 5.8 | 18.9 | 5.7 | 12,6 |
| BSA | $m^2$ | | 0.38 | 0.53 | 0.41 | 0.78 | 0.30 | 0.58 | 0.41 | 0.58 |
| LVMI | $g/m^2$ | $69\pm24$ [18,#] | 171 | 85 | 203 | 153 | 308 | 109 | 599 | 94,6 |
| LVPWd | mm | 6,5/6,3/6,2/5,3 [28*] | 10 | 6 | 10 | 10 | 11 | 10 | 16 | 7 |
| IVSd | mm | 7,4/7,5/7,4/6,5 [28*] | 9 | 10 | 12 | 14 | 14 | 10 | 18 | 8 |
| IVSd/LVPWd | | $\leq 1,5$ | 0,9 | 1,4 | 1,2 | 1,3 | 1,3 | 1,67 | 1,13 | 1,14 |
| RWT | | $\geq 0,45$ [19, 20, 22, 23] | 0,70 | 0,51 | 0,81 | 0,72 | 1,04 | 0,66 | 0,92 | 0,50 |
| LVPEP/LVET | | $(0.345\pm0.036)$ [29**] | 0,32 | 0,35 | 0,47 | 0,48 | 0,65 | 0,41(w168) | 0,72 | 0,42 (w84) |
| RVPEP/RVET | | $0,18\pm0,3$ [29**] | 0,53 | 0,25 | 0,47 | 0,31 | 0,27 | 0,5 | 0,36 | 0,26 |
| SF | % | $36\pm4$ [29**] | 55 | 45 | 41 | 37 | 25 | 46 | 12 | 39 |
| M E/A-ratio | | $(1.9\pm0.4)$ [30**] | 1,31 | 2,5 | 1,17 (w2) | 0,72 | 1,03 | 0,62 | 1,24 | 1,6 |
| M A-peak | m/sec | $0.49\pm0.08$ [30**] | 0.65 | 0.4 | 0.60 (w2) | 1,10 | 0.98 | 1.30 | 0.50 | 0.50 |
| M E-peak | m/sec | $0.91\pm0.11$ [30**] | 0.85 | 1.0 | 0.81 (W2) | 0.80 | 1.01 | 0.80 | 0.62 | 0.80 |
| T/m E/A-ratio | | 1.6 (0.6-2.6) [30,#,#] | 1,52 | 2,25 | 1,25 (w4) | 0,60 | 0,8 | 0,71 | 0,67 | 1,67 |
| T/m A-peak | m/sec | 0.4 (0.2-0.8) [30,#,#] | 0.50 | 0.40 | 0.40 (W4) | 1,00 | 0.74 | 0.70 | 0.30 | 0.30 |
| T/m E-peak | m/sec | 0.6(0.4-0.8) [30,#,#] | 0.76 | 0.90 | 0.50 (W4) | 0.60 | 0.59 | 0.50 | 0.20 | 0.50 |

[#] *Mean $\pm$ SD for the age of the patients at the end of follow up*

[*] *$P_{95}$ for the weight of the patients at the end of follow up*

[**] *Mean $\pm$ SD*

[,#,#] *Mean and range*

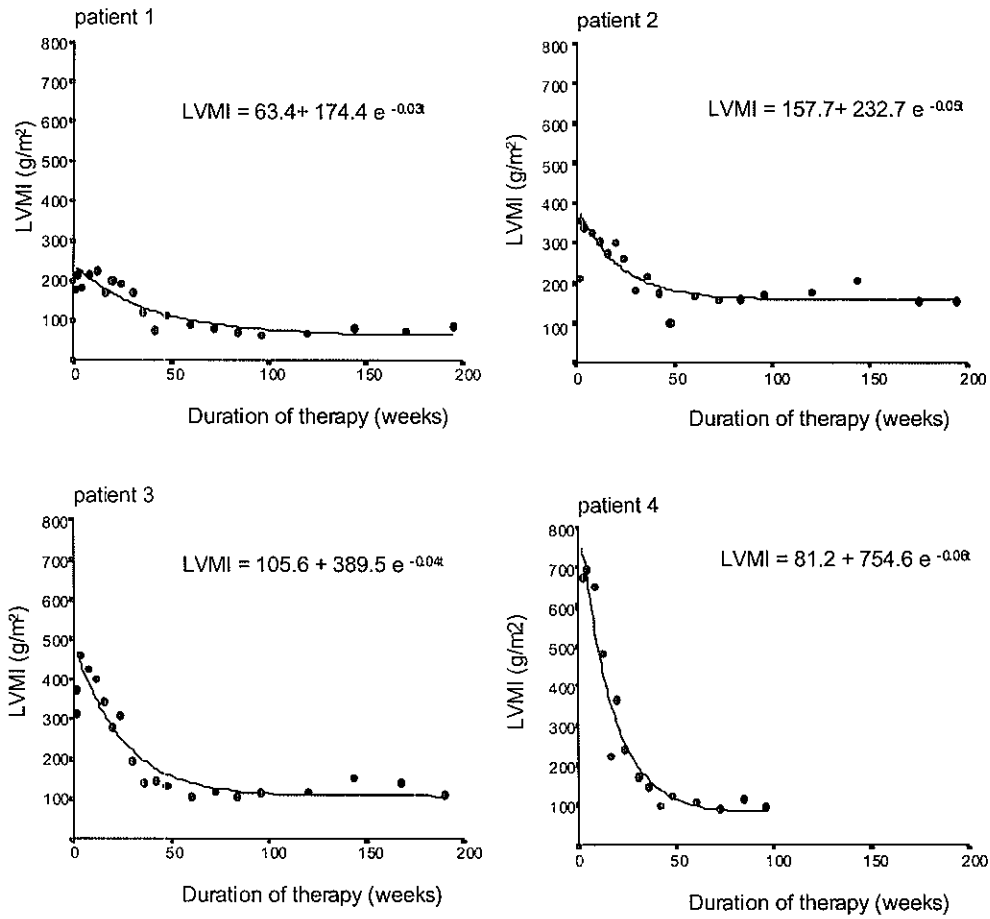


**Figure 2:** *Left Ventricular Mass Index (LVMI) during therapy. Formulas for fitted curves are included.*

*Systolic function*

Three of the 4 patients had a disturbed systolic function during the initial phase of treatment (table 1). Shortening fractions were decreased, whereas the LVPEP/LVET-ratio's were variably increased. During treatment the shortening fraction normalised in all. The LVPEP/LVET ratio remained elevated in one patient (patient 2).

*Diastolic function*

Diastolic function remained variably disturbed during treatment (Table 1).

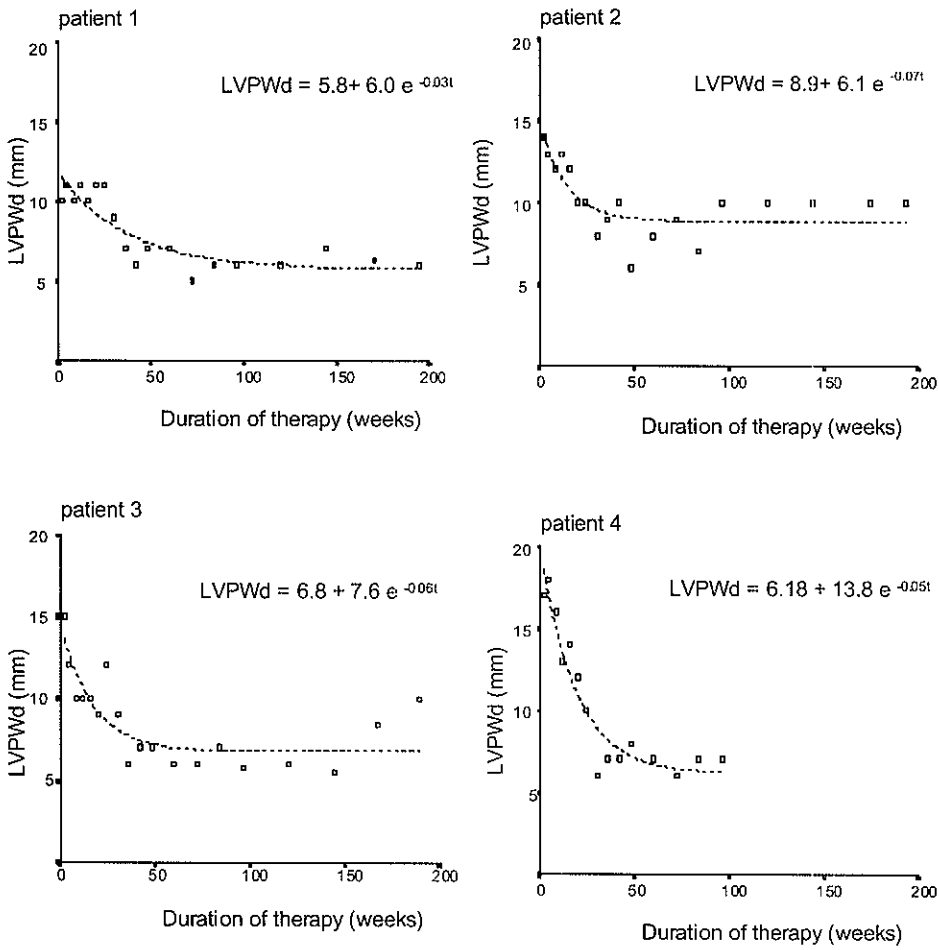


**Figure 3:** *Left Ventricular Posterior Wall (LVPWd) during therapy. Formulas for fitted curves are included.*

## Discussion

This study shows that the hypertrophic cardiomyopathy in Pompe's disease responds very well to enzyme replacement therapy. Cardiac hypertrophy in untreated Pompe patients is rapidly progressive as demonstrated by the significant increase of the LVPWd during the first months of life. The four patients in the study proved to be representative in this respect. The hypertrophy is much more pronounced than in other lysosomal storage disorders like Anderson-Fabry's disease (LVMI up to 599 g/m$^2$, $P_{97.5}$ 117 g/m$^2$). Despite this, we have shown that enzyme replacement therapy significantly improves ventricular geometry with a reduction of the LVMI up to 16% of pre-treatment levels.

The response on LVMI and LVPWd was not immediately seen after the first enzyme infusion. In the initial phase of treatment (4 weeks) the parameters still tended to increase. This may be explained in part by the fact that ventricular hypertrophy progresses rapidly and that baseline assessments were not performed immediately before but within a time frame of 2 weeks prior to the first administration.

Another explanation may be that the cardiac hypertrophy in Pompe's disease is not only caused by glycogen storage, but also by secondary changes which need time to respond. Intra-lysosomal glycogen storage in Pompe's disease only explains a negligible part of the increase in ventricular mass (1-10%)[3]. A similar finding was reported for the cardiac hypertrophy in Anderson Fabry's disease in which intra-lysosomal storage of globotriaosylceramide (GB3) causes < 1% of the increase in mass[19-22]. On the basis of these findings it was concluded that secondary phenomena play a role in the development of cardiac hypertrophy in lysosomal storage disorders leading to increase of muscular mass. The mechanism is not fully understood. For Fabry's disease it has been speculated[23] that the intra-lysosomal storage leads to disarray of myocardial fibres and that muscular hypertrophy occurs to overcome the structural disorganisation. Another mechanism proposed is that the intra-lysosomal deposits and cellular stress cause a trophic or immunological stimulus leading to proliferation of contractile elements and increase in myocardial cell volume. In later stages of disease hypertrophy may lead to fibre disarray and interstitial fibrosis.

The cardiac hypertrophy in Pompe's disease and Fabry's disease differ from the restrictive cardiomyopathy seen in amyloidosis. In the latter disorder the amyloid stores in the interstitium instead of intra-cellularly in lysosomes, which leads directly to fibrosis and hypertrophy. In amyloidosis diastolic dysfunction is present from the onset of disease and QRS complexes are normal. In the lysosomal storage disorders diastolic function is less impaired, while QRS complexes are large.

No matter what the mechanism may be, our study suggests that the secondary cardiac changes in Pompe's disease are reversible to a large extent. The LVMI became normal in 3 of 4 patients during the treatment period. Ventricular geometry changed from concentric ventricular hypertrophy to concentric ventricular remodelling.

The higher dose applied from week 12-24 after start of treatment may have attributed to a better response in the later stage of the study.

The improvement of cardiac geometry was accompanied by improvement of cardiac function and clinical condition. This was at least life saving for one patient. While cardiac failure is normally a major cause of death before the first year of life, patients now survived beyond 4 years of age and cardiac disease no longer played a role as life-threatening factor. Systolic function normalised in three of the four patients. Diastolic function remained mildly disturbed.

The effects on cardiac dimensions were reflected by changes of the EKG. A decrease of the left ventricular hypertrophy, calculated by accumulated voltages of the R in lead V6 and the S in V1, was found in all. The decrease over time was not significant indicating that cardiac ultrasound is more sensitive in monitoring effects of enzyme therapy on ventricular hypertrophy than the EKG. Other effects of enzyme therapy observed on EKG were those on repolarisation disturbances, right ventricular hypertrophy and PR interval. The latter effects were variable.

For both Pompe's disease and Anderson-Fabry's disease an abbreviated PR interval is one of the characteristic EKG findings reported, which has been attributed to progressive accumulation of storage material in cardiac tissue. The short PR time may lead to tachyarythmias and sudden death.

As in our study, Waldek et al.[24] recently reported an elongation of the PR time as an effect of 24 months of enzyme therapy with recombinant human alpha-galactosidase in a single patient with Fabry's disease. The finding was accompanied by an increase of the ejection fraction and a decrease of GB3 levels in the heart. Earlier Schiffmann and co-workers[25] had reported an effect of recombinant human alpha-galactosidase on the QRS-complex duration after 9 months of treatment. Eng et al.[26, 27] and Schiffmann et al.[25] both showed that the lysosomal storage product (Gb3) disappeared from vascular endothelium. In the initial phase of treatment this was not reported to be accompanied by an effect on cardiac geometry.

In our study the effect on LVMI became significant after 9 months of therapy[16]. Based on our results we expect that the cardiac hypertrophy in Fabry's and MPS I is also responsive to enzyme therapy.

The findings offer prospects for the treatment of cardiomyopathies in metabolic diseases.

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# CHAPTER 7

## Discussion

## Discussion

The 40-year history of investigations into the feasibility of enzyme replacement therapy for Pompe's disease is nearing its end. The chapters of this thesis give an overview of the historic developments and report the latest experiences leading to novel perspectives.
The core element of this thesis is a study on the safety and efficacy of recombinant human alpha-glucosidase (rhAGLU) in infantile Pompe's disease, an otherwise incurable and lethal lysosomal storage disorder. The study was innovative in two ways. It was the first regulator-approved study worldwide on the effect of enzyme replacement therapy for this disease. There above, it was the first application of a recombinant human medicine produced in the milk of transgenic rabbits. The elementary aspects of the study are discussed in the paragraphs of this chapter.

### 1. Design of the study

*1.1 The study population*

A Phase II study requires a thorough evaluation of the balance between benefit and risk for the patients. As no study had ever tested the safety and efficacy of therapeutic proteins from the milk of transgenic rabbits there was no reference point other than the outcome of preclinical studies in mice, toxicology studies in rat, and Phase I safety studies in healthy volunteers. Although the recombinant human alpha-glucosidase from rabbit milk (rhAGLU) had proven safe in the Phase I study, the risk of prolonged treatment of severely affected patients with Pompe's disease was unknown. Immunologic reactions, possibly leading to anaphylaxis could occur, especially in infants without natural (endogenous) alpha-glucosidase expression (so called, CRIM-negative patients). Furthermore, the results of treatment were largely unpredictable and could range from an unchanged course of disease to prolonged survival with severe handicap or full recovery. As patients with infantile Pompe's disease generally die within the first year of life, the potential benefit of treatment is high for this group of patients. Moreover, the infantile phenotype is rather homogeneous which facilitates the evaluation of clinical endpoints. On the other hand, we were concerned that infantile patients could develop progressive glycogen storage in the central nervous system (CNS), since the CNS is inaccessible for the drug due to the blood brain barrier. A literature search did not provide evidence for symptoms related to CNS damage in Pompe's disease, despite observations of lysosomal storage in various CNS cell types.
Patients with late-onset Pompe's disease have in theory a better chance to react positive to enzyme replacement than those with the infantile subtype. CNS involvement is definitely no issue. The deficiency of alpha-glucosidase is not complete and therefore easier to correct, the symptoms are milder, and the disease progresses more slowly. But, apart from therapeutic advantages there are disadvantages to start a Phase II study with patients suffering from late-onset disease. The group of patients is far more heterogeneous by age and degree of symptomatology. The rate of disease progression is slow. All this makes it difficult to set clinical endpoints for late-onset patients. Most of all, compared to infantile Pompe's disease, there is a

much higher risk to shorten rather than prolong the natural lifespan of patients with late-onset disease in case the treatment would prove unsafe and ineffective.
Carefully weighing the pros and cons, it was decided to start with a two-armed pilot study in four infants and three late-onset patients. In this thesis the infantile data are described.

*1.2 Study design, inclusion criteria, and endpoints*

As we found it unethical to administer weekly placebo infusions to patients with an early lethal disease, we choose for an open label design. In such a study two factors are of great importance. First, it is necessary to have thorough knowledge of the natural course in order to set endpoints and appreciate the effects of therapy. Second, to fully judge the effects care has to be taken to only include patients with the classic infantile form of Pompe's disease and to exclude clinical variants with a better prognosis beforehand. The survey of 20 Dutch patients and 133 cases from the literature was very helpful to establish the key elements defining the natural course of Pompe's disease in infants[1]. They present their first symptoms at a median age of 1.6 months and generally die before the first year of life. They typically have a progressive cardiac hypertrophy. Motor development is severely delayed as important motor milestones are not reached or lost shortly after acquisition. All patients have a virtually complete deficiency of alpha-glucosidase and fully deleterious mutations in each of the two alpha-glucosidase alleles.
The findings were taken into account for the formulation of the inclusion and exclusion criteria. Patients had to be younger than 10 months, they had to have symptoms characteristic of infantile Pompe's disease including a hypertrophic cardiomyopathy and a severe deficiency of alpha-glucosidase activity, as measured in an open muscle biopsy. The protocol did not include mutation analysis to verify the clinical and biochemical findings, but DNA analysis was carried out in second instance as data support. Guided by the conclusions of the survey, one-year survival after start of therapy was taken as the primary endpoint. A decrease of the LVPWd and LVMI and the achievement of developmental milestones were chosen as secondary endpoints. The increase of alpha-glucosidase activity in muscle and the improvement of muscle morphology were taken as additional indicators of effective treatment.

## 2. Effects of rhAGLU from rabbit milk

The stimulating and very important first finding was the safety of recombinant human alpha-glucosidase. None of the patients experienced serious reactions during the first five to seven weeks of treatment, despite the relatively high dose of 15 or 20 mg/kg. In the following weeks infusion reactions were recorded, and the patients were given anti-allergic drugs to suppress the effects. The mechanistic cause of the reactions remained uncertain, but the most likely explanation is IgG mediated complement activation. Reactions subdued several weeks later upon application of an adapted infusion scheme starting with low infusion rates. The pre-treatment schedule with anti-allergic drugs was no longer applied. By now, over eight hundred infusions have been administered without serious problems. This experience by itself shows

that a safe medicine can be produced in the milk of transgenic animals and opens the way to further explore the usefulness of this production platform for a whole series of protein drugs.
The question of therapeutic efficacy was equally crucial. The evidence for delivery of alpha-glucosidase to the muscle fibres is based on a combination of findings. The alpha-glucosidase activity in the biopsy specimens increased and later normalised at a dose of 40 mg/kg/wk per patient. Alpha-glucosidase species of 76 and 70 kDa were present in the muscle. They originate by lysosomal processing from the 110 kDa alpha-glucosidase precursor that was administered intravenously. Along with these findings, the muscle morphology of one patient improved substantially. The biopsies of the other patients revealed clearance of glycogen without long-term improvement of muscle morphology.
The clinical condition improved as well. The primary endpoint of one-year survival was reached without problems. All patients passed the age of four, whereas the natural course indicates a median survival of 7.7 months in the Dutch population and 6 months in the worldwide patient population. Although 8% of all reported patients with proclaimed infantile Pompe's disease survived for more than a year, only 2 of the 133 patients became older than 1.5 year and none passed the age of 3.
Taking the natural course as comparative standard, the cardiac parameters improved significantly as a result of the treatment. Instead of a progressive increase, the patients manifested a decrease of the LVPWd and LVMI. The improvement of cardiac anatomy was accompanied by clinical improvement. The exercise tolerance increased likewise.
Improvement of muscle function, taken as additional indicator of clinical efficacy, was also observed. Two of the patients achieved motor milestones that are not reached in the natural course.

### 3. Comparing two studies

Only limited information is available from other studies on enzyme replacement therapy with recombinant forms of human alpha-glucosidase. As a matter of fact, there is only one other peer-reviewed publication[2]. It concerns a 14-17 months long study in three infants who received recombinant human alpha-glucosidase from CHO cells.
The CHO enzyme is very similar to that from rabbit milk, but some structural differences were noted in pre-clinical studies with respect to the mannose 6-phosphate content of the carbohydrate chains. The difference comes to light when the enzymes are fed to cultured fibroblasts. The CHO form of recombinant human alpha-glucosidase is taken up more efficiently -in a mannose 6-phosphate dependent manner- than the form from rabbit milk. In vivo, however, both forms of alpha-glucosidase are taken up in almost equal quantity by heart and skeletal muscle of alpha-glucosidase deficient mice[3].
The three patients who were treated with enzyme from CHO cells initially received two doses of 5 mg/kg weekly. During the first 5 weeks muscle function improved in all patients. In one patient the response to treatment persisted. In the two other patients, however, the muscle function deteriorated after 7 to 13 weeks of treatment and the pulmonary function declined. Both patients became ventilator dependent after 5 months of treatment when they were 8

months old. As the decline of the response to treatment in these two patients was attributed to the formation of neutralising antibodies, the initial dose of 2 times 5 mg/kg per week was substantially increased. One patient received as much as 5 times 10 mg/kg per week. This intervention did not improve the condition of the patient but resulted in a nephrotic syndrome (Dr Y.T. Chen, personal communication).

The LVMI of two patients in the study with CHO enzyme decreased to approximately 65% of the pre-treatment value compared to 16% to 75% in our study. The third patient did not have a pronounced cardiac hypertrophy at start of treatment (the LVMI was 64 g/m$^2$ whereas the $P_{97.5}$ used by the author is 64.8 g/m$^2$), and did not develop this symptom either during the treatment. The results of both studies seem comparable but a proper judgement of the dose-response cannot be made due to the variable doses that were applied in the course of treatment. Notably, the patient without cardiac hypertrophy would not have met our inclusion criteria as this symptom is a key feature of infantile Pompe's disease. As it stands any conclusion about the effectiveness of alpha-glucosidase from CHO cells compared to rabbit milk is premature.

## 4. Experiences and questions

*4.1 Variable efficacy*

From the presently published data on the 7 infants participating in studies with alpha-glucosidase from rabbit milk or CHO cells it can be concluded that enzyme replacement therapy is safe and effective. However, the effects vary considerably between patients. The CHO cell study suggests that the variation is due to the development of neutralising antibodies in patients who, by the nature of their mutations, do not produce endogenous alpha-glucosidase. This explanation does certainly not hold for our study with alpha-glucosidase from rabbit milk. One of the two patients who reached important developmental milestones was homozygous for the delT525 mutation. The effect of this mutation was thoroughly investigated. It destabilises the messenger and prevents the formation of alpha-glucosidase completely. Besides, it is known from studies in other lysosomal storage diseases -Gaucher's disease, Fabry's disease and MPS I - that the formation of antibodies against the administered enzyme does not necessarily interfere with the efficacy of enzyme replacement therapy[4-8]. Further investigation of the relevance of antibody formation in relation to the genotype of the patients and the therapeutic outcome is advised.

A common finding in both studies is that the therapeutic effect is to a great extent determined by the condition of the patient at start of treatment. The patients who learned to walk had a relatively mild muscle weakness at start of treatment compared to the other patients who did not acquire this ability. A model in which the lysosomal glycogen storage can only be corrected in intact and functional muscle fibres provides a simple explanation. But, the actual mechanism is probably more complicated and likely involves clinical condition related differences in a complexity of factors acting in the processes of muscle damage and repair.

Independent of the mechanism, we have learned that the therapeutic window is small in the infantile form of Pompe's disease. As a consequence, physicians must be trained to be aware of the disease and the existing diagnostic tests in order to reach a rapid diagnosis and a timely decision about the start of treatment.

*4.2. What is the minimal effective dose?*

We noticed significant effects at a dose of 40 mg/kg per week. However, various degrees of residual disease remained. The two patients who had a very bad condition at start of treatment both developed respiratory insufficiency, one even before the first enzyme infusion, and the other after 10 weeks of low-dose treatment during a bout of pneumonia. Treatment with a higher dose probably would not have prevented the pulmonary problems. Both patients gained muscle strength in their arms, but not in their legs despite the fact that the alpha-glucosidase activities in the patient's muscle biopsies were normal.

The third patient had a moderate response to the dose of 20 mg/kg. After the dose elevation to 40 mg/kg once weekly, she could sit without support (at 19 months), and did not need respiratory assistance till the age of two. We believe that the dose increase in this patient was a prerequisite for her improvement. The fourth and best patient responded very well to the lower dose, but he too was transferred to the higher dose in order to keep the four patients in one protocol. He can walk and climb stairs, but his slightly waddling gait, hearing deficit and facial features are signs of residual disease and contra-indications for treatment with a dose lower than 40 mg/kg each week.

The dosing of 2 times 5 mg/kg per week in the CHO study was reported to be sufficient to improve the cardiac dimensions and the cardiac function of the two patients who had cardiac hypertrophy at the start of treatment. But, in practice a higher dose was given over a substantial period of time to counteract the immunologic response (Dr Y.T. Chen, personal communication). Respiratory insufficiency could not be prevented. The third patient was in good clinical condition at the start of treatment and did not show deterioration of functions during treatment. This is remarkable for a patient with infantile Pompe's disease. However, as mentioned above, further investigations are desired to proof that the patient has classic infantile Pompe's disease.

When compiling the data from both studies, the preferred dose for infants with a good clinical condition lies between 10 and 40 mg/kg per week, whereas the dose for severely affected patients must be 40 mg/kg per week. By the paucity of published data, it is at present impossible to make a comparison between the effectiveness of recombinant alpha-glucosidase from the one source versus the other. Due to the rapidly progressive nature of the disease and the difficulty to reverse the muscle pathology it is of utmost importance that patients are treated with an adequate dose from the beginning. When the initial dose is too low, valuable time is lost and irreversible muscle damage may occur.

*4.3 What is the best dosing regimen?*

The dosing regimen is dictated by the level of activity reached in the target organs after a single dose, and the tissue half-life of the drug. In vitro studies show that the half-life of the enzyme in cultured muscle cells is about 9 days[9], and studies in mice reveal a dose dependent uptake of enzyme by muscle in a dose range of 4-80 mg/kg. Based on these figures one can calculate, that it is certainly better to administer once 10 mg/kg per week than 2 times 5 mg/kg per week. At higher enzyme doses the dosing regimen is less critical, and double the dose every second week may elicit the same effect as a single dose every week. But, as it stands, much is still unknown about the real optimal dose and the optimal dosing regimen. The rate of infusion is of additional importance as it affects the steady state concentration of the therapeutic enzyme in the blood. In theory, uptake by heart and muscle is favoured after saturation of the receptor mediated endocytic pathway of the liver and the spleen. Much has still to be learned by clinical practice as ultimate measure for therapeutic efficacy.

*4.4 Residual disease*

Unfortunately, the treatment with recombinant human alpha-glucosidase from rabbit milk could not prevent all handicaps. This holds most for those patients who had a substantial loss of muscle function before the start of treatment. All four patients gained muscle strength at a dose of 40 mg/kg, but the increase of muscle strength stalled in three of the four patients after more than a year of treatment. They became ventilator dependent. From this experience we have learned that extensive muscle damage can be prevented but not cured by enzyme replacement therapy in infantile Pompe's disease. In future studies of enzyme replacement therapy investigators have to be aware that these problems can occur when severely affected infants are included. Milder signs of residual disease were also noted, like speech problems, emerging contractures and repeated airway infections. In addition, all four patients had a hearing deficit before they entered our study, and the hearing problem remained as residual disease by lack of response. The cause of hearing loss is currently under investigation. The problem doesn't seem related to CNS damage, as Brain Stem Audiometric Evoked Potential measurements show normal inter-peak conduction times. We haven't seen other signs either pointing to CNS involvement. The patients treated in the study seem to have a normal mental development, as they interact in a very social manner, have learned to speak either verbally or in sign language, and now attend pre-school if the circumstances allow. However CNS involvement might still occur and asks for specific attention, as the administered enzyme is too large to cross the blood-brain barrier. Our study shows that residual disease is hard to prevent, despite the significant effect of enzyme replacement therapy in infantile Pompe's disease. Therefore, a multidisciplinary approach is required for the treatment of patients with Pompe's disease.

## 5. How to design future studies?

The best response to treatment was seen in an infant who was diagnosed 2 weeks after birth and treated before 3 months of age, at a time that his muscle function was not yet severely impaired. The rapidly progressive course of infantile Pompe's disease limits the time to intervene. The slower course of late onset Pompe's disease leaves a larger therapeutic window. This was very evident from the experience with three patients with late-onset disease who also received enzyme replacement therapy in our hospital. Their condition stabilised or improved, and the youngest, least affected, patient walked after 2 years of treatment whereas he was wheelchair dependent when he entered the study. All patients reported less fatigue and more energy.

Therefore, future studies should be directed towards two groups of patients, those with delayed-onset disease and partial loss of skeletal or pulmonary function, and those with a very early stage of infantile Pompe's disease. In retrospect, it was not the best choice to take 10 months as maximum age of inclusion. Infants following the natural course of infantile Pompe's disease have at that age severe tissue pathology. As the symptoms manifest at a median age of 1.6 months, when tissue damage has already occurred, patients with the classic infantile form of Pompe's disease have to be included ideally before the age of two months and have to be treated with an immediate adequate dose. As mentioned above, we advise to perform a detailed molecular analysis to help distinguish patients with the classic form of Pompe's disease from those with a variant phenotype.

With regard to monitoring the efficacy of treatment, changes in the LVPWd and LVMI are sensitive parameters. However, these parameters do not measure the condition of the patient directly and therefore are surrogate markers.

A motor function test reflects the condition of the patient more directly. However we could not find a validated, evaluative motor test for patients under one year of age. In practice the Bayley Scales for Infant Development (BSID II[10]) and the Alberta Infant Motor Scales (AIMS[11]) are used as evaluative tools. However both were originally developed with a discriminative purpose and were not validated for evaluative use. Due to the lack of validated tests we decided to include the BSID II and AIMS in our protocol. We found that both tests adequately measure large changes such as the achievement of milestones, but we experienced that they are less useful for the measurement of subtle changes in the muscle function of patients with severe hypotony. An extra shortcoming of the AIMS is that it can only follow motor function in children until the age of 18 months. Currently the Gross Motor Function Measurement[12-14], a validated evaluative motor test designed for children older than 4 years, is adapted both for children under the age of five and for patients with Down's syndrome[15-17]. Possibly these ongoing developments will lead to a validated, evaluative test for hypotonic infants and children.

Other problems came to light when we used the mental scale of the BSID II. The hearing problem of the patients interfered with the questioning, and the hypotony prevented the patients to achieve the assignments.

## 6. Perspectives for the production of recombinant human enzyme

This thesis describes the first successful application of a recombinant human protein produced in the milk of transgenic animals. The currently used transgenic rabbit production line supplies approximately 10 gram of recombinant human enzyme per animal per year whereas 25-100 grams of enzyme are needed to treat a single patient (average weight 50 kg) year-long with a dose of 10-40 mg/kg per week. Given these figures, production of rabbit milk enzyme is feasible in the initial phase of product development, but falls short to supply the worldwide group of around 5000 patients. Weighing the costs and risks of introducing a brand new medicine-production technology, the industrial producer of recombinant human alpha-glucosidase decided to opt for further development of enzyme replacement therapy with the enzyme from CHO cells and stopped production of the transgenic enzyme. There are concerns, however, that the worldwide production capacity in CHO cells is rapidly used up by the growing line of new products[18, 19]. Moreover, it is believed that the transgenic production technology is both technically and economically better suited for production above the100 kg per year scale than the CHO-cell production systems. As production of recombinant human alpha-glucosidase in both rabbit milk as well as CHO-cells is costly and troublesome because of the high dose required to elicit therapeutic effects, we advertise production in the milk of cow, goat or sheep as attractive alternatives.

## 7. Protection of patients in Phase II trials

The position of patients in medical studies is very vulnerable, more so if these patients are minors. To protect patients the international guideline for good clinical practice[20] and the law on research with humans (Wet medisch-wetenschappelijk onderzoek bij mensen, WMO, 1998)[21] were instituted. The guideline for Good Clinical Practice is an international ethical and scientific standard of quality for the design, performance and evaluation of clinical research in which human subjects are involved. It gives a public guarantee that the rights, safety and well being of the subjects are protected in accordance with the principles of the declaration of Helsinki and that the data derived from the clinical study are reliable. In the Netherlands the law on medical-scientific research with humans (WMO) gives the guidelines on good clinical practice a juridical basis. The WMO prohibits research with humans if it does not lead to new insights in medicine or if answers can be obtained by other methods; furthermore benefits from participating in a study have to outweigh the risks. In all cases a study is only approved if the patient - in case the patient is older than 12 - and the parents - in case the patient is younger than 18 - are carefully informed in writing about the purpose and risks of the study and give their informed consent. The manner in which the above is made operational should be laid down in a protocol. The protocol should be approved by a medical ethical review board. Specific attention is given in the law (WMO) to research with minors. Research in minors is not allowed, unless the subject may benefit from the study or unless the study can only be performed in this age group and risks are low. The study has to be stopped when the patient resists against participation. At any time parents are allowed to withdraw their child from the study without reason. Therapeutic

studies can be reviewed by the local institutional review board. A Phase II study is not considered to be therapeutic unless it is reasonable to assume that the patient will have clinical benefit form participation in the study. Studies without demonstrable profit to the patient have to be approved by the central commission on human research (CCMO) in The Hague. All studies in humans are reported to the CCMO. Local Institutional Review Boards provide the CCMO with an annual report. If physicians do not comply with the law on medical-scientific research in humans (WMO) they can be put into custody.

However can the guideline for good clinical practice and the law on medical-scientific research in human, as outlined above, properly protect patients in Phase II trials in practice? At start of the study we anticipated two scenarios. The new enzyme replacement therapy with recombinant human alpha-glucosidase from rabbit milk would either be effective and become permanently available after registration, or it would appear ineffective leading to an early discontinuation of the study. However, a third situation occurred. The treatment was effective, but the sponsor formed a strategic alliance with a third party. The newly formed joint-venture decided to discontinue production of the medicine under study, to continue the production in CHO cells, and to lower the dose. Business decisions were made, while the academic world was still analysing the outcome of the clinical studies. The decisions were not reviewed by an independent committee, and if so, this committee would not have had any sanction to oblige the pharmaceutical industries to continue with the parallel development of the two products until proof of comparability. The law on medical-scientific research in humans (WMO) does not foresee in this situation and does not give any protection.

We have experienced that parents and patients learn to cope with the course of the disease, but that it is very difficult for them to cope with uncertainties about drug supply once the drug has proven to be effective.

Can decisions about the patient's welfare be left to the pharmaceutical industries, or should situations like this be assessed by unbiased commissions such as the CCMO? Parties should then be held by law to comply with the committee's decision. But looking from the other side, will the pharmaceutical industry be interested to develop a drug if they can be obliged to continue the production of an effective, but non-profitable drug.

I conclude that patients in Phase II studies are not sufficiently protected when situations occur as depicted above. The issue is difficult and deserves more attention.

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# *CHAPTER 8*

## Summary

**Summary**

Pompe's disease is a progressive myopathy caused by the deficiency of acid alpha-glucosidase. This enzyme deficiency leads to lysosomal glycogen storage and results eventually in the destruction of muscle tissue and loss of muscle function. Partial enzyme deficiency gives rise to a slowly progressive phenotype involving mainly skeletal muscle. Complete enzyme deficiency leads to the very severe and rapidly progressive, infantile form of Pompe's disease characterised by generalised muscle weakness and cardiomegaly. The symptoms present within the first 3 months of life. Loss of muscle strength prevents infants from ever achieving developmental milestones like sitting, standing and walking. Cardiac failure and respiratory insufficiency eventually lead to death, typically in the first year of life.

The studies described in this thesis are focused on the development of enzyme replacement therapy for this otherwise incurable disease.

As Pompe's disease is caused by a lysosomal enzyme deficiency, it was challenging to investigate the feasibility of enzyme replacement therapy. The principle of treatment is based on the capacity of lysosomes to engulf exogenous proteins via endocytosis. Since 1964 several attempts were made to treat patients with alpha-glucosidases derived from Aspergillus niger and human placenta. It is presently understood that these early attempts failed by the administration of too low doses of impure and immunogenic enzyme preparations. Once the human alpha-glucosidase gene was cloned two methods were developed for the large-scale production of recombinant human alpha-glucosidase (rhAGLU). One employs genetically engineered Chinese Hamster Ovarian Cells (CHO-cells), the other transgenic animals that produce the therapeutic enzyme in their milk. The latter method was used to obtain the recombinant human alpha-glucosidase for performing the clinical studies described in this thesis.

The first chapter serves as a general introduction into the field of Pompe's disease and reviews the historic development of enzyme replacement therapy. Chapters 2-6 present the results of research into the natural course of Pompe's disease and the outcome of a clinical study in 4 patients with infantile Pompe's disease who were treated for 3 ½ year with recombinant human alpha-glucosidase from rabbit milk. The work is evaluated and discussed in chapter 7.

The effect of enzyme replacement therapy cannot be appreciated without the precise knowledge of the natural course of Pompe's disease. Only two reviews dealing with the natural course of infantile Pompe's disease were published over the past 50 years. Chapter 2 presents the results of a study comprising 20 cases of infantile Pompe's disease seen by Dutch collaborative centres and an additional 133 cases from the literature. We identified five key elements defining the infantile subtype of Pompe's disease. i) Patients with infantile Pompe's disease typically die in the first year of life. ii) They have a rapidly progressive cardiac hypertrophy. iii) Their motor development is severely delayed and important motor milestones like turning, sitting and standing are not achieved or lost shortly after acquisition. iv) They gain significantly less weight than unaffected babies, and v) the levels of ASAT, ALAT, LDH, CK and CK-MB are elevated.

In addition acid alpha-glucosidase is severely deficient and patients have fully deleterious mutations.

Based on the key elements, we decided to take 'survival', 'reduction of the diastolic thickness of the left ventricular posterior wall and left ventricular mass index' and 'achievement of major motor milestones' as endpoints for a study on the safety and efficacy of enzyme replacement therapy in infantile Pompe's disease. A single-centre, open-label, study was started in the beginning of 1999 with four infants. It was originally planned to last for six months, but was extended several times with a six-month period till it was ended in 2001 and patients continued treatment under a compassionate use protocol. The results of this study are described in the chapters 3, 4, 5 and 6. The patients were treated weekly with recombinant human alpha-glucosidase (rhAGLU) from rabbit milk. The enzyme preparation was administered intravenously starting with doses of 15 mg/kg or 20 mg/kg, which was increased to 40 mg/kg after 3 to 5 months. The treatment was tolerated well, and the alpha-glucosidase activity in muscle biopsies normalised in the course of treatment. Targeting to lysosomes was evidenced by the conversion of the administered 110 kDa precursor to the mature 76/70 kDa lysosomal form of alpha-glucosidase and the clearance of glycogen filled vacuoles in muscle with concomitant improvement of muscle morphology in the best performing patient. All patients responded in terms of clinical improvement. They all reached the age of four, whereas the average natural life span in infantile Pompe's disease is less than one year. Two patients achieved major motor milestones like sitting and standing. The cardiac hypertrophy of all patients diminished as one of the first signs of therapeutic effect. Chapter 6 focuses particularly on the changing cardiac parameters. The infants had a concentric hypertrophy at start of treatment as indicated by the increased left ventricular posterior wall diameter (LVPWd) and left ventricular mass index (LVMI). The hypertrophy cannot be caused by glycogen storage alone as the glycogen content of the heart of patients with Pompe's disease is rarely increased by more than 10% of the wet weight. Therefore, we hypothesise that the hypertrophy arises by either disarray of the contractile elements or by a trophic reaction to the lysosomal glycogen storage. The systolic left ventricular function was seriously impaired and the diastolic function mildly disturbed, before the start of therapy. During therapy, the concentric hypertrophy diminished as the LVMI and the LVPWd decreased in all patients. The function of the heart improved and was considered to be lifesaving for at least one patient. The early and good response to therapy shows that the heart is particularly amenable to enzyme replacement therapy. This might be related to the fact that low residual activity is already sufficient to prevent cardiac damage and to the fact that the heart -compared to the muscle- receives the therapeutic enzyme effectively via the coronary system.

Despite the clear beneficial effects that we have seen in infants receiving enzyme replacement therapy, we have also encountered the limitations of the treatment in that it is difficult and not always possible to prevent residual disease. One of the four patients performs extremely well taking into account the devastating course of the untreated disease, but three of the four patients required at some point in the study artificial ventilation.

The studies do not give a clear answer on the question why some patients respond better than others, but indicate that both the severity of symptoms and age are predictive parameters for the efficacy of the treatment. It will be the future challenge of a team of specialists to evaluate the condition of affected infants, based on clinical, biochemical and genetic parameters in order to predict the natural course of the disease and to estimate the chances for successful treatment.

The studies described in this thesis demonstrate both the safety and efficacy of enzyme replacement therapy for Pompe's disease. In addition, they show for the first time the feasibility of producing medicines in the milk of transgenic animals. The results of these studies strongly encourage continuation of the current developments towards enzyme replacement therapy for Pompe's disease in its several clinical subtypes. Because of the high dosing requirement, they are a stimulus to fully explore the practicality of super-scale production of therapeutic proteins in the milk of transgenic animals, which is of utmost importance in an era that the capacity of more conventional production platforms for recombinant human proteins falls short.

## Samenvatting

De ziekte van Pompe is een progressieve myopathie veroorzaakt door een tekort aan het enzym zure alpha-glucosidase. Dit tekort leidt tot lysosomale stapeling van glycogeen en resulteert uiteindelijk in destructie van spierweefsel en verlies van spierfunctie. Een gedeeltelijk tekort aan dit enzym veroorzaakt een langzaam progressief fenotype, waarbij vooral de skeletspieren zijn betrokken. Een volledig tekort aan het enzym leidt tot de zeer ernstige en snel progressieve, infantiele vorm van de ziekte van Pompe, die wordt gekarakteriseerd door algemene spierzwakte en cardiomegalie. De symptomen presenteren zich binnen de eerste 3 maanden van het leven. Verlies van spierkracht belet de kinderen ooit belangrijke mijlpalen in de ontwikkeling, zoals zitten, staan en lopen, te bereiken. Hart falen en respiratoire insufficiëntie leiden uiteindelijk tot de dood, meest typisch in het eerste jaar van het leven.

De studies beschreven in dit proefschrift zijn gericht op de ontwikkeling van enzym vervangingstherapie voor deze anderszins ongeneeslijke ziekte.

Omdat de ziekte van Pompe wordt veroorzaakt door een lysosomale enzym deficiëntie was het een uitdaging om de haalbaarheid van enzym vervangingstherapie te onderzoeken Het principe van de behandeling is gebaseerd op de capaciteit van de lysosomen om exogene eiwitten op te nemen via endocytose. Sinds 1964 werden verschillende pogingen gedaan patiënten te behandelen met alpha-glucosidase verkregen uit Aspergillus niger en menselijke placenta.

Deze vroege pogingen faalden waarschijnlijk door de toediening van een te lage dosering of het gebruik van onreine en immunogene enzym preparaties. Na de klonering van het menselijk alpha-glucosidase gen, werden twee methodes ontwikkeld voor de productie van recombinant humaan alpha-glucosidase (rhAGLU) op grote schaal. De ene methode gebruikt genetisch gemanipuleerde ovarium cellen van de Chinese hamster (CHO-cellen), de andere transgene dieren die het therapeutische enzym produceren in hun melk. Deze laatste methode werd gebruikt om het recombinant humaan alpha-glucosidase te produceren voor de klinische studies beschreven in dit proefschrift.

Het eerste hoofdstuk dient als een algemene introductie over de ziekte van Pompe en geeft een overzicht over de historische ontwikkeling van de enzym vervangingstherapie. In hoofdstukken 2 tot 6 worden de resultaten gepresenteerd van een onderzoek naar het natuurlijk beloop van de ziekte van Pompe en de uitkomst van een klinische studie bij 4 patiënten met de infantiele vorm van de ziekte van Pompe die gedurende 3 ½ jaar werden behandeld met recombinant humaan alpha-glucosidase uit konijnenmelk. Het werk wordt geëvalueerd en bediscussieerd in hoofdstuk 7.

Het effect van enzym vervangingstherapie kan niet naar waarde worden geschat zonder nauwkeurige kennis van het natuurlijk beloop van de ziekte van Pompe. In de afgelopen 50 jaar werden slechts twee overzichten over het natuurlijk beloop van de ziekte van Pompe gepubliceerd. Hoofdstuk 2 presenteert de resultaten van een studie bij 20 casussen van de infantiele vorm van de ziekte van Pompe, gezien in de Nederlandse samenwerkende centra, en 133 casussen uit de literatuur. Wij identificeerden vijf karakteristieken van de infantiele vorm van de ziekte van Pompe. i) Patiënten met de infantiele vorm van de ziekte van Pompe overlijden typisch in het eerste jaar van het leven ii) Ze hebben een snel progressieve

hypertrofie van het hart. iii) Hun motorische ontwikkeling is ernstig vertraagd en belangrijke motorisch mijlpalen als draaien, zitten en staan worden nooit bereikt of worden verloren snel na het bereiken ervan. iv) Hun gewichtsgroei loopt significant achter ten opzichte van gezonde baby's en v) ASAT, ALAT, LDH, CK en CK-MB zijn verhoogd. Tevens is er een ernstige deficiëntie van alpha-glucosidase en hebben patiënten volledig deletatoire mutaties.

Naar aanleiding van deze karakteristieken, besloten we om 'survival', 'reductie van de diastolische dikte van de linker achterwand van het hart en de linker ventriculaire massa index' en 'het bereiken van belangrijke motorische mijlpalen' te kiezen als eindpunten voor een studie naar de veiligheid en het effect van enzym vervangingstherapie bij de infantiele ziekte van Pompe. In het begin van 1999 startten wij een open-label, single-centre studie bij 4 zuigelingen. Oorspronkelijk was gepland dat deze 6 maanden zou duren, echter de studie werd verschillende keren verlengd met een periode van 6 maanden tot deze werd beëindigd in 2001 en de behandeling werd gecontinueerd op basis van 'compassionate use'. De resultaten van de studie worden beschreven in de hoofdstukken 3, 4, 5 en 6. De patiënten werden wekelijks behandeld met recombinant humaan alpha-glucosidase uit konijnen melk. De enzym preparatie werd intraveneus toegediend, waarbij gestart werd met een dosis van 15 mg/kg of 20 mg/kg, welke later werd verhoogd tot 40 mg/kg na 3 tot 5 maanden. De behandeling werd goed verdragen en de alpha-glucosidase activiteit in de spier biopsie werd normaal. Lysosomale opname werd bewezen door de omzetting van de toegediende 110 kDa precursor naar de mature 76/70 kDa lysosomale vorm van alpha-glucosidase en het verdwijnen van de met glycogeen gevulde vacuoles in spier met gelijktijdige verbetering van de morfologie van de spier bij de beste patiënt

Alle patiënten toonden een klinische verbetering. Zij bereikten allen de leeftijd van 4 jaar, terwijl de gemiddelde levensverwachting bij de infantiele vorm van de ziekte van Pompe minder dan 1 jaar is. Twee patiënten bereikten belangrijke motorische mijlpalen als zitten en staan. De hypertrofie van het hart van alle patiënten verminderde als een van de eerste tekenen van effect van therapie.

Hoofdstuk 6 richt zich met name op de veranderingen van het hart. De patiënten hadden een concentrische hypertrofie bij start van behandeling, zoals te zien aan de toename van de dikte van de linker achterwand van het hart (LVPWd) en de linker ventrikel massa index (LVMI). Deze hypertrofie kan niet worden verklaard door de stapeling van glycogeen alleen omdat de hoeveelheid glycogeen in het hart van patienten met de ziekte van Pompe zelden meer is verhoogd dan 10%. Wij veronderstellen daarom dat de hypertrofie ontstaat door disarray van de contractiele elementen of als reactie op de lysosomale stapeling. Voor start van therapie was de systolische linker ventrikel functie ernstig verminderd en de diastolische functie mild gestoord. Gedurende de therapie nam de concentrische hypertrofie af, hetgeen wordt geïllustreerd door de daling van de LVMI en de afname van de dikte van de LVPWd bij alle patiënten. De functie van het hart verbeterde, hetgeen waarschijnlijk levensreddend was voor tenminste één patiënt.

De vroege en goede respons van het hart op therapie laat zien dat het hart gevoelig is voor enzym vervangingstherapie. Dit kan gerelateerd zijn aan het feit dat een lage restactiviteit reeds voldoende is voor het voorkomen van schade aan het hart en aan het feit dat het hart – vergeleken met andere spieren- het therapeutisch enzym effectief ontvangt via het coronaire systeem.

Ondanks het duidelijke effect dat we zagen bij kinderen die enzym vervangingstherapie krijgen, hebben we ook de beperkingen van de therapie ervaren. Het is moeilijk en niet altijd mogelijk rest schade te voorkomen. Een van de 4 patiënten in het onderzoek presteert opvallend goed, rekening houdend met het vernietigende beloop van de onbehandelde ziekte. Echter 3 van de 4 patiënten hadden beademing nodig op enig punt in het onderzoek.
De studies geven geen duidelijk antwoord op de vraag waarom sommige patiënten beter reageren dan anderen, maar laten aanwijzingen zien dat de ernst van symptomen en leeftijd predictieve parameters zijn voor het effect van therapie. Het zal de toekomstige uitdaging van een team van specialisten zijn de conditie van aangedane zuigelingen te evalueren, gebaseerd op klinische, biochemisch en genetische parameters, om zo het natuurlijk beloop te voorspellen en de kans op succesvolle behandeling in te schatten.

De studies beschreven in dit proefschrift demonstreren de veiligheid en de effectiviteit van enzym vervangingstherapie. Bovendien, laten zij voor het eerst de mogelijkheid zien van de productie van medicijnen in de melk van transgene dieren. De resultaten van deze studies moedigen de continuatie van de huidige ontwikkelingen in de enzym vervangingstherapie bij de ziekte van Pompe in de verschillende subtypen aan. Vanwege de hoge dosis behoefte, vormen zij een stimulus om de praktijk van grootschalige productie van therapeutische proteïnen in de melk van transgene dieren volledig te onderzoeken. Dit is van het grootste belang in een tijdperk waarin de capaciteit van de meer conventionele productie platformen te kort schiet voor de productie van humane eiwitten.

# *Epilogue*

## Epilogue

On March 17, 2003 one of the four patients in our study died (patient 3, in chapters 3 to 6). Her death came quite unexpectedly and touched all the people who were intimately involved for more than 4 years. This sad event lays emphasis on the experimental nature of the treatment and places the joy of success next to the reality of a lethal disease. The causes and consequences of the event need to be evaluated to safeguard the well being of patients in the present and future studies on the safety and efficacy of enzyme replacement therapy in Pompe's disease, and to direct further developments.

The clinical course and the response to therapy of patient 3 are described in the chapters 3, 4, 5 and 6, covering the period from March 1999 to October 2002. The period from January 2002 to February 2003, was marked by intermittent respiratory infections and a gradual decline of the patient's overall condition. On March 3, 2003 she was admitted to the hospital because of fever, increased sputum production and increased ventilatory oxygen need. Upon physical examination she was not critically ill. There was decreased air-entry of the right lower lobe. A chest X-ray revealed atelectasis of the right upper and lower lobes of the lung. Laboratory investigations showed an increased value of C-reactive protein (65 mg/l) and an elevated leukocyte count (19.5 x 10 9/l with 65 % neutrophiles). The diagnosis pneumonia was made and the patient was started on broad-spectrum IV antibiotics. Sputum cultures only revealed a low-grade pseudomonas aeruginosa colonisation; no viruses could be isolated. In the days after admission the fever remained variably present, but the condition of the patient improved.

On March 8, there was a sudden clinical deterioration. The patient developed a high intractable fever up to 42.5 °C, which did not respond to extensive antipyretic treatment (paracetamol, diclofenac, and dantrolene sodium combined with active cooling). The fever was accompanied by periods of very high blood pressures (systolic pressures up to 220 mm Hg), followed by periods of normal and very low blood pressures for which volume expansion was required. The patient became subconscious and later comatose.

As additional therapeutic measures a switch in IV antibiotics was performed and anti-mycotic drugs were started. All cultures remained negative for bacteria and yeast. From the stools an adenovirus was isolated.

Blood chemistry revealed a low level of C-reactive protein (27mg/l); leukocyte count increased shortly to 31 x 10 9/l. CK levels raised mildly from 306 to 540 U/l on the first day of hyperthermia; there was no myoglubinuria excluding rhabdomyolisis as cause of hyperthermia.

The combination of coma and instability of temperature and blood pressure raised concerns about possible damage to the central nervous system (CNS), and more specifically damage of the brainstem. Brain imaging was performed via a computed tomograpy (CT) scan and magnetic resonance imaging (MRI). The CT-scan showed abnormalities of the white matter both in the peri-ventricular as well as in the subcortical areas and in the capsula interna. The MRI confirmed the abnormal signal intensity of the white matter. Thalamus, nucleus caudatus and putamen appeared normal. Dubious changes were seen in the region of the mesencephalon and pons.

The patient died on March 17, 2003. The parents did not give permission for autopsy.
The question remains: 'Was the hyperthermia and the instability of the blood pressure caused by progressive glycogen storage in the brain?'
There is one other publication on hyperthermia in Pompe's disease[1]. Two five and three years old children had a high and intractable fever shortly before they died. The authors pointed in these cases to the association of high fever with muscle wasting as seen in malignant hyperthermia. As an alternative explanation they suggested that the hyperthermia was possibly due to glycogen storage affecting the brain's centres for temperature regulation.
The CT and MRI images that were obtained from our patient did not reveal an extent of brain damage that could naturally explain the hyperthermia. The images seemed more consistent with changes that were caused by the hyperthermia, but firm conclusions can not be drawn.
In conclusion, since damage to the central nervous system can not be ruled out in patient 3 monitoring of the CNS function needs to remain part of the present and future study protocols. Fortunately, no neurological problems were found in patients 1, 2 and 4. This may implicate that even if the hyperthermia in patient 3 was caused by glycogen storage in the thermo-regulatory centres of the brain this does not have to be a uniform problem in patients with infantile Pompe's disease.

## References

1. Martini C, Ciana G, Benettoni A, Katouzian F, Severini GM, Bussani R, Bembi B. Intractable fever and cortical neuronal glycogen storage in glycogenosis type 2. Neurology 2001; 57:906-8.

# *Addendum*

Figures

## Chapter 4

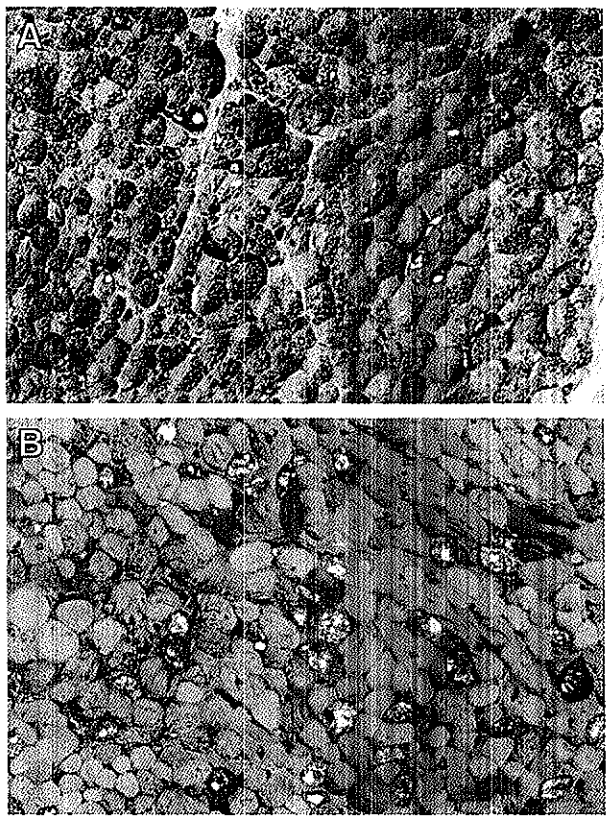


**Figure 2:** *Muscle biopsy (cross section) from patient 1 obtained before start of treatment with rhAGLU (A) and after 12 weeks of treatment with 40 mg/kg (B). Sections were stained with PAS to visualise lysosomal glycogen.*

## Chapter 5

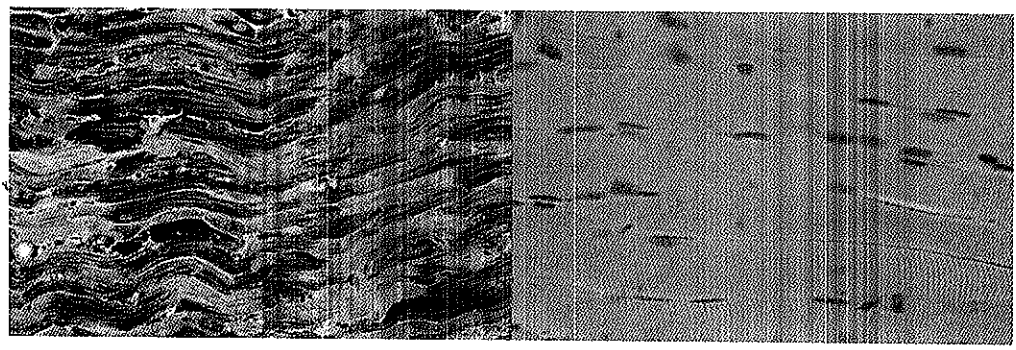

**Figure 3:** *Correction of muscle pathology. Longitudinal sections of a muscle biopsy from patient 1 at baseline (left) and after 72 weeks of treatment (right). Sections are stained with PAS to visualise lysosomal glycogen.*

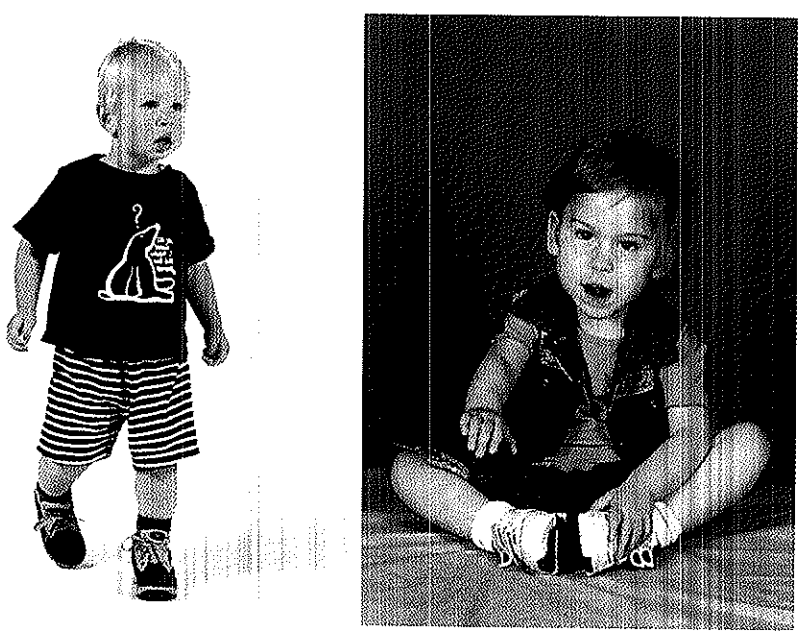

**Figure 5:** *Motor development. Patient 1 shown walking at 16 months of age (left), and patient 3 sitting without support at the age of 19 months (right).*

# Dankwoord

En zo komt een einde aan een drukke periode. De "Pompe-studie" kenmerkte zich door steeds weer nieuwe verwikkelingen. Ondanks de hoop op rustig vaarwater werd dit nooit bereikt. Toch bleef temidden van al deze verwikkelingen een grote groep mensen uit velerlei disciplines hun bijdrage leveren aan het project. Uiteindelijk prijkt slechts één naam op de voorkant van dit boekje. Ondanks het feit dat het onmogelijk is iedereen die bijdroeg aan dit proefschrift te bedanken, wil ik toch graag een aantal mensen met name noemen. Mocht ik mensen ongenoemd hebben gelaten, weet dan dat mijn dank ook hen geldt.

Allereerst de kinderen en ouders die meededen aan de studie. De impact die de ziekte van Pompe op hun leven had, maakte grote indruk op mij. Ik heb groot respect voor de kracht waarmee patiënten, ouders en kinderen, zich staande hielden en houden in de onzekerheid van dit onderzoek. Ik hoop dat er een dag komt dat ook voor mensen met de ziekte van Pompe een goede behandeling beschikbaar is.

Ans, jij gaf me de kans te komen werken in een uniek project op de drempel van laboratorium en kliniek. De vasthoudendheid en ongebreidelde energie waarmee jij dit project voortstuwt is bewonderenswaardig. Ik heb enorm veel geleerd in deze periode zowel over basaal en klinisch onderzoek, als ook over alle andere dingen die te maken hebben met fase II geneesmiddelen-studies. Ik kijk met spanning uit naar het boek, dat je echt ooit moet schrijven over deze periode. Het wordt zeker een spannende bestseller.

Arnold, met de start van het fase II onderzoek verhuisde het project van jouw laboratorium naar het Sophia. Desondanks bleef je betrokkenheid groot. Kritisch en actief bleef je het project volgen. Ik bewonder je gedrevenheid in de wetenschap die de basis was voor dit project. De levendige, niet aflatende, discussies waren een sterke stimulus om de gegevens steeds vanuit alle invalshoeken te bekijken.

Prof. Büller u stond zowel aan het begin als aan het einde van dit project. U maakte mij attent op het onderzoek. Door de jaren heen volgde u het project en in deze laatste fase was uw hartelijke steun een belangrijke stimulus voor het afronden van het boekje.

Leon, ajacied uit Amsterdam. Met jouw komst werd de Pompe kamer opgefleurd met Ajax-accesoires. Je nam het klinische werk over en gaf er snel je eigen stempel aan. Steeds was je weer bereid tot hulp, wanneer ik aan het schrijven was. Volgens jouw planning kan ik snel iets voor je terug doen en dan kun je op me rekenen! Joep, aanstaande promovendus, optimist, levensgenieter en wetenschapper. Jij ziet altijd licht, ook in woelige tijden. Ik hoop dat het licht aan het einde van de 'promotie-tunnel' dichtbij is, zodat je verder kunt met de vele ideeën die borrelen in dat hoofd van jou! Laat me weten wanneer ik jou kan helpen met het opzoeken van foto's en opsporen van onze co-promotoren! Marloes, de nieuwste promovendus in het Pompe team. De combinatie van vastberadenheid en nauwgezet werken zijn een nieuwe drijvende

kracht achter het Pompe team. Ook jij bleef vrolijk als ik weer eens belde met een verzoek uit Leiderdorp.

Voor het vele onderzoekswerk was de hulp van research nurses van groot belang. Marja, Mariella en Karen jullie legden de basis voor een solide organisatie van dit project en brachten me de eerste beginselen van fase II onderzoek bij. Angela, jouw optimisme, vrolijkheid en levenslust maakten de lange serieuze infuusdagen gezellig. Je aanwezigheid wordt nog steeds gemist.
Lizeth en Geertien, eindeloos maakten jullie afspraken, gingen naar testen en vulden jullie CRF'en in. Als buitenstaanders lieten jullie, samen met Leon, een nieuw verfrissend licht schijnen over het Pompe project. Toch bleef er tijd voor gezelligheid wat ertoe leidde dat we steeds met plezier weer aan het werk gingen.

De studenten. Danielle die met mij het hele land afreisde op zoek naar de Nederlandse Pompe patiënten. Joris en Maaike die een enorme hoeveelheid literatuur verzamelden en met groot enthousiasme delen van het onderzoek uitwerkten.

Op vele afdelingen in het Sophia genoten wij van de gastvrijheid. Na een bewogen periode op de ICP werd de Pompe zaal op 2NKG onze thuisbasis. Naast Angela, werden Marit, Asha en Leo al snel onze steun en toeverlaat tijdens de infusen. De zorg die alle artsen en verpleegkundigen op de ICP en 2NKG droegen voor de patiënten in de trial was van groot belang.

Het 'Pompe team'. Adri Cromme-Dijkhuis, met ongebreidelde energie genereerde en interpreteerde jij de cardiologische gegevens. Na al je klinische werk, had ik je veel rust gegund. Dat dit niet zo heeft mogen zijn voelt op zijn minst onrechtvaardig. De vanzelfsprekendheid waarmee Prof. Helbing de interpretatie van de cardiologische data van je overnam heb ik zeer gewaardeerd. Joke, jouw bijdrage aan de echo's, je flexibiliteit, en de precisie waarmee je alle echo's bewaarde, mogen niet ongenoemd blijven. Nynke Weisglas en de fysiotherapeuten, Margriet Bruning en Lianne van der Giessen. Ik heb veel geleerd van de manier waarop jullie met kinderen werken. Het was inspirerend om te zien hoe je spelend een kind kunt bewegen zich maximaal in te spannen. Christa Loonen, ik bewonder de manier waarop jij betrokken blijft bij het onderwerp van je promotie! Je grote verstand van zaken is een grote hulp geweest in het onderzoek. Prof. Arts, samen met Christa Loonen voerde u vele neurologische onderzoeken uit, en volgde u als lid van het 'strategie-team' kritisch de gang van zaken in het project, tenslotte nam u zitting in de leescommissie. Arnold Vulto, als apotheker bekeek je rhAGLU vanuit een andere invalshoek. Dat is voor mij, als dokter, erg leerzaam geweest. De apotheek waar een enorme hoeveelheid infusen werd klaargemaakt. Dat was geen makkelijke klus. Ontelbare ampullen werden met zorg opengemaakt. Steeds stond de medicatie op tijd klaar. Hans van Hirtum met grote deskundigheid ontwierp jij het protocol voor de histopathologische verwerking van de biopten. Het is mede dank zij jou dat we nu mooie coupes hebben! L.A. Severijnen, met enthousiasme nam je het stokje van Hans over. Ook na

het vertrek van Hans, bleef het verwerken van de coupes met behoud van kwaliteit mogelijk. G.A. van Zanten, J. Ponsen en het personeel van het neurofysiologische laboratorium maakten ondanks de krappe bezetting, toch steeds weer plaats voor de patiënten van het Pompe onderzoek. Otto van Diggelen en Marijke de Boer, bepaalden steeds de alpha-glucosidase activiteit. De precisie waarmee jullie dit deden en alle monsters bewaarden zijn bewonderenswaardig. Simon Robben, Jan Huijmans, Peter Merkus en Els van de Wiel maakten de vele foto's en echo's en verrichtten vele testen. Niet alle data zijn verwerkt, dus blijft er gelukkig voldoende te doen. Jan Lindemans, Yolanda de Rijke en het personeel van het CKCL waren altijd bereid onze monsters te verwerken. Het Pompe project kreeg een vaste plek in de koelkast en met precisie werden de monsters bewaard.
Naast het vergaren van de gegevens was het verwerken daarvan van groot belang. Wim Hop, jouw hulp hierbij was van groot belang.
De leden van het 'strategie-team', waarvan een aantal mensen reeds genoemd is, hadden ieder hun eigen inbreng. Nog niet genoemde zijn Hans de Klerk, Pieter van Doorn, Gerard de Jong, Barbara Sibbles, Ewin van der Voort, Prof. van den Anker, Prof. Sauer en Prof. Galjaard. Door de multi-disciplinaire inbreng werden de problemen van de patiënten vanuit vele invalshoeken bekeken.

Alle onderzoekers op het 'Pompe-laboratorium' op de 24$^{e}$ verdieping. Agnes, Marian, Dik, Jan en Monique. Door jullie maakte ik kennis met de wereld van de PCR's en blotjes.

J. Smeitink, B.T. Poll-The, H. Bakker, J. van Hove, C. Lincke, W. van der Zijde en P. Smit stuurden patiënten naar ons door en stelden gegevens beschikbaar van hun infantiele Pompe patiënten. Deze gegevens vormden de basis voor het therapie onderzoek en waren dus van groot belang.

Leden van de kleine commissie, Ron Wanders, Ben Oostra en Willem-Frans Arts, maakten tijd om mijn boekje te lezen en voorzagen het van commentaar. Het enthousiasme waarmee u allen dit deed, was een grote stimulans.

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Mede-assistenten in opleiding, een collega hebben die nog moet promoveren, valt niet altijd mee! Toch bleef er interesse in mijn promotie. Het was jullie collegialiteit die het mogelijk maakte dat er tijd kwam om aan het boekje te werken. Prof. van der Heijden, Peter, Dana-Anne en Ester, jullie inspanning hiervoor was van groot belang.
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Hanneke

## Curriculum Vitae

Hannerieke van den Hout was born in Berkel-Enschot, the Netherlands, on August 24th, 1968. In 1987 she graduated from high school (Cobbenhage College in Tilburg) and started to study medicine at the University of Maastricht (formerly Rijksuniversiteit Maastricht). During her study she spent 3 months at the department of gynaecology and obstetrics of the University College Hospital Galway (Galway, Ireland) and 3 months in a project on mother and child care at the department of medicine of the Federal University of Minas Gerais (UFMG) (Belo Horizonte, Brazil). After graduating in medicine in 1995 she worked for 20 months as a junior registrar at the department of paediatrics of the Reinier de Graaf Gasthuis in Delft and the Erasmus Medical Centre. In 1998 she joined the Rotterdam research team of dr. A.J.J. Reuser and dr. A.T. van der Ploeg, which was working on the development of enzyme replacement therapy for Pompe's disease. She started her training in paediatrics in 2002 under Prof. H.A. Buller and Prof. A.J. van der Heijden. Presently Hannerieke is working at the "Zuiderziekenhuis", Rotterdam, where she continues her training in paediatrics under dr. A. Oudesluys-Murphy. Hannerieke lives in Leiderdorp together with Harald, Iris and Suzanne.

## List of Abbreviations

| | |
|---|---|
| AIMS | Alberta Infant Motor Scale |
| ALAT | Alanine aminotransferase |
| ASAT | Aspartate aminotransferase |
| Asn | Asparagine |
| Asp | Aspartic adic |
| BSIDII | Bayley Scales of Infant Development |
| CHO-cells | Chinese Hamster Ovary cells |
| CK | Creatine kinase (=CPK, creatine phosphokinase) |
| CK-MB | Creatine Kinase (MB fraction) |
| CRIM | Cross Reactive Immunologic Material |
| BAEP | Brainstem Auditory Evoked Potentials |
| EKG | Electrocardiogram |
| EM | Electronmicroscopy |
| EMG | Electromyography |
| GMA | glycol-methacrylate |
| GSD II | Glycogen Storage Disease type II |
| IVSd | Intra Ventricular Septum measured at diastole |
| kb | kilo base |
| kDa | kilo dalton (kD) |
| LDH | Lactate dehydrogenase |
| LVIDd | Left Ventricular Internal Dimension measured at diastole |
| LVPEP | Left Ventricular Pre-ejection Period |
| LVEP | Left Ventricular Ejection Period |
| LVM | Left Ventricular Mass |
| LVMI | Left Ventricular Mass Indexed by body surface area |
| LVPWd | Left Ventricular Posterior Wall measured at diastole |
| M6P | Mannose 6-phosphate |
| MPS | Mucopolysaccharidosis |
| (m)RNA | (messenger) Ribonucleic Acid |
| PAS | Periodic Acid Schiff |
| RWT | Relative wall thickness |
| rhAGLU | recombinant human alpha-glucosidase |
| SF | Shortening Fraction |

# List of Publications

## Articles

1. Van den Hout H, Reuser AJ, Vulto AG, Loonen MC, Cromme-Dijkhuis A, Van der Ploeg AT. Recombinant human alpha-glucosidase from rabbit milk in Pompe patients. Lancet 2000; 356:397-8.

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4. Reuser AJ, Van den Hout H, Bijvoet AG, Kroos MA, Verbeet MP, Van der Ploeg AT. Enzyme therapy for Pompe disease: from science to industrial enterprise. Eur J Pediatr 2002; 161:S106-11.

5. Van den Hout JMP, Hop WJC, Van Diggelen OP, Smeitink JAM, Smit GPA, Poll-The BT, Bakker HD, Loonen MCB, De Klerk JBC, Reuser AT, Van der Ploeg AT. The natural course of infantile Pompe's disease; 20 original cases compared with 133 cases from the literature. Pediatrics 2003; 112.

6. Winkel LP, Kamphoven JH, Van den Hout HJ, Severijnen LA, Van Doorn PA, Reuser AJ, Van der Ploeg AT. Morphological changes in muscle tissue of patients with infantile Pompe's disease receiving enzyme replacement therapy. Muscle Nerve 2003; 27:743-51.

7. Van den Hout HMP, Kamphoven JHJ, Winkel LPF, Arts WFM, De Klerk JBC, Loonen MCB, Vulto AG, Cromme-Dijkhuis AH, Weisglas-Kuperus N, Hop WJC, Van Hirtum H, Van Diggelen OP, Kroos MA, Van Doorn PA, Van der Voort E, Sibbles B, Van Corven EJJM, Brakenhoff JPJ, Van Hove J, Smeitink JA, De Jong G, Reuser AJJ, Van der Ploeg AT. Long term IV treatment of Pompe's disease with recombinant human alpha-glucosidase from milk. Submitted 2003.

8. Van den Hout JMP, Cromme-Dijkhuis AH, Winkel LPF, Van Hove J, Hop WJC, Helbing WA, Reuser AJJ, Van der Ploeg AT. Cardiac response to enzyme replacement therapy in Pompe's disease. Submitted 2003.

**Abstracts**

1. Van den Hout H, Reuser A, Vulto A, Arts WF, Cromme-Dijkhuis A, Hop W, Van der Ploeg A. First clinical test with recombinant human alpha-glucosidase from rabbit milk shows therapeutic effect in Pompe patients. Am J Hum Gen 2000; 67:6.

2. Van den Hout HJMP, Smeitink JAM, Smit GPA, Poll-The BT, Bakker HD, De Klerk JBC, Van der Ploeg A. Het natuurlijk beloop van de infantiele vorm van de ziekte van Pompe. Tijdschr voor Kindergen 2001; 69:35.

3. Van den Hout HJMP, Winkel LPF, Arts WFM, Cromme-Dijkhuis A, Remmerswaal M, Disseldorp J, Reuser AJJ, Van der Ploeg AT. De eerste klinische test met recombinant humaan alpha-glucosidase uit konijnenmelk bij Pompe patienten. Tijdschr voor Kindergen 2001; 69:36.

4. Vulto AG, Van den Hout H, Reuser AJJ, Van der Ploeg AT. Successful treatment of infantile Pompe's disease with recombinant human alpha-glucosidase from rabbit milk: results after 72 weeks treatment. Clinical Pharmacology & Therapeutics 2001; 69:P31.

5. Winkel LPF, Van den Hout, JMP, Kamphoven, JHJ, Reuser, AJJ, Van Diggelen, OP, Arts, WFM, Van Doorn, PA de Jong, G, Vulto, AG, Van der Ploeg, AT Preliminary findings in patients with juvenile Pompe's disease treated with recombinant human alpha-glucosidase from rabbit milk. Am J Hum Genet 2001:674.

6. Cromme-Dijkhuis AH, Van den Hout H, Reuser A, Vulto A, Hop W, Van der Ploeg AT. Recombinant human alpha-glucosidase from rabbit milk has effect on cardiac muscle in patients with Pompe's disease. Cardiology in the young 2001; 11:59.

Stellingen behorend bij het proefschrift:

**Enzyme Therapy in Infantile Pompe's Disease**

a clinical study on the effect of recombinant human alpha-glucosidase produced in the milk of transgenic rabbits

1. Intraveneuze toediening van recombinant humaan alpha-glucosidase corrigeert de hypertrofie van het hart, verbetert de spierfunctie en verlengt het leven van patiënten met de 'klassiek-infantiele' vorm van de ziekte van Pompe.
   *(Dit proefschrift)*

2. Patiënten met de 'klassiek-infantiele' vorm van de ziekte van Pompe hebben mutaties die leiden tot een volledige deficiëntie van alpha-glucosidase activiteit, een hypertrofie van het hart, een vertraagde motorische ontwikkeling en overlijden typisch in het eerste levensjaar.
   *(Dit proefschrift)*

3. Enzymvervangingstherapie bij patiënten met de infantiele vorm van de ziekte van Pompe dient te worden gestart voordat verlies van spierfunctie in de extremiteiten klinisch duidelijk wordt.
   *(Dit proefschrift)*

4. Patiënten met de 'klassiek-infantiele' vorm van de ziekte van Pompe zullen naar verwachting ook indien zij vroeg starten met enzymvervangingstherapie milde restsymptomen van de ziekte houden.
   *(Dit proefschrift)*

5. Bij open-label onderzoek naar het effect van enzymvervangings-therapie in lysosomale stapelingsziekten is mutatie-analyse onmisbaar voor een nauwkeurige definitie van de patiëntengroep.
   *(Dit proefschrift)*

6. De bewering van Amalfitano et al. dat recombinant humaan alpha-glucosidase uit CHO-cellen ten opzichte van recombinant humaan alpha-glucosidase uit konijnenmelk bij een lagere dosering hetzelfde effect heeft, is gebaseerd op de publicatie van een incomplete en selectieve set gegevens.
   *Amalfitano et al. Genet. Med. 2001; 3: 132-138*
   *Hunley et al. Genet. Med. 2003; 5: 209 (abstract no.38)*

7. Gezien de toenemende vraag naar complexe humane eiwitten, de technische beperkingen van de productie in celkweeksystemen en de aan deze productiemethode gerelateerde hoge kosten, verdient de verdere ontwikkeling van het transgene productieplatform meer aandacht.
   *Andersson et al. In Vivo: The Business and Medicine Report 2001; 72: 1-5*
   *The Economist, 2002;365: 5-6*
   *DePalma Genetic Engeneering News 2003: 23, 11: 40-43*

8. Patiënten die participeren in fase II of III studies worden onvoldoende beschermd als de ongebruikelijke situatie zich voordoet dat de productie van het medicijn waar zij baat bij hebben gestopt wordt.

9. Het aantonen van effecten van medicatie is niet gelijk aan het aantonen van de effectiviteit daarvan.

10. Wanneer wij als samenleving een goede gezondheidszorg willen, gevoed door goed onderwijs en onderzoek, zullen wij bereid moeten zijn hiervoor de prijs te betalen.

11. Kinderen spelen met onzichtbare dingen.
    Zij gaan met minder dan een schaduw om.
    Wat voor ons stom is heeft voor hen geluid.
    Waar wij niets zien zien zij de mooiste ogen.
    *(Adriaan Morriën, 5 juni 1912-7 juni 2002)*

Rotterdam 24 september 2003 Hannerieke van den Hout