Enzyme Therapy in Non-classic Pompe's Disease

Safety and efficacy of recombinant human α-glucosidase from milk of transgenic rabbits and from Chinese hamster ovary cells

Léon P.F. Winkel

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Enzymtherapie voor de niet klassieke vorm van de ziekte van Pompe

Veiligheid en effectiviteit van recombinant humaan α-glucosidase uit melk van transgene konijnen en uit Chinese hamster ovarium cellen

Proefschrift

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Contents

Wijs is hij die anderen kent;
Maar verlicht is hij die zichzelf kent

Vertaald uit de Tao Te Tjing van Lao-Tse

Voor Roos, Raphaël en Jonathan

Objectives and Scope

Pompe's disease is a progressive muscle disorder caused by the deficiency of lysosomal acid α-glucosidase. Severe and milder forms of the disease are recognized. No registered treatment is available. The experimental work of this thesis was performed to define the natural course of the milder form of Pompe's disease and to evaluate the safety and efficacy of enzyme replacement therapy with recombinant human α-glucosidase from rabbit milk and from Chinese hamster ovary cells.

This thesis consists of the following elements. Chapter 1 serves as introduction and gives an overview of lysosomal storage disorders in general and the current status of enzyme replacement therapy for the various lysosomal storage disorders. Chapter 2 reviews the existing literature containing case reports on Pompe's disease, in order to describe the natural course of the milder forms of Pompe's disease. This study was conducted to fully recognize signs and symptoms of the disease; to set guidelines for further subtyping and to establish clinical endpoints for enzyme replacement therapy studies. Chapter 3 evaluates the long-term clinical effects of 3 years of treatment with recombinant human α-glucosidase from rabbit milk in three patients with non-classic Pompe's disease.

This is followed by the description of a 1.5 year-period of treatment with varying doses of recombinant human α-glucosidase from Chinese Hamster Ovary cells in chapter 4. In chapter 5, 6 and 7 specific effects of enzyme therapy are discussed in more detail. Chapter 5 describes the effects of enzyme replacement therapy on skeletal muscle tissue morphology, the feasibility and limitations. Chapter 6 reports the pharmacokinetics of different α-glucosidase preparations and the effects of applying different doses and infusion rates. In chapter 7 the significance of an early marker of the efficacy of enzyme replacement therapy, as observed in lymphocytes, is discussed. Chapter 8 gives an overall discussion of the findings of this thesis.

Introduction
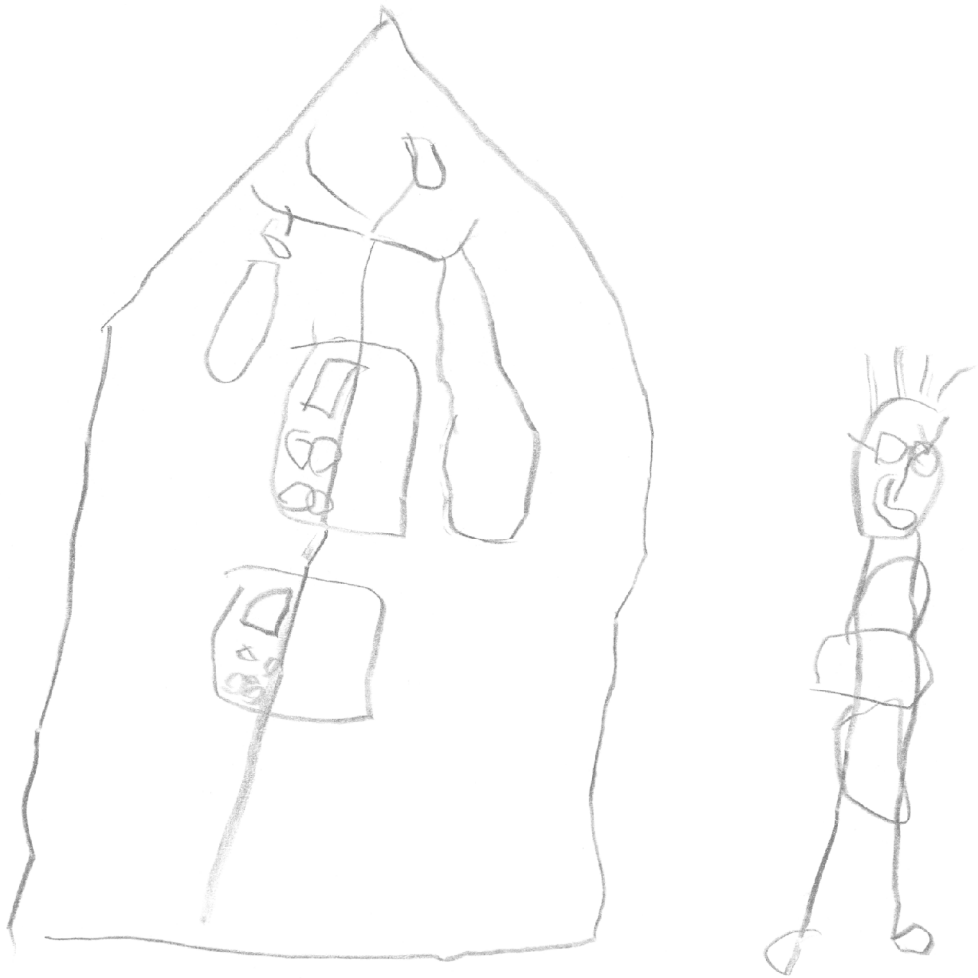
1.1 Lysosomal storage disorders

The lysosomal storage disorders (LSD's) comprise a group of more than 40 diseases (fig.1). Each disease results from storage of a substrate in the lysosomal compartment of the cells. The majority of lysosomal storage disorders is caused by the deficiency of a single lysosomal protein like in Gaucher's, Fabry's and Pompe's disease [1-3]. Others are caused by a defect in lysosomal enzyme phosphorylation and thereby improper targeting of the lysosomal enzymes to the lysosomes (mucolipidosis II and III) [4], by a defective lysosomal transporter, like in cystinosis [5] and Salla disease [6], or a deficiency of a lysosomal activator protein [7]. Each LSD is caused by a different genetic defect and is inherited as an autosomal recessive trait. Exceptions are Fabry's disease and mucopolysaccharidosis (MPS) type II, which show X-linked inheritance [2].
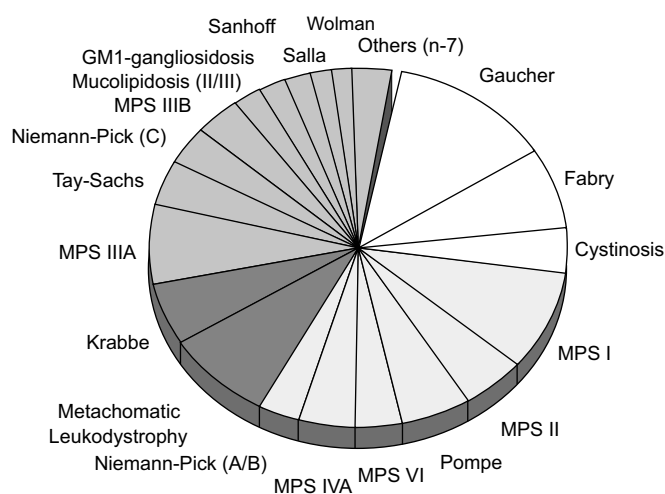
Figure 1. Pie chart showing the relative incidence for LSD's in Australia. Pie slices represent the relative prevalence of each disorder. [8]

Many LSD's have been classified into clinical subtypes (such as Hurler-Scheie definition, or the three different types of Gaucher's disease), but it is clear that most LSD's have a broad continuum of clinical severity and age of presentation [9]. It has been demonstrated for various LSD's that clinical diversity is linked to residual enzyme activity of the deficient enzyme, such as Pompe's disease, Tay Sachs, Niemann Pick and Mucolipidosis [10-15]. This variation is ascribed to the different mutations within the same gene [16, 17]. However this genotype-phenotype correlation is not strict [18, 19]. Environmental factors or (epi-) genetic background may play a role in these differences.

Although the different LSD's result from mutations in different genes and subsequently have a different deficient enzyme activity or protein function, all LSD's share the common biochemical aspect that the disorder results in an accumulation of otherwise normally degraded substrates within the lysosomes. The difference is expressed first in the substrates that accumulate.

The tissue-location of the non-degradable substrates determine the symptoms of the disorder. For instance in the group of mucopolysaccharidoses enzymes involved in the degradation of glycosaminoglycans (mucopolysaccharides)

are deficient. This results in the accumulation of glycosaminoglycans in liver and spleen, cartilage and bone tissue and in the brain. Organomegaly, joint immobility, coarse facial features and mental retardation are the consequences [20]. In the milder variants of Pompe's disease the symptoms are mostly limited to the muscles, because glycogen is present in large quantities in muscles. It can not be degraded in the lysosomes due to the deficiency of α-glucosidase, which causes a cascade of muscle cell damage, leading to muscle weakness [3].

The prevalence of LSD's is estimated to be 1 per 5-7,000 births for all disorders together [9, 21]. Generally, Gaucher's disease is considered to be the most prevalent LSD. The exact prevalence was determined in new born babies in Australia, being 1 per 57,000 births [8]. There are two studies that indicate that Pompe's disease occurs with a similar frequency. For both the Netherlands and the USA the incidence was calculated to be 1 in 40,000 [16, 22]. In some populations or countries specific LSD's occur more frequent, such as GM2 Gangliosidosis type 1 (Tay Sachs) and Gaucher's disease in the Ashkenazi Jewish population, with a reported incidence of 1 per 855 for Gaucher's disease [1]. GM2 gangliosidosis type 2 (Sandhoff's disease) has a higher incidence in Portugal [23] and MPS IV, GM2 gangliosisidosis type 2 (Sandhoff's disease), and ceroid lipofucsinosis in Saudi Arabia [24].

Most lysosomal disorders can be (easily) diagnosed prenatally via the determination of enzyme activity in chorionvilli/amniocytes. However, these types of diagnostic procedures will only be initiated if a sibling was born with the disease. Postnatal diagnosis can be performed in fibroblasts/leukocytes or in biopsies of specific tissues (for example muscle tissue in Pompe's disease).

Currently, the possibilities of newborn screening are investigated in order to track down these disorders in a very early state, before severe tissue damage has occurred. This is relevant in light of the growing number of newly available therapeutic options for LSD's [25]. However, it has to be realized that some disorders will remain untreatable.

Mutation analysis is not necessary for the diagnosis of LSD's but can sometimes be required for carrier detection and recognition of pseudodeficiencies. It can also help to provide more information on the clinical subtype and clinical course of the disease. For example the N370S mutation in Gaucher's disease indicates that there will be no neurological involvement [1, 26].

1.2 Enzyme therapy for lysosomal storage disorders

For most patients with LSD's optimal supportive care is the only therapeutic option. The available causal treatments for LSD's are based upon two major strategies.

The first is to reduce substrate accumulation by intervening in metabolic pathways through substrate inhibition therapy or enhancement of enzyme activity. The latter has been theoretically described to promote normal trafficking through the secretory pathway of the ER and increases enzyme activity in the lysosomes [27]. This approach has been used for Fabry's disease by Frustaci *et al.* They treated a single patient with the cardiac variant of Fabry's disease for two years with galactose infusions in a dose of 1.0 mg/kg bodyweight every other day. The authors reported that the infusions were well tolerated and safe, as was deduced by normal liver function tests. Increased α-Galactosidase A activity was found in lymphocytes and endomyocardial cells. Echocardiographic studies showed improved cardiac contractility and reduction of myocardial-wall thickness. Most importantly, the patients' New York Heart Association functional status improved from class IV to

class I, taking him off the cardiac transplantation list [28]. Unfortunately, a report of larger groups of patients responding equally well to this treatment is not available and the method still has to prove its general applicability and efficacy. A possible advantage of chaperone therapy may be that the blood brain barrier can be crossed [29, 30]. There is currently one substrate reduction therapy that has been regulatory approved, N-butyldeoxynojirimycin (NB-DNJ; Zavesca). This treatment has proven to be effective in patients with mild to moderate type 1 Gaucher's disease. Reduction of glucocerebrosides and liver and spleen volumes have been noted. Hematological response was less impressive than with Enzyme Replacement Therapy (ERT) [31-33]. This therapy is now reserved for patients who are unable or unwilling to receive ERT.

The second strategy is to increase the deficient enzyme activity, either by replacing the enzyme itself (ERT) or by replacing deficient cells through bone marrow transplantation or human stem cell therapy.

A third option would be to repair or replace the defective genes causing the enzyme deficiency that leads to accumulation of storage material in the lysosomes (Gene therapy). However there is no successful application yet in humans with LSD's. This thesis will focus on ERT as therapeutic option.

The basic principle of enzyme replacement therapy

De Duve laid out the basic principle for enzyme replacement therapy in the 1960's through his description of the structure and function of the endosomal-lysosomal apparatus. He suggested that the therapeutic enzyme could reach the lysosome through heterophagy, after (intravenous) administration [34]. It was further demonstrated that cultured fibroblasts derived from Hunter and Hurler patients were able to correct each other mutually by exchanging enzymes [35]. Later was found that certain receptors play an important role in the uptake of enzymes *in vitro*, such as the mannose 6-phosphate receptor for fibroblasts [36], and skeletal muscle [37], and the mannose receptor for macrophages [38].

It took however many years to get from *in vitro* studies to successful *in vivo* trials. Early preclinical studies showed uptake of purified enzymes in fibroblasts such as β-hexosaminidase A from urine [39]. The first clinical tests were performed in infants with Pompe's disease receiving intramuscular and intravenous α-glucosidase extracted from Aspergillus Niger. These studies showed limited success, the α-glucosidase was taken up by the liver, but limited decrease of glycogen storage was shown and clinical effects could not be sustained [40, 41]. Similar therapeutic interventions were attempted in other LSD's [42, 43].

Several problems put ERT on hold. These problems in the 1970's were that there was not enough enzyme to treat patients, purified from natural human resources like placenta and urine, and there was no possibility to produce recombinant enzymes either, because no LSD-genes had been cloned at that stage. Detailed knowledge about glycosylation and tissue specific targeting of lysosomal enzymes was not available, and finally there were few (small sized) animal models at that stage to test the feasibility of ERT, so the step from *in vitro* to human experiments was rather large.

This situation has been changed through several developments. Genes coding for enzymes involved in LSD's were cloned [44-46], animal models were developed [47-51], and production methods for lysosomal enzymes were initiated [52, 53]. In the early 1990's the first proof of principle for ERT was obtained by Barton *et al.* [54].

They proved that placental acid β-glucosidase was effective in patients with type I Gaucher's disease.

The production of recombinant human lysosomal enzymes is complex, because proper glycosylation is required. For example the explored prokaryotic production systems did not provide the correct post-translational modifications. In other words, the enzyme was not functional in humans and needed to be produced either in animal or human cells or in transgenic animals [52, 53]. Production of therapeutic proteins in transgenic plants [55] and in the milk of transgenic animals [56] is still under investigation. No transgenically produced proteins have received market approval at this stage. The safe and successful application of transgenically produced recombinant human α–glucosidase is described in this thesis and was earlier reported by Van den Hout *et al.* [57, 58]

For clinical trials recombinant human lysosomal enzymes have been produced in Chinese hamster ovary cells, human fibroblasts and the milk of transgenic rabbits. These enzymes have been used for enzyme replacement therapy in Gaucher's, Fabry's and Pompe's disease, and mucopolysaccharidoses types I, II and VI [57, 59-65]. Recombinant human sphingomyelinase is currently tested in preclinical studies in mice with Niemann Pick type B. Further, preclinical studies are performed for MPS VII, and Wolman's disease.

1.3 Enzyme replacement therapy in Gaucher's disease

Gaucher's disease is an autosomal recessive condition caused by the deficiency of glucocerebrosidase, leading to the accumulation of glucocerebrosides. This results in a systemic disorder consisting of progressive visceral enlargement and gradual replacement of the haematopoietic system with lipid-laden macrophages. Symptomatic anaemia, thrombocytopenia, hepatosplenomegaly and skeletal damage are prevalent in most patients.

Clinically, three subtypes are recognized. Type 1 or the non-neuropathic form is most common. This type of disease presents mostly during adulthood, but also occurs in children. Type 2, or the acute neuropathic form, is characterized by infantile onset and severe neurological involvement. Type 3 or the subacute neuropathic form, is characterized by progressive neurological symptoms, leading to death in the first decade of life [1].

Barton *et al.* described the first patient in whom ERT for Gaucher's disease appeared effective. Weekly intravenous infusions with 167 International Units (IU) macrophage targeted human placental glucocerebrosidase/kg bodyweight in a child with type 1 Gaucher's disease resulted in an increased hemoglobin level, increased platelet count, decreased phagocytic activity in the spleen, and skeletal improvement. These effects gradually disappeared when treatment was discontinued for 25 weeks. He concluded that these observations documented clinical response to ERT [54].

The positive results of this pilot study led to the first open label clinical trial in twelve patients with type 1 Gaucher's disease [59]. They were treated with 60 IU macrophage targeted human placental glucocerebrosidase/kg bodyweight every two weeks for 9 to 12 months. The hemoglobin concentration increased in all 12 patients, and the platelet count increased in 7 patients. Serum acid Phosphatase decreased in 10 patients and plasma glucocerebrosides decreased in 9 patients. Splenic volume decreased in all patients and hepatic volume in five of them. Early signs of skeletal improvement were seen in three patients. This led to the conclusion that ERT was effective in Gaucher's disease, but that hematological and

visceral improvements preceded skeletal response to therapy.

After a few years of experience with placental glucocerebrosidase, production of glucocerebrosidase in CHO-cells was initiated (imiglucerase) [66, 67]. This recombinant human enzyme has gradually replaced the placental enzyme.

A review of the results of the treatment of 175 type 1 Gaucher patients showed that liver size decreased by 16-20% over a 1-2 year treatment period. The spleen decreased in size by 40-50%. Anaemic patients showed about 1.5 gm% increase of haemoglobin level, and about 40% of these patients attainined (near) normal haemoglobin values. Thrombocytopaenia resolved in mildly affected patients and improved in severely affected patients [29].

Lessons learned from ERT in Gaucher's disease are that there are some necessary ingredients to make ERT a successful treatment for LSD's. Development of successful therapy was only possible after achieving sufficient understanding of phenotype definition, genetic cause and biology of the disease, enzyme structure and purification of the enzyme, and how to deliver the enzyme efficiently to the target organs. These items are the fundaments of the successful application of ERT for LSD [68].

Currently ERT is considered to be the standard therapy for type 1 Gaucher's disease. Ten years of experience in over 3000 patients has led to the following conclusions. ERT results in reversal of anaemia, thrombocytopenia and hepatosplenomegaly and it clears the storage in bone marrow. When the treatment is initiated early in life, it results in normal bone structure and skeletal development [69]. The frequency of painful bone crises and bone fractures appear to have markedly decreased in adult patients, but this has not been formally demonstrated in a comparative trial [70]. The addition of biphosphanates to ERT may have additional value as was shown in several patients [71, 72].

ERT for Gaucher's disease is usually given as an intravenous treatment every other week. Treatment has appeared very safe, but seroconversion occurs in 13% of the patients, from whom 75% had no detectable levels of circulating inhibitory antibodies. 90% of the 13 % who seroconverted became tolerant over time [73].

There are still some issues to be addressed for Gaucher's disease, such as which dose-regimen is the most suitable [74, 75]. Elstein *et al.* reported that low dose low-frequency imiglucerase as a starting regimen of enzyme replacement therapy for patients with type 1 Gaucher's disease worked well [76]. However, general consensus states that mild disease should be treated with 15-30 Units/kg bodyweight every two weeks and that severe disease should be treated with 60-120 U/kg bodyweight. Patients with type 1 Gaucher usually benefit from initial treatment with 60 U/kg from 6-12 months, which is then, reduced to a lower maintenance dose [29, 30, 70]. Surrogate markers such as chitotriosidase can be used to monitor therapeutic efficacy [77].

In a series of 95 type 1 patients, 68% of them had some abnormality of lung function, but clinical lung disease was rare [78]. The most serious complication was pulmonary hypertension, which sometimes improved during treatment, but worsened in other cases. The mechanism behind this pulmonary hypertension is still unknown.

The neurological symptoms of type 3 Gaucher's disease can not be treated by ERT. Still ERT is advised for the treatment of systemic effects of type 3 Gaucher's disease. ERT is not effective and therefore not advised for patients with type 2 Gaucher's disease, even when high doses are applied. [79].

1.4 Enzyme replacement therapy in Fabry's disease

Fabry's disease is an X-linked disorder resulting from mutations of the α-galactosidase A gene. The defect results in accumulation of glycosphingolipids systemically because of the deficiency of α-galactosidase A (α-Gal A). Accumulations of globotriaosylceramide (Gb3) are found in vascular endothelium and smooth muscle cells, myocardium (cardiomyocytes), renal epithelium (podocytes) and the dorsal root and autonomic ganglia in the peripheral nervous system and neurons in the central nervous system. Symptoms that develop because of these accumulations are painful small fiber neuropathy, hypohydrosis, progressive renal, cardiac and cerebrovascular degeneration.

Patients can generally be divided into two major groups on the basis of absence or presence of residual α-Gal A activity. There are patients with so called classic disease and there are milder, later onset, atypical variants. Presentation and clinical course can vary within these phenotypes [2, 70, 80].

Two different groups have been developing ERT for Fabry, leading to the approval of gene-activated α-gal A (ga-hαGal A, agalsidase α, Replagal, produced by TKT, Cambridge, Massachusetts) and recombinant human α-gal A (r-hαGal A, agalsidase β, Fabrazyme, produced by Genzyme, Cambridge, Massachusetts) [80].

Agalsidase α
A phase I trial in 10 male subjects who were given a single injection of agalsidase α, was the first one to study efficacy and safety of this treatment in humans. Increasing doses from 0.3 to 4.7 Units / kg of bodyweight (0.007–0.1 mg/kg) were used. Maximum plasma levels were determined by the dose. The liver appeared to absorb more than 50% of the administered enzyme, resulting in a 31% decrease of the accumulated Gb3. Urinary Gb3 levels decreased with 38% within 28 days after the single injection, indicating that the injected enzyme reached the renal tubular cells and reduced the amount of Gb3. From this study it was concluded that agalsidase α was both safe and biochemically active in patients with Fabry's disease [60].

The results of this study led to the conduction of a 6-month placebo controlled clinical trial, in which the efficacy and safety of 0.2 mg agalsidase α/kg/2 weeks was studied. It was not reported why this dose was twice the maximum dose of the original study. A significant reduction of neuropathic pain and improvement of pain-related quality of life was found in the treatment group. This did not occur in the placebo group. Significant improvement of renal pathology was found in the treatment group, compared to 16% deterioration in the placebo group. There was also a significant reduction in lysosomal inclusions in kidney vascular endothelial cells. Inulin clearance decreased in both the treatment group (6.2 ml/min) and the placebo group (19.5 ml/min) without significant difference. Creatin clearance increased 2.1 mL/min in the treatment group, and decreased 16.1 ml/min in the placebo group (p=0.02). In the treatment group plasma glysphingolipid-levels decreased approximately 50%, cardiac conduction improved and bodyweight increased. None of this was found in the placebo group [81].

In the same group of patients cerebral blood flow was studied. Abnormal cerebral blood flow was found in the patients before start of treatment, which normalized after they received ERT. Also blood flow dermal blood vessels showed improvement. Finally it was demonstrated that there was an increase in bodyweight in the treatment group [82].

Mild infusion associated reactions did occur but these tended to diminish and even disappear with repeated infusions. Increasing the infusion period from 20 to 40 minutes also decreased the number of infusion associated reactions [81, 83]. Currently the treatment (0.2 mg/kg/2 weeks) is regarded safe enough to be administered at home [70].

The crossing over of the placebo group to the treatment group showed similar results as were found in the initial treatment group. After three years of treatment neuropathic pain had decreased in both the original treatment group as well as the original placebo group. This was accompanied by an increase of pain related quality of life. An improved ability to sweat was observed 24 −72 hours after an infusion with agalsidase α. Three years of treatment resulted in a moderately reduced sensory threshold for warm and cold[83].

From these studies it can be concluded that agalsidase α can safely be administered to patients with Fabry's disease. The treatment has a beneficial effect on the metabolic and morphological level, as demonstrated by reduction of Gb3 accumulation. Clinical efficacy is so far limited to reduction of pain (at its worst) and the possible slowing down of renal deterioration.

Agalsidase β

Agalsidase β was first administered in a dose finding phase I/II study to 15 patients. Enzyme doses of 0.3, 1.0 and 3.0 mg/kg bodyweight biweekly were well tolerated, and plasma, liver and renal Gb3 was reduced in a dose dependent manner. Histological clearance of lysosomal inclusions was observed in the liver, heart and skin. Clearance of Gb3 was seen mostly in vascular endothelial cells. IgG antibodies were found in 54% of the patients, no neutralizing antibodies were detected. Patients anecdotally reported decrease of pain and increased ability to sweat. From this study it was concluded that agalsidase β was both safe and enzymatically active for patients with Fabry's disease [61].

The results formed the basis for a phase 3 trial, a multicenter, placebo-controlled study. A dose of 1.0 mg/kg/2 weeks was chosen based on the results of the first study. After 20 weeks (11 infusions) 20 of 29 patients in the treatment group had reached the primary endpoint, which was no microvascular endothelial deposits of Gb3 in kidney biopsies. None of the 29 patients in the placebo group reached this endpoint. Similar results were obtained in skin and heart biopsies. Plasma Gb3 and urinary Gb3 decreased. Reduction of pain and improvement of quality of life was reported for both the treatment and the placebo group.

The placebo group crossed over into a 6 months open label extension study. After this period 98% (42 of 43) of the patients had cleared Gb3 from renal microvascular endothelium. Similar clearance was obtained in 96% of the skin specimens (45 of 47) and in 75% of the heart specimens (24 of 32). Renal function, as measured by glomerular filtration rate, did not change substantially from baseline for the whole group after 1 year. The infusions were well tolerated. No changes were recorded in electrocardiogram or echocardiogram or other safety assessments. The patients did show mild to moderate infusion associated reactions, that were managed by prolonged infusion time and the use of premedications (acetaminophen, ibuprofen and/or prednisone). IgG seroconversion occurred in 51 of the 58 patients [84]. The conclusion of this study is that agalsidase β can safely be administered to patients with Fabry's disease and the treatment has a beneficial effect on the molecular level, as demonstrated by the reduction of Gb3 accumulations. Clinical efficacy has not yet been proven [85].

A comparison of both enzymes *in vitro* and in a mouse model by two groups showed that both proteins were structurally and functionally similar, with comparable specific activity and glycosylation [86, 87]. The rationale for different doses could not be explained by these studies. It should be noted, however, that these preclinical studies might not fully reflect the clinical studies on enzyme therapy in humans.

There are several studies that have investigated the effect of agalsidase α or β on target organs. Agalsidase β was found to significantly improve small fiber neuropathy [88]. Both Agalsidase α and β were reported to improve cardiac dimensions [89, 90]. Agalsidase α was reported to reverse (at least some) of the hearing deterioration found in Fabry patients. This improvement was very slow and only noted after 18 months of treatment [91, 92]. ERT did not have any effect on the ocular manifestations of Fabry (cornea verticillata) [93]. The effect of agalsidase α on glomerular filtration rate was investigated in the Fabry Outcome Survey. They reported that ERT can improve renal function of patients with only a mild decrease in GFR, and it might be able to slow down the natural decline in renal function in patients with a moderate decline of GFR [94]. The number of descriptions of children and adolescents with Fabry's disease treated with ERT is limited, the first reports show similar findings as in the adult patients [95-97].

Although market approval has been given to both products in Europe and to agalsidase β in the United States, long-term studies are required to evaluate the efficacy of ERT in Fabry's disease over time on endstage renal disease, cardiac insufficiency, stroke, quality of life and life span [85], and expanded clinical trials are necessary to evaluate the efficacy in children with Fabry's disease.

1.5 Enzyme replacement therapy in Mucopolysaccharidosis (MPS)

Mucopolysaccharidosis type I is caused by α-L-iduronidase deficiency, which results in the accumulation of glycosaminoglycans in liver, spleen, heart, cornea, brain, and 'joints'. The severity of the disease varies. Three main subtypes are recognized i.e. Hurler's, Hurler-Scheie's and Scheie's disease. Symptoms of the Hurler subtype are most severe. Patients present in there first years of life with developmental delay, corneal clouding, coarse facial features, airway obstruction, obstructive sleep apnea syndrome (OSAS), cardiac disease, hepatosplenomegaly, and severe joint restrictions. Most children die before the age of 10 years. The intermediate Hurler-Scheie and Scheie variants show a milder course of disease and express less to no central nervous system involvement [20].

In 2001 the first results were published on enzyme therapy with recombinant human α–L-Iduronidase (rh-IdU), produced in CHO-cells. The drug was administered to 10 patients in a dose of 125.000 Units/kg/week (= 100 SI U /kg = 0.58 mg/kg). The infusion was given in 3 hours. This time was prolonged to 6 hours when hypersensitivity reactions occurred. In general, the infusions were well tolerated. After 52 weeks liver and spleen size had decreased by a mean of 25% and glycosaminoglycan (GAG) excretion in urine by a mean of 63%. Growth rate increased in the 6 prepubertal patients and the mean maximal range of motion increased significantly in shoulder and elbow joints. The number of apnoea and hypopnoea during sleep decreased by 61%. Cardiac disease as defined by the New York Heart Association improved 1 or 2 functional classes, and the patients were reported to be able to perform better on daily life activities. All patients developed IgG antibodies to Chinese hamster ovary cell proteins (impurities of the preparation) and four patients developed IgG antibodies for rh-IdU. Five patients

had transient urticaria or facial swelling during infusions, which disappeared after each infusion. In patients with recurrent urticaria the infusion rate was decreased, the dose was reduced and they received pre-medications before the infusion such as diphenhydramine [63].

These promising results of the open label phase I-II trial were reason to start a phase 3 multicenter, multinational, placebo-controlled study. Twenty-two patients were treated for 6 months with 100 IU rh-IDU/kg bodyweight (=0.58 mg/kg) per week and 23 others received a placebo. In the following open label extension study all 45 patients received 100 IU rh-IDU/kg/week. During the first 6 months Forced Vital Capacity (FVC) increased 5.9% in the treatment group compared to the placebo group. With the "six minutes walk test" an increase of 38.1 meters was recorded. In addition it was observed in the treatment group that hepatomegaly and urine GAGs decreased, while the apnoea index and shoulder flexion improved. The improvement of shoulder flexion was most evident in the most severely affected patients (9.6 degrees). Adverse events occurred in both groups and nearly all patients developed IgG antibodies (91%) [98, 99].

During the 72 weeks extension study in which the placebo (placebo/rhIDU) and treatment group (rhIDU/rhIDU) received rhIDU, both groups showed significant improvements in mean percent of predicted normal FVC compared to baseline values. The rhIDU/rhIDU group improved 10.2%, and the placebo/rhIDU group improved 4.9%. These data suggest stabilization of the rate of lung volume growth with treatment, as opposed to the decline over time as expected for untreated patients. With the six minutes walk test a mean increase of 43.0 meters was recorded in the original rhIDU/rhIDU group and 27.6 meters in the placebo/rhIDU group. Liver volume decreased with 20% reaching normal values in 75% of all patients and the urine GAGs decreased with 70% reaching normal values in 38% of all patients. The range of motion in shoulder and knee joints improved 5-10 degrees in both treatment groups. Approximately 50% of all patients experienced mild to moderate infusion associated reactions, such as rash and flushing. These reactions were easily managed with adjustment of infusion rate and/or medication [99, 100]. This long-term ERT trial for MPS I suggests a favorable risk-benefit profile for rhIDU. Patients demonstrated sustained clinical improvements that were related to efficient clearance of GAG-substrate. The positive results of the studies led to market approval for rhIDU, or Aldurazyme (Genzyme corp., Massachusetts, USA), in Europe and the USA in 2003. Most patients included in the studies so far had the milder Hurler-Scheie and Scheie phenotypes. For patients with the severe Hurler phenotype additional studies are currently performed in patients under five years [101]. Since Aldurazyme is not capable to penetrate the blood-brain barrier it is expected that central nervous system involvement will not be affected by this treatment in Hurler patients. On the other hand positive effects on the many other tissues and organs involved may have a positive effect on the quality of life and wellbeing of patients. Results of this study have to be awaited.

MPS II or Hunter syndrome is an X-linked recessive disorder caused by the deficiency of the lysosomal enzyme iduronate-2-sulfatase (I2S) [20]. The disease is a progressive disorder with GAG accumulation throughout the body. Coarse facial features, short stature, skeletal deformities and joint stiffness and mental retardation characterize the severe form of Hunter's disease. Onset of disease occurs usually between 2 and 4 years with progressive neurologic and somatic involvement. Patients usually die between 10 and 15 years. Milder forms of Hunter's

disease are characterized by preservation of intelligence and survival into adulthood. Somatic features are similar to those seen in the severely affected Hunter patients, but at a reduced rate of progression. Recombinant human iduronidate suphatase (I2S) was produced in human cells for clinical application in enzyme therapy trials by TKT, Cambridge Massachusetts. In March 2001, 12 patients were enrolled in a randomized placebo controlled trial. They received 0.15 mg/kg, 0.5 mg/kg, 1.5 mg/kg I2S or placebo by IV infusion biweekly for 6 months. Thereafter all 12 were enrolled in an open label extension study. Preliminary results show that urinary GAGs decrease by 51% in the patients receiving I2S. I2S-treatment also results in significant reductions of liver and spleen size. Infusion related reactions occurred after infusions with 0.5 and 1.5 mg/kg, and these reactions were easily managed by slowing down the infusion rate and by the administration of premedications [64]. A phase 3 trial has started in 2003, but no results have been reported yet.

MPS VI or Maroteaux–Lamy is caused by deficiency of N-acetylgalactosamine-4-sulfatase (arylsulfatase B). This syndrome was first described as a Hurler like syndrome. The severe form of MPS VI is characterized by an enlarged head, deformed chest, umbilical and/or inguinal hernia's, growth retardation, joint deformation and stiffness. Patients have a normal intelligence. Most reported patients with the severe form have died of heart failure in their second or third decades. Milder cases have been described and have a slower disease progression [20].

Recombinant human arylsulfatase B (rh ASB) was produced by Biomarin Pharmaceuticals, Novato, CA, USA. Seven patients were enrolled in a randomized, two-dose (0.2 and 1.0 mg kg), double blind study. One patient withdrew after 3 weeks for personal reasons, 6 completed the first 24 weeks, 5 patients completed another 24 weeks after withdrawal of one patient because of lack of benefit. All patients received diphenhydramine before each infusion. No study drug related adverse events, laboratory changes or allergic reactions were noted.

Patients on both doses rh ASB showed a reduction of total GAG excretion of 51% (low dose) and 63% (high dose) respectively. In 4 of 5 patients walking improved over the 48-week period. In one patient breathing was reported to improve, as measured by increase of Vital Capacity and by improvement of apnoea-hypopnoea index, the latter was also reported for one other patient. Shoulder movements improved in the 5 patients completing the 48 weeks treatment period. Shoulder movement and joint pain improved in the most severely affected patients. After 48 weeks the dose was equalized in all patients to 1.0 mg/kg [65, 102].

It was concluded that the treatment was well tolerated and that clinical responses were present in all patients, but most so in the most severely affected patients receiving the high dose of ERT [102]. A phase 3 trial was started in 2003 for both MPS II and MPS VI, but no (preliminary) results have been reported yet.

1.6 Enzyme replacement therapy in Pompe's disease
Pompe's disease is a neuromuscular, autosomal recessive disorder caused by lysosomal α-glucosidase deficiency. The deficiency results in accumulation of glycogen in the lysosomes of all cells, but especially in the muscle.

Pompe's disease is generally subdivided into early and late-onset subtypes [3]. The first one is known as the classic (infantile) form of Pompe's disease and consists of a rather homogeneous group of patients. Patients with classic Pompe's disease typically have a rapidly progressive cardiac hypertrophy, and important motor milestones like turning, sitting and standing are not achieved or lost shortly after

acquisition. They gain significantly less weight than unaffected babies do and the levels of ASAT, ALAT, LDH and CK are elevated, acid α-glucosidase is severely deficient and they have fully deleterious mutations. The onset of symptoms is at a median age of 1.8 months, the median survival is 6.0-7.7 months and patients rarely survive beyond 18 months [103].

The first attempt at ERT for classic Pompe's disease was performed in 1964 with α-glucosidase derived from Aspergillus Niger [40]. Later two patients were treated with α-glucosidase from human placenta [104]. There were no sustained clinical effects.

In 1999 our group started the first study with recombinant human α-glucosidase in patients. The product was produced in the milk of transgenic rabbits. Before clinical application the enzyme was tested first for its efficacy in Pompe knockout mice. Safety was demonstrated in a phase I study in healthy human beings [105]. In the first clinical study 7 patients were enrolled, 4 patients with classic Pompe's disease and 3 with a milder form. The details of the results obtained in the latter patients are discussed in detail in chapter 3 of this thesis.

The clinical effects in the 4 patients with classic Pompe's disease 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 in muscle tissue normalized in all four patients after treatment with 40 mg/kg/week and muscle-morphology improved markedly in one patient. Cardiac hypertrophy improved in all four patients. Two patients achieved motor milestones that are unmet in classic Pompe's disease, but only one patient learned to walk. The long-term clinical response was variable. One patient responded very well to treatment and is at age 5.5 years still ambulant, with a normal cardiac size and function. One patient showed initially a good response to treatment, but her clinical condition deteriorated after an infection of the Port A Cath, she finally died at age 4.5 years after a period of intractable fever and unexplained coma. The two other patients were 7 and 8 months old when treatment started. They initially showed improvement of muscle function, but they are fully ventilator dependent at age 6 years and muscle function is minimal. It was concluded that the therapeutic effect is largely dependent on the clinical condition of the patient at start of treatment [57, 58, 106].

In the same year that we started our study a second trial started, in which 3 infants with Pompe's disease were treated with recombinant human α-glucosidase derived from CHO-cells. The dose they received was 2 times 5 mg/kg/week initially, which was later increased in two patients. The cardiac dimensions of all three patients decreased and their cardiac function was maintained and they survived for more than a year [62]. One patient responded very well to treatment, but recently it was communicated that this patient did not have the classic infantile form of Pompe's disease (dr. P Kishnani, personal communication, Heidelberg, Germany 2003). The other two patients became ventilator dependent at 8 months (5 months of treatment). New studies with 10 mg/kg per week and 20 or 40 mg/kg/2 weeks have been initiated, but no definite reports have been published.

Apart from classic infantile Pompe's disease there are other clinical subtypes with skeletal muscle weakness as the main symptom, but with a slower disease progression and minimal or no cardiac involvement. The first symptom may present in infancy, childhood, adolescence or adulthood. The studies described in this thesis are focused on the safety and efficacy of ERT for these patients.

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The natural course of non-classic Pompe's disease; a review of 225 published cases

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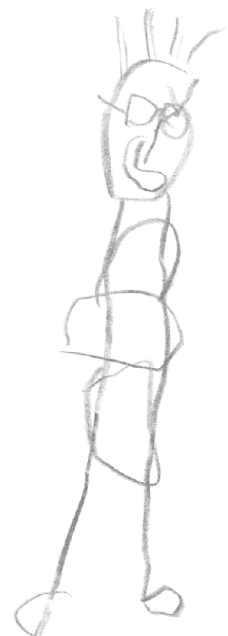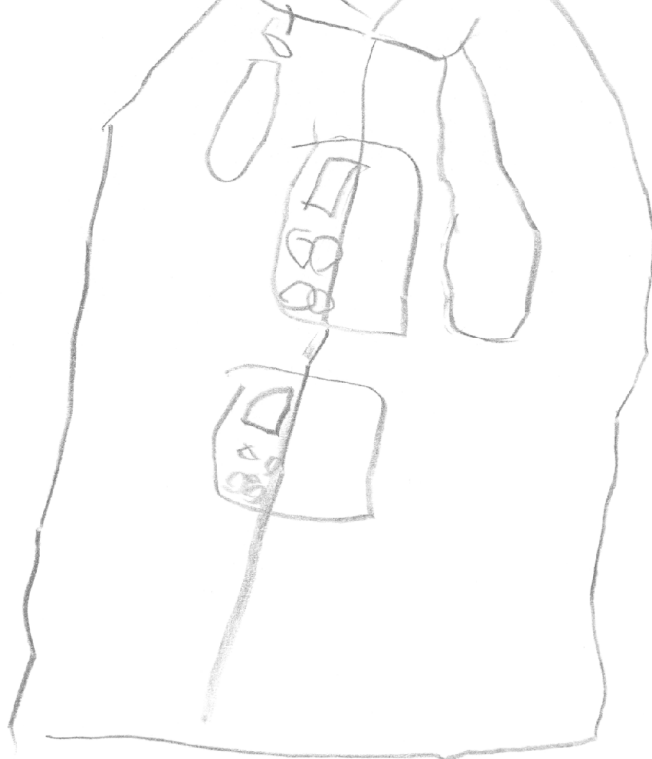
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Abstract

Pompe's disease is a neuromuscular disorder caused by the deficiency of lysosomal acid α-glucosidase. Recombinant human α-glucosidase is under evaluation as therapeutic drug. In light of this development we studied the natural course of cases not fitting the definition of classic infantile Pompe's disease. Our review of 107 reports including 225 cases shows a continuous spectrum of phenotypes. The onset of symptoms ranged from 0 to 71. We were unable to establish criteria to delineate sub-types. For clinical practice, we propose to use the term non-classic Pompe's disease for this spectrum of phenotypes and speak about 'infantile', 'childhood', 'juvenile' or 'adult' cases, to focus on the current age.

Common denominators of these cases are that symptoms are related to or caused by muscle weakness, early onset of symptoms seems to be associated with faster disease progression, and respiratory failure is the most frequent cause of death. CK, LDH, ASAT, ALAT and muscle glycogen levels are frequently elevated, but normal levels do not exclude non-classic Pompe's disease, neither does normal morphology of a muscle biopsy. Measurement of deficient α-glucosidase in fibroblasts or skeletal muscle confirms the diagnosis. The use of leukocytes is error prone.

Clinical studies in non-classic Pompe's disease should focus on skeletal muscle strength and function, disability, handicap, quality of life and pulmonary function. Prospective studies on these aspects are important for setting endpoints in future clinical trials.

Introduction

Pompe's disease is a metabolic myopathy caused by the deficiency of acid α-glucosidase needed for the degradation of lysosomal glycogen [1, 2]. The disease was so far untreatable but enzyme therapy, implying the intravenous administration of recombinant human α-glucosidase, is currently under investigation and the first results are promising. Patients with early and late onset forms of Pompe's disease may regain muscle strength and function if therapy is started in time [3-5].

With studies on enzyme therapy well underway it becomes increasingly important to recognise signs and symptoms of the disease properly and to establish the diagnosis without delay. Accurate knowledge on the natural course of the disease is further required to set endpoints for pivotal clinical trials and to decide in each individual case at what moment enzyme therapy should be started once it is generally available.

In 1932 J.C. Pompe presented the first case report. It concerned a patient with a hypertrophic cardiomyopathy and progressive generalised muscle weakness [6]. The child died at eight months of age. This severe form of the disease is quite well delineated [7-10]. Symptoms start at a median age of 1.6 months, patients die at a median age of 6-8 months, a hypertrophic cardiomyopathy is characteristically present, and developmental milestones like rolling-over sitting and standing are not achieved. It is called the classic infantile subtype of Pompe's disease.

Milder subtypes were described later, these were called non-classic infantile, childhood, juvenile, adolescent, adult and late-onset forms of Pompe's disease [11-13]. The knowledge about the natural course of these subtypes is fragmentary. Most reviews describe a small number of cases and cover a relatively short period in time. Guidelines for sub-classification are not clearly set [14-22].

To delineate the features of these subtypes that do not fit the description and course of classic infantile Pompe's disease, we performed a literature search

on clinical descriptions of patients and tried to bring to light as many clinical descriptions of patients as possible. This resulted in a compilation of 225 case reports from 107 articles. Information was gathered about disease specific signs and symptoms, age at onset of disease, age at diagnosis, and diagnostic criteria and procedures. All data were entered into a database. We subdivided the patients in four categories on the basis of reported age of onset and then compared the clinical parameters by the category.

Methods

We included all case reports identified via Pubmed by a search for "Late (-) onset Pompe's disease", "Acid Maltase deficiency", "Glycogenosis type II/ 2", "Glycogenosis type 2a" and "Childhood -", "Juvenile -", "Adult -" and "Non(-)Classic Infantile Pompe's disease". Case-reports cited in the collected articles, and case-reports dating before 1965 and not identified via Pubmed were added to the list. Articles written in English, French, German or Dutch were included.

Excluded were publications lacking clinical information, cases with normal acid α-glucosidase activity in muscle tissue or fibroblasts and/or cases described as Danon's disease [23-29]. We further excluded all cases that fulfilled the criteria of classic infantile Pompe's disease, which were earlier included in a review on the natural course of infantile Pompe's disease [9]. This led to a collection of 225 cases in 107 articles [12-22, 30-127].

The following variables were scored for each case described: Gender, nationality/country, age at diagnosis/onset of disease, age of the patient at time of description in article, age at death, first symptoms, all symptoms and signs described, developmental milestones (mental/motor), wheelchair use, pulmonary function, respiratory failure and/or use of artificial ventilation, laboratory findings including acid α-glucosidase activity in cells and tissues, mutations in the α-glucosidase gene, muscle pathology, muscle glycogen content and other diagnostic tools.

If particular symptoms or signs were not reported, they were scored as negative. Laboratory findings were scored as abnormal (high) or normal, and by exact value, according to what was reported by the authors.

All findings were entered into a database and analysed using SPSS 10.1. This led to a list of over 50 variables. We used descriptive statistics / frequencies for all calculations in this report. The data presented are medians, unless otherwise indicated.

In order to identify subtypes of Pompe's disease we divided the cases in four categories by age of onset: Group 1, first year; group 2, from 1 – 6 year; group 3, from 6 –18 year; group 4, 18 years and older. We then compared the patients in the four categories with regard to symptoms and signs, age at time of description or age at death, and use of wheelchair and/or respiratory support.

Results

Population

We collected 225 case reports of patients with Pompe's disease from a total of 107 articles, published between 1965 and 2003. The course of the disease of the patients did not fulfil the criteria of classic infantile Pompe's disease. The case reports originated from 19 countries, as indicated in Table 1.

Country	Frequency	Percentage
United States	67	29.8
France	36	16
The Netherlands	34	15.1
United Kingdom	16	7.1
Japan	14	6.2
Germany	13	5.8
Italy	9	4
Belgium	5	2.2
New Zealand	5	2.2
Spain	5	2.2
SouthAfrica	5	2.2
Switzerland	5	2.2
Greece	3	1.3
Austria	2	0.9
Canada	2	0.9
Albania	1	0.4
China	1	0.4
Israel	1	0.4
Sweden	1	0.4

Table 1 The list of countries from which the case reports originated

Ninety-six patients were female (43%), 128 patients were male (57%), in one case gender was not described. Median age at time of description was 33 years (n=189), and the age range varied from 0.8 to 71.0 years (Fig. 1).
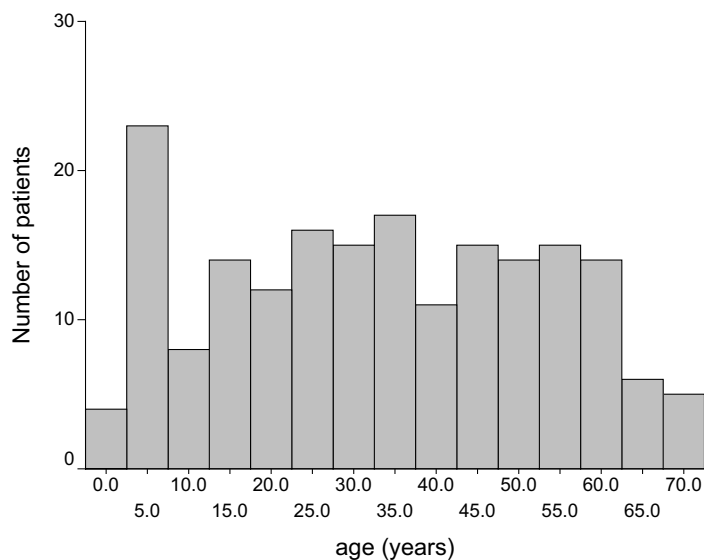
Fig. 1 The age-distribution of the patients at description in the original articles

In 36 case reports (6%) the patient had deceased at the time of description. The median age at death of these patients was 24.5 years (range 0.9 – 66 years) (Fig. 2).
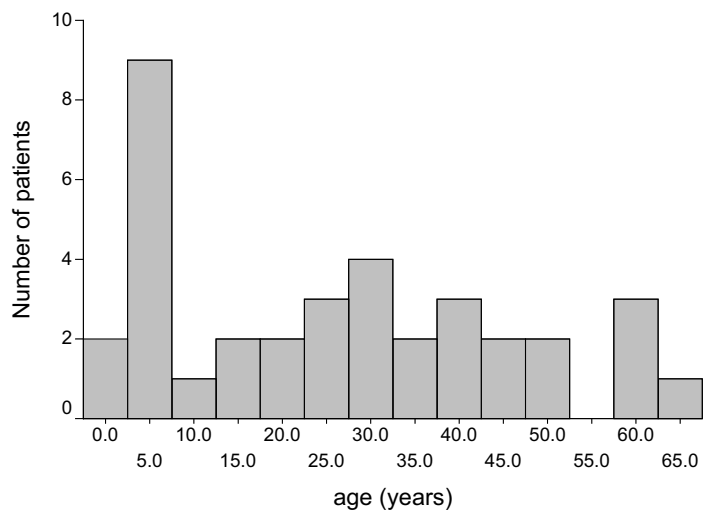
Fig. 2 The age-distribution of the patients when they died

Course of the Disease
The age at first symptoms was mentioned in 172 of the 225 cases. The median age of onset was 24.0 years (range 0.0–68.0 years). Thirty-two patients presented

symptoms in the first year of life. The time between onset of symptoms and diagnosis varied greatly from 0.0-47.0 years (median is 7 years, n=157). The diagnosis was established at a median age of 33 years (range 0.1-71.0 years).

In 62 of 225 patients (28%) use of artificial ventilation was reported. In these patients ventilation was started at a median age of 34.0 years (range 3.0-59.0 years, n=49). In only 18 case reports it was indicated that the patient used a wheelchair (8%), and in six cases it was also mentioned when the patient started using it (range 3.0–49.0 years).

Further analysis showed that the deceased patients had experienced their first symptoms significantly earlier (at the age of 7.0, range 0 - 60 years vs. 24.0, p=0.018, Mann-Whitney test) and were also significantly younger at the time of diagnosis (24.0 years, range 0.7 – 65.0 years versus 33.0 years, p=0.037, Mann-Whitney test) than for the total group. The time between the onset of symptoms and the diagnosis did not differ, neither did the percentage of patients using artificial ventilation or the age at which they started using it (Table 2).

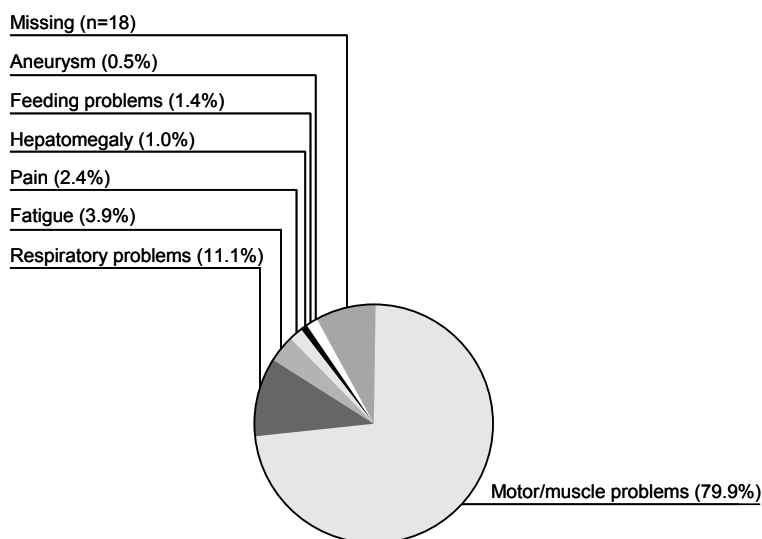| Age of Onset:		Whole Group n=225 (100%)	0-1 years n=32 (14%)	1-6 years n=24 (11%)	6-18 years n=30 (13%)	18 years and older n=139 (62%)
Symptoms						
Muscle weakness		213 (95)	31 (97)	23 (96)	26 (87)	133 (96)
Walking problems		106 (47)	23 (72)	20 (83)	10 (33)	53 (38)
Respiratory Problems	Respiratory symptoms	99 (44)	17 (53)	6 (25)	12 (40)	64 (46)
	Artificial ventilation	62 (28)	13 (41)	3 (13)	5 (17)	41 (30)
Problems climbing stairs		58 (26)	18 (56)	9 (38)	5 (17)	26 (19)
Problems rising		59 (26)	20 (63)	8 (33)	5 (17)	26 (19)
Lordosis/Kyphosis/Scoliosis		45 (20)	3 (9)	6 (25)	7 (23)	29 (21)
Problems sporting/running		40 (18)	7 (22)	9 (38)	5 (17)	19 (14)
Low or absent reflexes		37 (16)	6 (19)	5 (21)	3 (10)	23 (16.5)
Delayed motor milestones		31 (14)	26 (81)	5 (21)	0	0
Hypotonia		25 (11)	17 (53)	2 (8)	2 (7)	4 (3)
Cardiac involvement		25 (11)	16 (50)	2 (8)	1 (3)	6 (4.3)
	Hypertrophic cardiomyopathy	12(5)	12(38)	0	0	0
	Cor Pulmonale	4(2)	0	0	1(3)	3(2)
	other heart abnormalities	7(3)	4(1)	2(8)	0	2(1)
Fatigue		23 (10)	2 (6)	2 (8)	3 (10)	16 (12)
Large tongue		20 (9)	13 (41)	1 (4)	1 93)	5 (4)
Pain		13 (6)	0	1 (4)	1 (3)	11 (8)
Feeding problems		14 (6)	12 (38)	1 (4)	0	1 (1)

Age of Onset:		Whole Group n=225 (100%)	0-1 years n=32 (14%)	1-6 years n=24 (11%)	6-18 years n=30 (13%)	18 years and older n=139 (62%)
Hepato(spleno)megaly		8 (4)	5 (16)	3 (13)	0	0
Weight problems	Underweight	9 (4)	1 (3)	3 (13)	1 (3)	4 (3)
	Overweight	3 (1)	0	0	0	3 (2)
Aneurysms		6 (3)	1 (3)	1 (4)	2 (7)	2 (1)
Difficulty lifting object		5 (2)	0	0	0	5 (4)
Difficulty combing hair		3 (1)	0	0	0	3 (2)
Hyperparathyreoidism		1 (0.4)	0	0	1 (3)	0
Scapula alata		5 (2)	1 (3)	3 (13)	1 (3)	0
Foot abnormalities		5 (2)	1 (3)	1 (4)	2 (7)	1 (1)
Abnormal speech		9 (4)	2 (6)	1 (4)	0	6 (4)
Epilepsy		2 (1)	0	0	0	2 (1)
Abnormal mental development		3 (1)	1 (3)	1 (4)	0	1 (1)
Female		96 (43)	12 (38)	5 (21)	11 (37)	68 (49)
Age at onset		24 (0-68) n=172	0.25 (0-1) n=31	2.0 (1.1-4.5) n=19	12 (6-17) n=20	35 (18-68) n=102
Age at diagnosis		33 (0.1-71) n=206	3.75 (0.1-17) n=20	7 (1.6-32) n=32	17 (6-61) n=32	43 (18-71) n=134
Doctors'delay		7 (0-47) n=157	3.7 (0.9-17) n=19	6 (0-28) n=19	1 (0-47) n=20	7 (9-34) n=99
Age at description		33 (0.8-71) n=189	4.1 (0.8-28) n=20	(2.5-32) n=20	17 (6-61) n=25	44 (19-71) n=124
Start of ventilation		34 (3-59) n=49	7.5 (3-18) n=6	7 (7-24) n=3	18.5 (15-31) n=6	38 (21-59) n=34
Start of wheelchair		16 (3-49) n=6	3.5 (3-4) n=2	7 n=1	32 (25-39) n=2	49 n=1
Age at death		24.5 (0.9-66) n=36	6.1 (0.9-24 n=12	22.6 (6.5-28) n=4	25.1 (15-40.5) n=5	44.9 (25-66) n=15

Table 2 Symptoms and findings related to age of onset

Clinical History: First Symptoms and Signs

First symptoms were described in 207 (92%) of the 225 cases (Fig. 3). Most often mentioned were symptoms and signs related to muscle weakness (165 of 207 patients; 80%). These comprised abnormal walking, difficulty with climbing stairs, delayed motor development and hypotonia. Second most frequent were respiratory problems. These were described in 23 of 207 patients (11%). Respiratory failure was the presenting symptom in five patients (2% of all cases). Fatigue, muscle-pain, feeding problems, hepatomegaly, and (the rupture of) a cerebral aneurysm were further reported as first symptom.

Missing (n=18)

Aneurysm (0.5%)

Feeding problems (1.4%)

Hepatomegaly (1.0%)

Pain (2.4%)

Fatigue (3.9%)

Respiratory problems (11.1%)

Motor/muscle problems (79.9%)

Overall Symptoms and Signs

All symptoms and signs with their frequency as reported for the 225 patients are listed in Table 2. During the course of the disease virtually all patients experienced symptoms related to loss of muscle function (95%). Problems rising from a chair or from the floor, problems in participating in sports and difficulties in performing daily life activities, such as combing hair or lifting objects, were reported in addition to the muscle problems documented as first symptoms. Forty-four percent of the patients were reported to have respiratory difficulties. Initiation of artificial ventilation was documented for 28% of all cases. Cerebral aneurysms were found in 6 cases (3%).

Lordosis /kyphosis /scoliosis, absent or decreased deep tendon reflexes, cardiac involvement, large tongue, abnormal speech, and low body weight were reported as additional features.

Cause of Death

The most frequent cause of death (26 out of 36 cases, 72%) was respiratory failure. Nine patients died despite the initiation of artificial ventilation. Pneumonia as cause of respiratory failure was reported six times. Other causes of death were rupture of a cerebral aneurysm (n=4); cardiac failure (n=1) and intractable fever (n=2).

Age-related problems
We further investigated whether the age of onset as documented in the case reports was related to the occurrence of a particular subset of symptoms and the rate of disease progression. For this purpose case reports were subdivided in four groups by age of onset of symptoms (see Table 2).

There appeared to be an overlap between the four groups when time between onset of symptoms and diagnosis, and the ages at description, start of ventilation or death were compared. For example, among the patients who presented in the first year of life there was a patient who was diagnosed at the age of 17 years and was still alive at the age of 28 years. Another patient who experienced first symptoms between 6 and 18 survived beyond 61. The oldest patient described in the literature, 71 years old, presented with symptoms after 18 years.

Symptoms related to muscle and respiratory function were reported in similar frequency in all groups. An enlarged tongue, a scoliosis, and hypotonia were most often reported in the group of patients that presented before the age of one, but were also documented for patients who presented later. Delayed motor milestones, feeding problems and hepatomegaly were only reported for patients with first symptoms under 6 years of age. Presence of cardiac hypertrophy was only found in a subset of patients presenting before the age of one year.

Biochemical findings
Creatine kinase, LDH, ASAT and ALAT were frequently elevated (Table 3), but not in all. All patients with normal CK levels (n=12) presented with symptoms after 18 years of age. In four cases with normal CK levels, levels for ALAT and ASAT were also available, ASAT and ALAT appeared elevated in two of these cases.

Diagnostic Tools:	n	median value (range)	% result fitting the diagnosis Pompe	% false negative
AGLU in fibroblasts	24	3% (0-25)	100	0
AGLU in muscle	153	4% (0-35)	100	0
AGLU in leukocytes	89	14% (0-125)	90	10
Muscle glycogen	74	N.A.	80	20
Muscle morphology	74	N.A.	97	3
CK	138	354 IU (3-4270)	91	9
LDH	46	561 IU (76-3175)	96	4
ALAT	34	79 IU (29-1331)	94	6
ASAT	55	82 IU (14-580)	95	5

Table 3 Diagnostic tools and biological markers and their sensitivity

Diagnostic tools
In 208 of the 225 cases the diagnosis Pompe's disease was confirmed by measurement of α-glucosidase deficiency in leukocytes, fibroblasts and/or in muscle tissue (Table 3). Various substrates were used (i.e. the artificial substrate 4-methylumbelliferyl α-D-glucopyranoside or the natural substrates glycogen and maltose). Measurement in fibroblasts or muscle biopsies always showed deficiency of α-glucosidase. Nine patients were reported to have a normal α-glucosidase

activity in leukocytes. They all presented with symptoms after the age of 18 years.

In the 17 cases that the α-glucosidase activity was not measured the diagnosis Pompe's disease was based on the finding of an increased glycogen content and/or abnormal tissue morphology of a muscle biopsy.

Twenty percent of 74 patients had a normal muscle glycogen content. Thirteen of these patients presented with symptoms after the age of 18 years. Muscle tissue specimens were mostly reported to be heterogeneously affected. PAS-positive (diastase sensitive) vacuoles were observed using light microscopy. With electron microscopy membrane bound vacuoles filled with glycogen were visualised. In some cases type I fibres were reported to be more affected than type II. In 2 cases the muscle had normal morphology. Besides in skeletal muscle, glycogen accumulation was reported to be present in eccrine glands, pericytes, endothelial cells, fibroblasts, smooth muscle cells, and Schwann cells of small nerve fibres of skin and muscle [47]. Glycogen was also found in smooth muscle of the basilar artery in a patient who died of a ruptured cerebral aneurysm [90].

Other diagnostic tools
Electromyography performed in patients with Pompe's disease may typically show profuse myotonic discharges, sometimes combined with fibrillations. In a subset of patients presenting before the age of one a hypertrophic cardiomyopathy was found. Echocardiography data were scarcely available for the older patients. Measurement of pulmonary function was only reported in 94 out of 225 cases; in most cases only a single measurement was reported. Evaluation of pulmonary function over time could not be extracted from the data.

Molecular characterisation
In 40 patients one or both mutant alleles were identified (Table 4). The most common mutation found in the α-glucosidase gene was the IVS1-13T->G (n=32). The age range of patients having this mutation was 2-53 years; median age 35 years. In 10 of the latter cases the other allele expressed the completely deleterious mutation 525delT. One patient was found to be homozygous for the IVS1-13T->G mutation.

No Patients	Allele 1	effect	Allele 2	effect	Age at onset in articles	IVS1 combined with other mutation
13	IVS1	mild	?		34 (range 2.0-53.0)	35 (range 2.0 - 53.0)
10	IVS1	mild	DelT525	severe	30 (range 18.0-52.0)	
1	IVS1	mild	L208P	severe	29	
1	IVS1	mild	G643R	severe	34	
1	IVS1	mild	P161Shift	severe	34	
1	IVS1	mild	R40X	severe	43	
1	IVS1	mild	IVS1	mild	35	
1	IVS1	mild	R585M	severe	36	
1	IVS1	mild	R854X	severe	41	
1	IVS1	mild	delta6aa607>612	severe	43	
3	del exon18	severe	?		55	
1	w490X	severe	p324L	less severe	2	
1	H308	severe	R672T	less severe	39	
1	G643R	severe	?		49	
1	Tyr292Cys	mild	null	severe	1.1	
2	p361L9exon7	?	R437C(exon8)	?	0.8 / asymptomatic	
40						

Table 4 Mutations related to severity found in database and age of onset

Discussion

Our study shows that Pompe's disease may present at any age from infancy to late adulthood. We investigated whether we could delineate subtypes of "non-classic" Pompe's disease by the course of disease and the type and severity of symptoms. To this end we subdivided patients by age of onset in four categories and compared these subjects. It appeared that some features, like a hypertrophic cardiomyopathy, hepatomegaly and developmental delays, occurred more often in the younger patients. However, we could not identify specific subsets of symptoms and/or different rates of disease progression that could serve as criteria for sub-typing. In all age groups there were patients with a more rapid and severe course compared to patients with a slower and milder course of disease. All in all there appears to be a tendency that an early start of symptoms leads to a faster progression of disease. On basis of these findings, we conclude that there are no criteria for sub-typing of "non-classic" Pompe's disease. Similarly, in a recent retrospective questionnaire study in 58 adult Pompe patients from the Netherlands we found that 58% of patients already experienced symptoms during childhood (unpublished results).

Taken these observations we propose to use the term classic Pompe's disease for all infants who develop symptoms in the first year of life, manifest hypertrophic cardiomyopathy, suffer from severe hypotonia and demonstrate developmental delay. These patients typically die within the first year of life [9]. We further propose to use the term non-classic Pompe's disease for all remaining cases and speak about 'infantile', 'childhood', 'juvenile' or 'adult cases' of non-classic Pompe's disease to maintain the possibility to sub-classify the patients by their current age.

Our study shows that non-classic Pompe's disease, like classic Pompe's disease [9], occurs worldwide. No cases from South America were incorporated, possibly because we excluded publications written in Spanish and Portuguese. As expected for an autosomal recessive disease, we found an equal male-female distribution for the patients with onset of symptoms above the age of 18 years. Below the age of 18 there were slightly more affected males than females. The

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latter was also the case in the group of patients with classic Pompe's disease [9]. We do not have an explanation for this phenomenon.

Symptoms and signs that point to non-classic Pompe's disease are: hypotonia, delayed motor development, muscle weakness, difficulties in walking and climbing stairs, problems in rising form a chair or floor, difficulties in participating in sports, fatigue, muscle pain or cramps, scoliosis, lordosis, respiratory difficulties, unexplained respiratory failure, a hypertrophic cardiomyopathy, a thick tongue, and a cerebral aneurysm, or a combination of these signs.

Biological indicators of non-classic Pompe's disease are increased CK, LDH, ASAT and/ or ALAT levels in blood, but normal levels do not exclude the disease. For CK this confirms the findings of Ausems et al. who reported earlier that measurement of CK is a sensitive marker for Pompe's disease. In the latter study CK levels were increased in all patients, including 5 patients who were still asymptomatic [128]. From the results of our review it does not become evident whether measurement of ASAT, ALAT and LDH has additional value, although in two cases increased ALAT and ASAT levels were reported while CK was normal. For patients with classic infantile Pompe's disease we found earlier that the serum ALAT, ASAT and LDH levels increased with progression of disease and were more sensitive follow-up parameters than CK [9].

Membrane bound glycogen observed by electron microscopy, or PAS positive vacuoles visualised by light microscopy may further direct to the diagnosis, and are theoretically better diagnostic markers than increased glycogen levels in muscle tissue sections. Importantly, muscle glycogen may be normal in patients with non-classic Pompe's disease (20% of all cases in which it was measured) like CK, ALAT, ASAT, LDH. Also the muscle morphology may not disclose abnormalities. In literature this was most often reported in patients presenting with symptoms after the age of 18years.

We conclude that the diagnosis Pompe's disease can not be established or excluded on basis of clinical testing or biological markers alone, and should always be confirmed by demonstrating α-glucosidase deficiency or deleterious mutations in both α-glucosidase alleles. The enzymatic assay on fibroblasts is the most sensitive, but can also be done on a muscle biopsy specimen. The median residual enzyme activity reported here is much lower, with a larger range, than was reported earlier [129]. This can be first explained by the fact that here all non-classic patients are taken as one group (non-classic through the adult patients). Second in several adult cases deficient or 0 enzyme activity is reported, which might be explained by measuring methods that are not sensitive enough.

Measurement of α-glucosidase activity in leukocytes may show normal results due to the presence of neutral maltases. In the literature, normal activities in leukocytes were reported only for patients over 18 years of age. If glycogen is used as substrate, there is the possibility of finding a pseudo-deficiency as 1 in 16 individuals statistically carries the GAA2 allele [96, 130]. As reported by Whitaker et al, the use of lymphocytes may also give rise to unreliable results [131].

The most common mutation in non-classic Pompe's disease is IVS1-13T$\rightarrow$ G. Patients with this mutation, identified in the current review, had an onset of symptoms between the age of 2 and 53 years, but the finding is hard to interpret by incompleteness of the α-glucosidase genotypes. Most case reports reviewed in this study did not include mutation data. A list of known mutations and their effect in both classic and non-classic Pompe's disease can be found at www.Pompecenter.nl.

The most frequently reported cause of death in non-classic Pompe's disease

is respiratory failure. Less than 40% of the patients who had died were mentioned to have had artificial ventilation. Especially the older literature will not reflect the current situation, but follow up and support of the respiratory function in patients with non-classic Pompe's disease certainly deserves attention [132].

The second most common cause of death was a ruptured cerebral aneurysm (4 out of 36). The prevalence of a cerebral aneurysm among all patients with non-classic Pompe's disease was 2.7% (6 out of 225), which is similar to the prevalence of 1.9%, found in the general population (range 1.5-2.4%) [133]. The question remains whether the patients with Pompe's disease were sufficiently examined for the presence of a cerebral aneurysm. A higher frequency of (cerebral) aneurysms can be explained, as glycogen accumulation was demonstrated to occur in smooth muscle cells of both humans and mice with Pompe's disease [47, 134]. Makos et al. specifically reported the presence of glycogen accumulation in the medial smooth muscle of the basilar artery of two patients with non-classic Pompe's disease, who died from a cerebral aneurysm [90]. In mice lysosomal glycogen storage was found in smooth tissue of bowel, bladder, uterus, arteries and veins [135]. Noteworthy, prolonged intravenous administration of recombinant human α-glucosidase to both humans and mice resulted in clearance of glycogen from smooth muscle cells [5, 134, 136].

Not all information appeared well documented in literature. The time frame in which the disease progresses from first symptoms to various disease specific events, like for example difficulties in climbing stairs, rising from a chair, need of walking aids and wheelchair dependency could insufficiently be extracted from the literature. Sequential measurements of pulmonary function were also rarely reported. Ventilator use was documented for 28% of the patients, but most reports lacked information on whether the patient was partially or completely ventilator dependent and whether ventilation was performed only during the night (in supine position) or also during the day, via nose hood or via a trachea canula. Wheelchair use was only documented in 18 case reports and use of walking aids was rarely recorded. More detailed information should also be obtained on how progressive loss of muscle function affects daily life activities and quality of life.

Our study addresses the importance to study these aspects of the natural course of non-classic Pompe's disease in a well-defined cohort of patients, before enzyme replacement therapy becomes generally available. In conclusion, for future clinical studies only two subtypes of Pompe's disease should be recognised being the classic and the non-classic form of Pompe's disease. Clinical trials in non-classic Pompe's disease should focus on skeletal muscle strength and function, disability, handicap, quality of life and pulmonary function.

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Enzyme replacement therapy in late-onset Pompe's disease: a three-year follow up

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Abstract
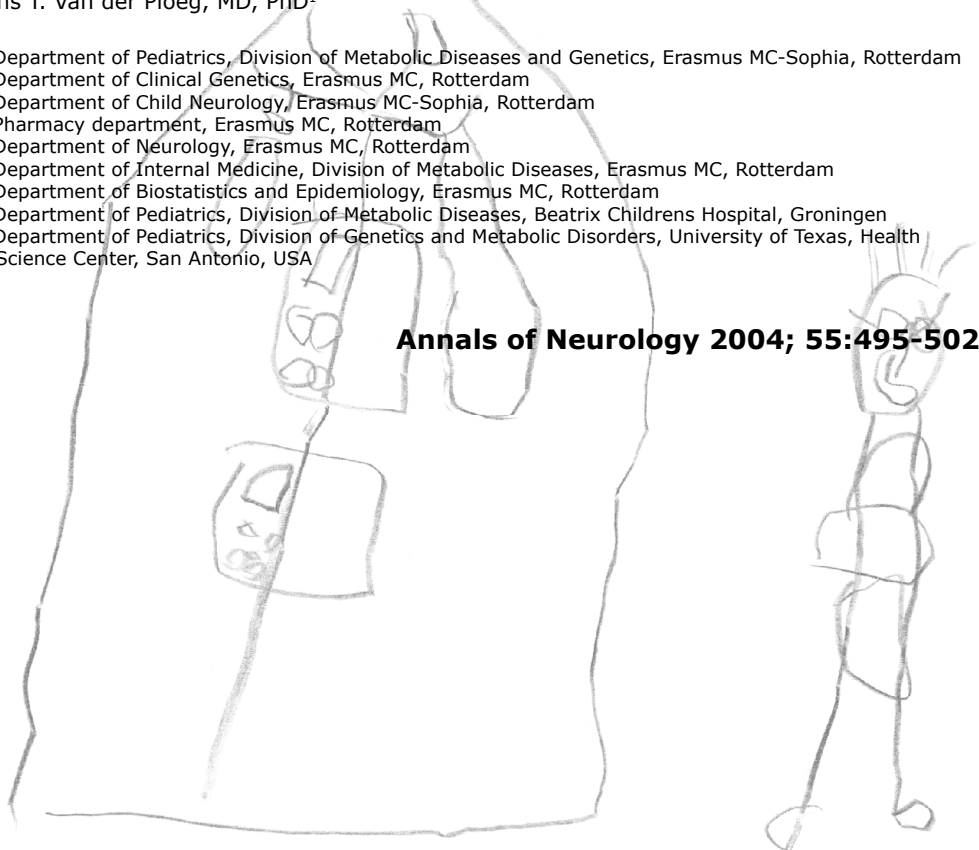
Pompe's disease is an autosomal recessive myopathy. The characteristic lysosomal storage of glycogen is caused by acid α-glucosidase deficiency. Patients with late-onset Pompe's disease present with progressive muscle weakness also affecting pulmonary function.

In search for a treatment we investigated the feasibility of enzyme replacement therapy with recombinant human α-glucosidase from rabbit milk. Three patients (aged 11, 16 and 32 years) were enrolled in the study. They were all wheelchair bound and two of them were ventilator dependent with a history of deteriorating pulmonary function.

After three years of treatment with weekly infusions of α-glucosidase the patients had stabilized pulmonary function and reported less fatigue. The youngest and least affected patient showed an impressive improvement of skeletal muscle strength and function. After 72 weeks of treatment he could walk without support and finally abandoned his wheelchair.

Our findings demonstrate that recombinant human α-glucosidase from rabbit milk has a therapeutic effect in late-onset Pompe's disease. There is good reason to continue the development of enzyme replacement therapy for Pompe's disease and to explore further the production of human therapeutic proteins in the milk of mammals.

Introduction

Pompe's disease or glycogen storage disease type II is an inherited myopathy characterized by lysosomal accumulation of glycogen and caused by acid α-glucosidase deficiency. Differences in the age of onset and rate of disease progression distinguish infantile from late-onset subtypes [1, 2].

Infants with classic infantile Pompe's disease manifest feeding-difficulties, generalized muscle weakness, cardiomyopathy and respiratory insufficiency. They have a median life span of 6-8 months and usually succumb by cardiorespiratory failure [3].

Late-onset Pompe's disease presents as a proximal myopathy with symptoms restricted to skeletal muscle. Limb-girdle weakness is often the first sign and may lead to scoliosis. Most patients become wheelchair dependent and may require artificial ventilation later in life [1, 2].

Enzyme replacement therapy (ERT) is currently under investigation as treatment for Pompe's disease. This therapeutic approach aims to supplement the deficiency of acid α-glucosidase by intravenous administration of highly purified enzyme, finding its way to the lysosomes via endocytosis [4-6]. The same type of treatment has been utilized in other lysosomal storage disorders, whereby recombinant human enzymes are employed, produced in genetically modified animal or human cells [7-10]. The first clinically applicable recombinant human α-glucosidase became available through production in the milk of transgenic rabbits. After successful completion of pre-clinical investigations we started clinical studies with this enzyme in early 1999 [11-14]. The first pilot study included four patients with classic infantile Pompe's disease. The procedure appeared to be safe, and positive effects were seen after 36 weeks of treatment [5, 15]. At present, three of the four patients are still alive at an age of 5.5 years [6]. Several other studies in infants were started with recombinant human α-glucosidase from CHO-cells [16].

We expanded our study to include individuals with late-onset Pompe's disease as they represent the largest group of patients. This is the first report on

three patients with juvenile Pompe's disease, who have received weekly infusions of recombinant human α-glucosidase from rabbit milk over a 3-year period.

Patients and Methods

Study Design
The study was conducted as a single center, open label, pilot study and approved by the Institutional Review Board of the Erasmus MC. Written informed consent was obtained from the patients and the parents, if required. The study objective was to evaluate safety and efficacy of recombinant human α-glucosidase from rabbit milk (rhAGLU).

Inclusion Criteria
Clinical and laboratory findings had to be consistent with late-onset Pompe's disease. The diagnosis had to be established before the age of 15, and confirmed by acid α-glucosidase deficiency and lysosomal glycogen storage in an open muscle biopsy. Patients had to be older than 4 and younger than 35 years at inclusion. Developmental delays not explained by Pompe's disease, allergies, and other conditions that could potentially interfere with the evaluation of the study objectives, were exclusion criteria.

Treatment
RhAGLU was provided by Pharming-Genzyme LLC. Enzyme purification and characterization was performed as previously described [13, 14]. The enzyme was administered intravenously as a 1-2 mg/ml solution in saline with 5 % glucose and 0.1% human serum albumin, initially in single weekly doses of 10 mg/kg, and later 20 mg/kg with a transition period of 15 mg/kg (Fig 1).
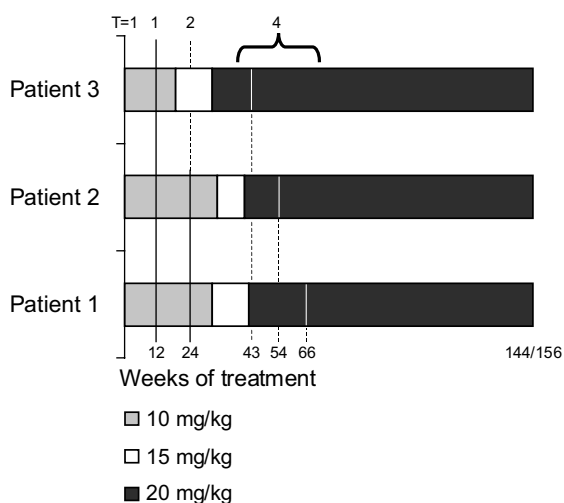

Figure 1
Overview of the dosing regimen and the time of biopsy for each patient. Biopsies were taken before treatment (t=1), after 12 weeks (t=2), and after 24 weeks (t=3) with a dose of 10 mg/kg/week, and after 12 weeks of 20 mg/kg/week (t=4). The total treatment duration of patient 1 and 2 was 156 weeks and 144 weeks for patient 3.

Muscle biopsy
Open muscle biopsies were taken at baseline, 12 and 24 weeks after start of treatment with 10 mg/kg/week, and minimally 12 weeks after increasing the dose to 20 mg/kg/week (Fig 1). The biopsies were performed 24 hours after the rhAGLU infusion. Tissue specimens for measurement of acid α-glucosidase activity and histology were prepared as described [6, 17, 18].

Assessments
The pulmonary function (EVC/FEV1) was measured with spirometry. Historic data were used for comparison. Muscle strength was measured with the Citec hand held dynamometer [19, 20] by trained physical therapists and with the MRC score by neurologists [21]. The scores given to each muscle group were added to obtain a total score for upper-, lower- and total body. The muscle groups tested were neck flexion, neck extension, shoulder abduction, elbow flexion and extension, wrist extension, jaw chuck (MRC only), hip flexion and abduction, knee extension and flexion, and ankle dorsiflexion and plantar flexion. The maximum MRC-score is 114 (normal strength of all muscles), and the minimum score 0 (complete paralysis).
        Muscle function was evaluated using the Gross Motor Function Measure (GMFM) and via timed tests (10 meter walk, the nine-hole peg, and rising from a chair and from the floor) [22]. Disability was evaluated with the Pediatric Evaluation of Disability Inventory (PEDI) [23]. ASAT, ALAT, CK and LDH were measured according to routine procedures[3]. Patient interviews and physical examinations documented clinical follow-up.

Statistical Analysis
The slopes of fitted curves were calculated using least-squares regression. In the evaluation of CK, outcomes were logarithmically transformed. Piece-wise linear regression ('broken stick' method) was applied to visualize and calculate changes in pulmonary function. Correlation coefficients given are Spearman's. P-values $\leq$ 0.05 were considered significant.

Patients
Table 1 summarizes the clinical histories. Patient 1 (16-y. female) and 2 (32-y. male) were in a far-advanced stage of the disease. They were wheelchair-bound and partially (patient 1) or fully (patient 2) dependent on artificial ventilation. The clinical condition of patient 1 was complicated by a progressive scoliosis, which had started at the age of 13 years. It progressed rapidly from a 30° right thoracic curve at age 14 to a 60° right thoracic curve and a left lumbar curve of 74° at the time of initiation of the study. Patient 3 was moderately affected. He had a normal pulmonary function at initiation of treatment, but used a wheelchair since two years.
        All three patients had mutations (Table 1) and enzyme-deficiencies (Table 2, column t=1) consistent with the diagnosis of late-onset Pompe's disease.

Characteristic	Patient 1	Patient 2	Patient 3
Age at inclusion (yr)	16	32	11
First symptom (age)	Difficulty climbing stairs (10 yr)	Difficulty lifting head during sports (7 yr)	Feeding difficulties (6 mo)
Age at diagnosis (yr)	11	10	2.5
Developmental milestones	Normal	Delayed	Delayed
Early motor development	Clumsy	Clumsy	Clumsy
Age walking	14 mo	2 yr	2.5 yr
Frequent airway infections	Yes	Yes	No
Ventilator dependency (age)	12 yr	15 yr	—
Ventilator use	18 hr/day	24 hr/day	—
Fatigue	Yes	Yes	Yes
Use of wheelchair since (age, yr)	16	22	9
Scoliosis/correction (age, yr)	13	14/15	—
Contractures	Yes	Yes	Yes
Genotype	[271G>A + 877G>S] "unknown"	IVS1-13t>g 1548G>A	IVS1-13t>g 525delT

Table 1. Characteristics of the Patiens at Start of the treatment

Results

This report describes a 3 years period wherein three patients with juvenile Pompe's disease received weekly infusions with recombinant human α-glucosidase from rabbit milk. Two patients did not experience an infusion-related reaction. The third patient had a three-month period of mild and transient skin reactions and received pre-medication with corticosteroids, antihistamines and cromoglycate.

Muscle strength and function
The least affected patient (patient 3) showed a dramatic gain of muscle strength and function. The total HHD score increased from 392 to 4684 Newton (r=0.97 p<0.001), and the total MRC sum score from 74 to 114 (maximum score) (r=0.92 p<0.001) over 144 weeks of treatment (Fig 2). The GMFM-score improved from 56.5% to 100% (r=0.99 p<0.001).

At the start of treatment, patient 3 was wheelchair bound and could not stand or walk (Fig 3A). After 72 weeks of therapy he could rise with difficulty from a chair employing the Gowers' maneuver and managed 10 steps on tiptoes. His muscle strength and function continued to improve after an Achilles tendon release procedure at 75 weeks of treatment. He performed the 10-meter walk test in 41 seconds in week 84, and 24 weeks later in only 3 seconds (Fig 3B). Further improvement was recorded by cycling against resistance, from 140 Watt after 108 weeks to 180 Watt (within the 10th percentile) after 132 weeks.

Patient 2 also showed a significant increase of muscle strength (HHD r=0.72 and MRC r=0.87, p<0.001) (Fig 2). At start of treatment he was virtually tetraplegic, but during treatment his leg, arm and neck muscles became a little bit stronger. This led to higher scores for self-care items in the PEDI questionnaire (dressing and washing).

Patient 1 lost muscle strength of the lower body during the first 53 weeks of treatment, mainly due to a progressive scoliosis. The right thoracic curve progressed from 60 to 68 degrees and the left lumbar curve from 74 to 90 degrees. Lumbar pain and the appearance of a Babinski reflex accompanied this. She lost the ability to walk without assistance (r=-0.84, p=0.010), but kneeling, crawling and sitting improved (r=0.90, p=0.002), leading to a significantly higher total GMFM-score (r= 0.86, p=0.007) (Fig 2).

Between week 64 and 66, the right thoracic and left lumbar curve were surgically corrected to 28 degrees. Thereafter the patient regained strength and function, more so in the upper than in the lower limbs. She learned to walk between parallel bars, but requires a wheelchair in daily functioning. Scores on self-care items of the PEDI questionnaire improved gradually (washing and dressing).
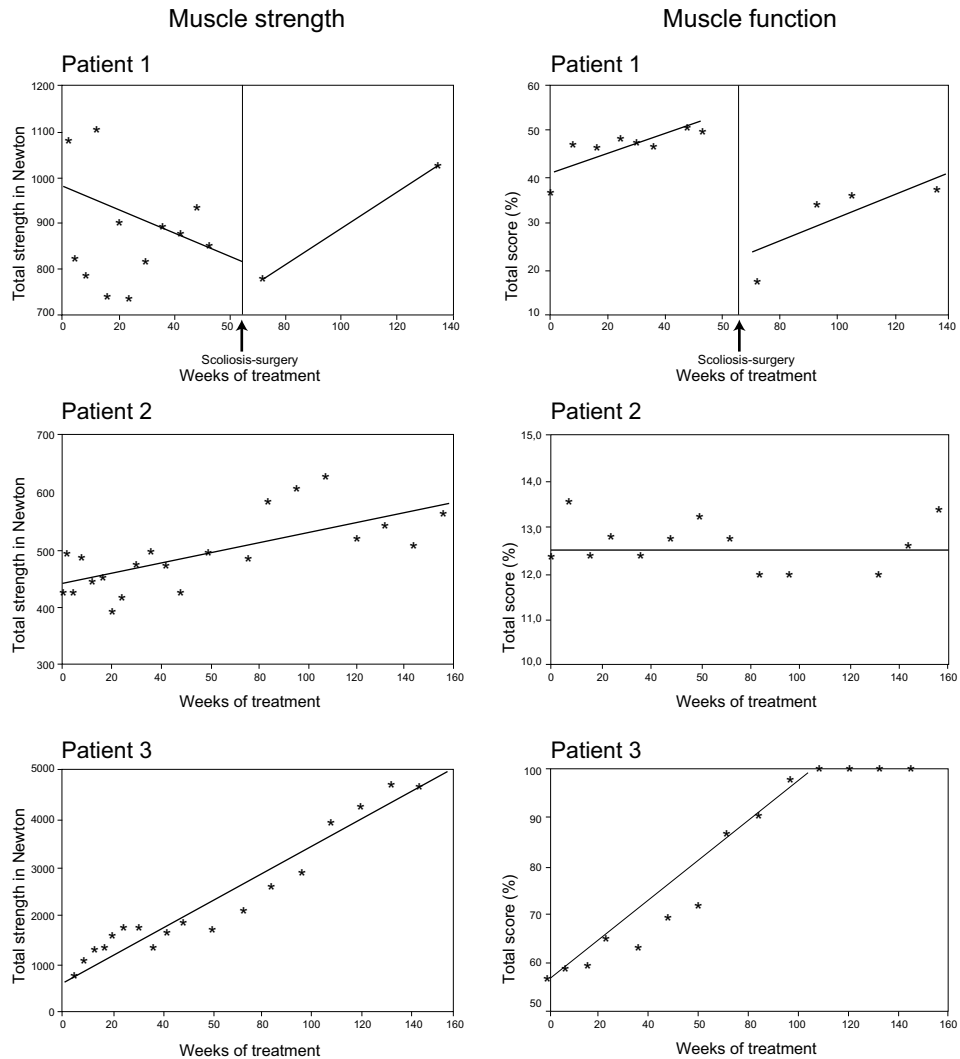

Figure 2
Effects of treatment on muscle strength and function. The muscle strength was measured with Hand Held Dynamometry (left panels); the GMFM was used as a measure for muscle function (right panels).
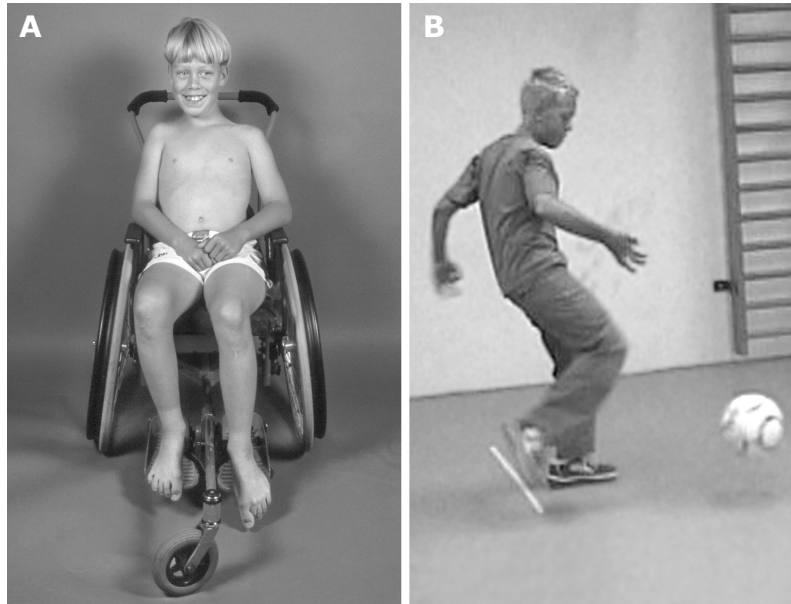
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Figure 3
Patient 3 before (A) and after 100 weeks of treatment (B).

Pulmonary function
Both patients 1 and 2 had a significant decline of vital capacity (VC) in the 6–9 years before the start of treatment, down to 14% and 9% of normal, respectively (patient 1, r=-0.99, p<0.001; patient 2, r=-0.98, p=0.021). Using the 'broken-stick' method, the slope of VC changed significantly after the start of treatment for both patients (patient 1, p=0.002; patient 2, p<0.001) (Fig 4). The VC of patient 2 increased significantly to 16% (r=0.58, p=0.024).

Patient 3 had a normal age-related increase of VC (p<0.001) during treatment, both in supine and sitting position (values between 87% and 98% of normal), whereas his VC remained constant in three years preceding treatment. The change was significant (sitting p=0.023, supine p<0.001).
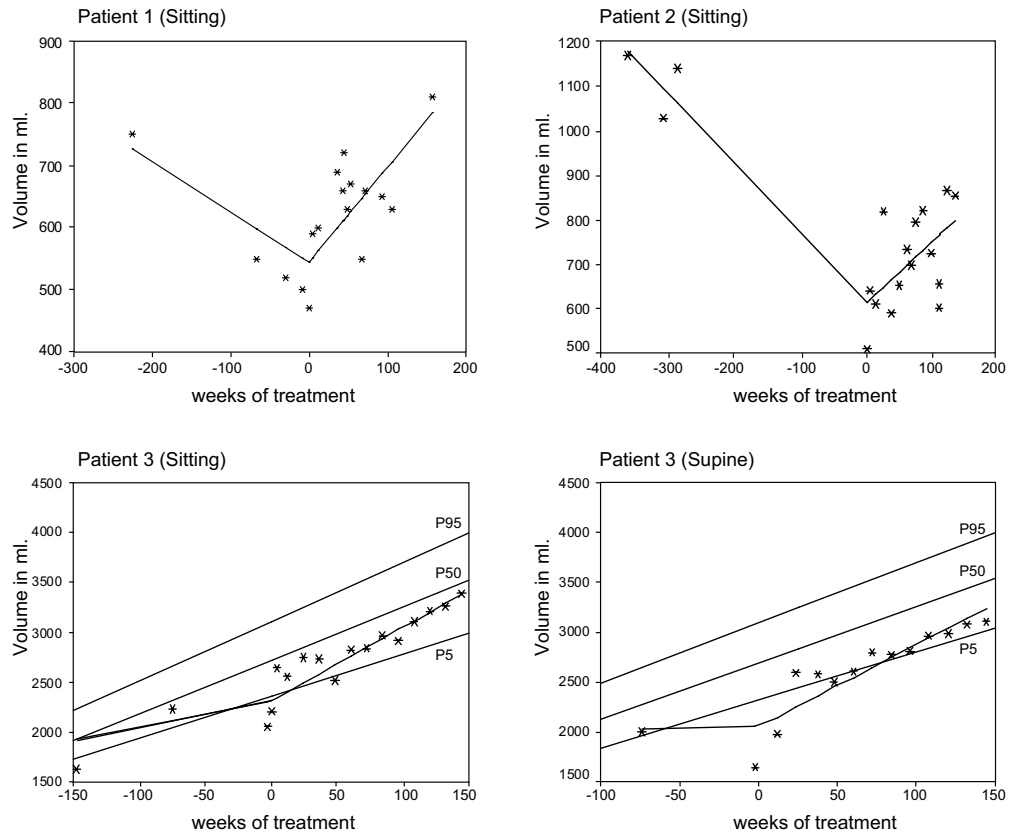
Figure 4
Expiratory vital capacity before and during treatment. The change after start of treatment was calculated with the broken stick method.

Quality of life
All three patients gained energy and quality of life during treatment. Patient 1 and 2 needed less ventilation. Patient 1 resumed her education, started courses at a university, and participates in social life. Her ventilator need decreased from 18 to 10 hours per day. Patient 2 was frequently hospitalized before the start of treatment because of airway infections. During treatment the infections usually resolved without antibiotics, and admissions were no longer required. Instead of being bedridden for most of the day (21 hours at start of treatment) he could now stay up for 13 hours a day and go out. Telephone conversations became possible, as his speech improved.

Patient 3 was the best responder. He used to ride a wheelchair in the two years preceding and the first 2 years after start of treatment. He can ride his bicycle for more than 25 km and plays sport with friends. He now attends school for four days a week, and receives his medication on the fifth day.

Alpha-Glucosidase uptake and glycogen degradation

Table 2A shows the α-glucosidase activities in skeletal muscle (see Fig 1 for timepoints). After 12-24 weeks of treatment with 10 mg/kg we measured a slight increase of α-glucosidase activity compared to baseline. To optimize the therapeutic effect, the rhAGLU dose was then increased to 20 mg/kg/week. Twelve weeks later, α-glucosidase activities were 2.9-8.4 nmol MU/mg/hour and substantially above baseline (0.82-2.6 nmol MU/mg/hour), but still below normal (8-40 nmol MU/mg/ hour). The glycogen content decreased slightly at the higher dose (Table 2B).

| Patient No.	t = 1	t = 2 (12 X 10mg)	t = 3 (24 X 10mg)	t = 4 (12 X 10mg)
α-Glucosidase activity				
1	2.6	3.1	4.9	3.0
2	1.3	5.7	7.9	8.4
3	0.8	1.1	ND	2.9
Reference range	8-40nmol 4MU/h/mg protein			
Late-onset patients	0.6-2.6nmol/h/mg			
Glycogen content				
1	1,045	1,415	1,445	745
2	276	503	424	232
3	141	99		45
Reference range	3-180µg glycogen/mg protein			

ND not determined.

Table 2

Routine laboratory

The CK levels of all patients decreased significantly during treatment, particularly of patient 1 (1560-> 545 IU, p<0.001) (Fig 5). ALAT, ASAT and LDH activities also decreased (not shown), and patient 3 reached near normal values for his age after 144 weeks of treatment. Other biochemical parameters measured for safety reasons did not change during the treatment.

Muscle morphology

Muscle sections of the quadriceps showed a lower PAS staining intensity as a result of treatment. PAS-stained vacuoles had disappeared from the endothelium after 12 weeks of treatment with 10 mg/kg, and gradually also disappeared from the smooth muscle of the arteries and veins. Glycogen storage in peripheral nerves was also corrected. The muscle fibers remained variably affected in patient 1 and 2, but regained a near normal morphology in patient 3 after 43 weeks of treatment (not shown).
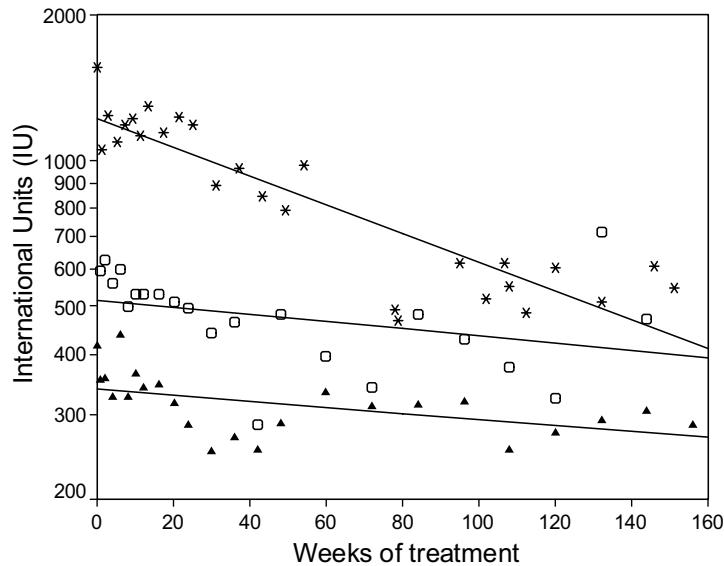
Figure 5
Effect of treatment on Creatine Kinase levels.   ✳ = patient 1,   ▲ = patient 2,   ▢ = patient 3

Discussion

This study shows for the first time that patients with late-onset Pompe's disease can benefit substantially from long-term intravenous administrations of recombinant human α-glucosidase from rabbit milk, like patients with infantile Pompe's disease [5, 6].
As the risks accompanying a new form of therapy can overrule the benefits, we limited our study to three patients in different stages of the disease. As a consequence the patients responded differently in pulmonary and muscle function tests so that the effects needed to be evaluated individually.

Efficacy
The effect of treatment was most significant in the least affected patient. He gained normal muscle strength and function, and his pulmonary function increased steadily according to his age. The two severely affected patients benefited from the treatment mainly through a lower degree of disability and improvement of quality of life, however they remained wheelchair-bound. Their pulmonary function stabilized. In parallel with these clinical accomplishments, a decrease of the CK, ALAT, ASAT and LDH levels was recorded. We noticed that the best responding muscle groups were those that were actively used. The distal muscles responded better than the proximal. The same was observed in the infantile study-group [18]. We assume that this relates to the higher blood flow in active compared to resting muscle and to the percentage of muscle fibers with sufficiently preserved structure to actively capture and deliver the administered enzyme to the lysosomes. Moreover, muscle activity leads to an increase of IGF-1 and enhanced satellite-cell proliferation, needed for muscle cell regeneration [24].

Safety
Producing recombinant human α-glucosidase in transgenic rabbits and extracting it

from the milk results in a remarkably safe product for intravenous administration. The three patients tolerated the weekly infusions with the relatively high dose of 10-20 mg/kg, generally without pre-medication. A similar tolerance was observed in the four infantile patients [5, 6, 15]. It must be noted that food and protein allergy was an exclusion criterion. Pre-medication is frequently used for the treatment of Fabry's disease with recombinant human enzymes from CHO or human cells in much lower doses [7-9].

We did measure an IgG type of antibody response, despite the fact that all three patients had residual synthesis of endogenous α-glucosidase. The immune response did not interfere with the effect of treatment as reported in two patients with infantile Pompe's disease receiving recombinant human enzyme from CHO cells [16].

Patient selection and clinical endpoints
Our findings provide a solid basis for further development of enzyme replacement therapy for late-onset Pompe's disease. Improvement of muscle strength, muscle function, and vital capacity appear to be suitable endpoints for a future pivotal trial.

The MRC works better for measuring the strength of weak muscles than the HHD. Gain of muscle function is more relevant to the patient and can be measured reliably with the GMFM. If patients have sufficient mobility, timed tests can be added. The PEDI demonstrated to be a useful instrument to record subtle improvement of the patients, at the level of self-care and mobility.

With regard to patient selection, our study illustrates that it is desirable to work in future with a rather homogeneous group of moderately affected patients. Their age may differ, but it is important that they have similar residual muscle strength and function, and/or similar pulmonary function. Patients should be older than 6 years to perform the tests adequately.

We noticed in our studies that the process of recovery is slow and therefore recommend that a pivotal study should last for at least one year. It is advisable to collect historic data that can serve as intra-patient control.

Decrease of plasma-CK, reduction of muscle-glycogen, and improvement of muscle morphology can be used as surrogate markers. But, muscle pathology can vary substantially between muscle bundles and fibers [18].

Dosing and production capacity
The poor accessibility and the poor regenerative capacity of muscle tissue are obstacles for successful treatment of Pompe's disease. The circulating therapeutic enzyme must cross the capillary wall to reach the myocytes and can in this process be trapped by the lysosomal system of the endothelial and the interstitial cells. This is probably why the amount of enzyme needed to treat Pompe's disease exceeds the dose needed for treatment of Gauchers disease and Fabry's disease [7-9]. Our studies in infantile and late-onset Pompe's disease indicate that 20 mg/kg is the minimal dose to target α-glucosidase to the muscle and obtain a clinical effect [5, 6, 18]. From this perspective it is expected to be more effective to give high doses weekly or biweekly than low doses more frequently.

At a dose of 20 mg/kg/week the need of recombinant human α-glucosidase for an estimated 3000 patients in the western world is 150 kg of enzyme formulation and approximately double this amount of crude preparation. With present day technology it is a great challenge to produce this large amount in CHO cells [25].

The economic burden of health care forces our society to search for alternative production platforms to keep up with the ever-increasing demand of sophisticated products [26, 27]. Our studies advocate more focus on transgenic technology as we have demonstrated that a product purified from milk of transgenic animals can be safe and effective for the treatment of human diseases.

Acknowledgements

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Enzyme therapy in Pompe's disease: from rabbit milk to CHO-cell derived α-glucosidase

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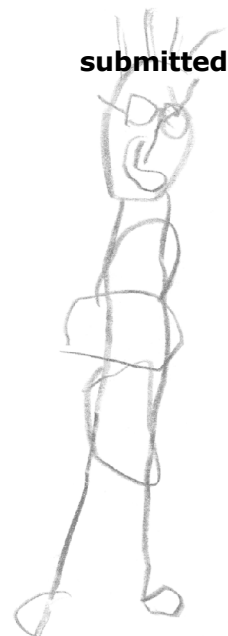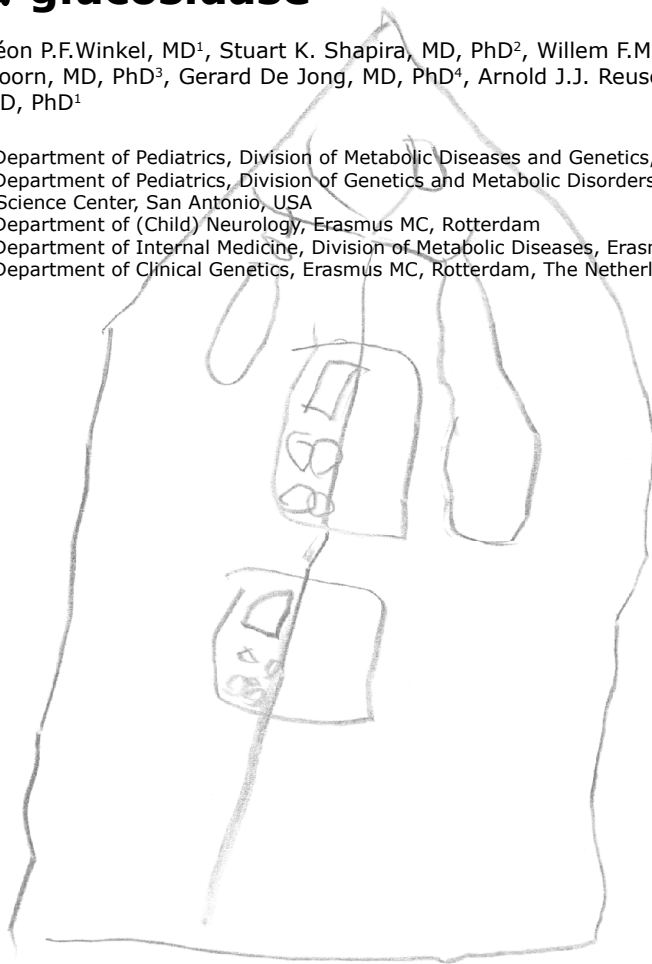
Abstract

Three patients with late-onset Pompe's disease were treated for 3 years with recombinant human α-glucosidase from rabbit milk (20 mg/kg/week) and later with the same enzyme from Chinese hamster ovary cells in doses of 10 mg/kg/week (3 months), 20 mg/kg/biweekly (next 3 months) and finally 30 mg/kg/biweekly (1 year). Both products were well tolerated.

During treatment with the lowest doses of CHO-cell derived α-glucosidase the patients reported fatigue, and loss of (muscle) function. This was corroborated by objective measurements in only one patient. These symptoms disappeared after dose-increase to 30 mg/kg/biweekly. We conclude that patients with late-onset Pompe's disease can be treated safely and effectively with α-glucosidase from rabbit milk and CHO-cells.

Introduction

Pompe's disease is caused by acid α-glucosidase deficiency leading to lysosomal glycogen storage. The classic infantile form of Pompe's disease is rapidly progressive and characteristically results in cardiorespiratory failure within the first year of life. The late-onset form is slowly progressive and eventually results in respiratory failure and loss of ability to walk [1]. Enzyme therapy is under investigation.

In January 1999 we started the first application of enzyme therapy with recombinant human α-glucosidase from rabbit milk in four infants (age 2.5-8 months) with the severe classic form of Pompe's disease and in three patients with late-onset disease (age 11, 16 and 32 years). The treatment appeared successful. In the infants cardiac hypertrophy and function improved and survival increased significantly [2, 3]. In the older patients the treatment resulted in stabilization of pulmonary function and improvement of muscle strength and function [4].

In the same year, a second pilot study commenced in three infants (age 2.5-4 months) with α-glucosidase produced in Chinese hamster ovary (CHO) cells [5]. The applied dose and infusion regimens were different. The results of both studies could not be compared.

In April 2000 the sponsors of the studies decided to focus on the development of recombinant human α-glucosidase from CHO cells and to discontinue production in milk. The three patients with late-onset Pompe's disease were transitioned first in July 2002. Here, we report on the transition and 1.5-year follow-up.

Methods

The study was conducted as extension of an open label pilot study to evaluate the safety and efficacy of transgenic α-glucosidase and approved by the institutional review board [4]. Written informed consent was obtained from the patients and the parents. Pharming/Genzyme LLC (Leiden, The Netherlands) provided recombinant human α-glucosidase from rabbit milk (rabbit enzyme) and Genzyme Corporation (Cambridge MA) provided recombinant human α-glucosidase from Chinese hamster ovary (CHO) cells.

The applied dose and infusion regimens are given in Table 1. At the time of transition, enzyme administrations were discontinued for 4 consecutive weeks due to shortage of both products.

As follow-up parameters were used: safety measurements (blood chemistry and physical examination), pulmonary function (VC), muscle strength (HHD [6] and MRC-score [7]) and function (GMFM [8], PEDI [9]), essentially as described before [4].

SPSS 10.1 was used for statistical analysis, p-values ≤ 0.05 were considered significant.

Results
Transition
At the time of transition, CHO-cell derived α-glucosidase had not been administered to patients in doses higher than 10 mg/kg. One infant who received this dose five times per week over a period of more than a year developed an immune nephritis [10].

We had learned from our previous studies that IgG-mediated infusion-associated reactions can be prevented by starting the infusions slowly, but also that high plasma peak levels are essential to obtain enzyme-uptake in skeletal muscle [3, 11] These contradicting terms led us to apply the infusion regimens as depicted in Table 1.

Initially, α-glucosidase from CHO-cells was made available in a dose of 10 mg/kg/week or 20 mg/kg biweekly. For safety reasons we started with 10 mg/kg/week and then switched to the latter regimen. The applied infusion regimens and the increase of dose and infusion rate were tolerated well by the patients. One patient occasionally experienced chills during part of the infusion, but this phenomenon disappeared. No other infusion-associated reactions were observed. Premedications were not required.

Patient reports (Table 2)
Patient 1 was 16 years old when she started to receive transgenic α-glucosidase. In the preceding 4.5 years her pulmonary function had deteriorated from 34% to 14%. Walking had become increasingly difficult through muscle weakness and a progressive scoliosis (60-degree right thoracic curve and 74-degrees left lumbar curve). During the 3 years of treatment with transgenic enzyme her pulmonary function had stabilized and she needed less ventilation during daytime. Her progressive scoliosis required surgery. Thereafter, muscle strength, function and disability scores showed some improvement, but wheelchair dependency remained. Quality of life increased as indicated by the patient. She was less tired and resumed courses at the university [4].

During the first 6 months on CHO-cell derived α-glucosidase her ventilator need increased and her complaints about fatigue and intestinal problems that she had before the start of treatment, reappeared. Three months after dose-increase from 20 to 30 mg/kg biweekly the latter complaints disappeared and her condition became similar as before transition. Pulmonary function remained stable and muscle strength and function showed mild improvement over the treatment period.

Patient 2 was 32 years old when he started to receive transgenic α-glucosidase. He was completely ventilator dependent and wheelchair bound. He was bedridden most of the day resulting in social isolation. His pulmonary function had deteriorated from 47% to 9% in the 18 years preceding treatment, but improved slightly during the 3 years he received enzyme from rabbit milk. Moreover, he could manage for 30 minutes per day without ventilator. His muscle strength and disability scores improved slightly, but his muscle function as assessed by the GMFM did not improve. He gained quality of life. He resumed social activities, could stay out of bed and regained the ability to have telephone conversations [4].

During the first 6 months that he received CHO-enzyme, he complained of fatigue, pain in the neck and headache. He could no longer pick up things from the floor and his ability to be without ventilation decreased from 30 to 5 minutes per day.

Table 1 Dose regimen and infusion schedule

Scheme:	Applied for transgenic	Advised for CHO	Applied for CHO first 6 weeks	Applied for CHO week 7-12	Applied for CHO week 13-26	Applied for CHO since week 26
first hour	0.1-0.2 mg/kg/hour	2 mg/kg/hour[1]	0.1-0.2 mg/kg/hour	0.1-0.2 mg/kg/hour	0.1-0.2 mg/kg/hour	0.1-0.2 mg/kg/hour
second hour	0.7-0.8 mg/kg/hour	NA	0.7-0.8 mg/kg/hour	0.7-0.8 mg/kg/hour	0.7-0.8 mg/kg/hour	0.7-0.8 mg/kg/hour
rest of infusion	15-17 mg/kg/hour	3.6 mg/kg/hour[2]	3.6 mg/kg/hour	gradually increased[3]	15-17 mg/kg/hour	15-17 mg/kg/hour
Dose	20 mg/kg/week	10 mg/kg/week	10 mg/kg/week	10 mg/kg/week	20 mg/kg/2weeks	30 mg/kg/2weeks

[1] duration 30 minutes instead of 60 minutes
[2] duration 2.5 hours (rest of infusion)
[3] rate was gradually increased from 3.6 to 15-17 mg/kg/hour

Table 2 Patient parameters before and during treatment

Patient	1				2				3				Reference values
gender	Female				Male				Male				
Age at diagnosis	11				10				2.5				
After treatment	Baseline	3 yr Rabbit	0.5 yr CHO	1.5 yr CHO	Baseline	3 yr Rabbit	0.5 yr CHO	1.5 yr CHO	Baseline	3 yr Rabbit	0.5 yr CHO	1.5 yr CHO	
Age at	16.5	19.5	20	21	32.9	35.9	36.5	37.5	11.9	14.7	15.3	16.3	
GMFM (%)	36%	38%	40%	42%	12%	12%	12%	12%	57%	100%	97.8%	100%	100%
HHD (total, Newton)	720#	782#	952#	1074#	428	562	558	636	392	4684	4719	5007	NA
MRC (Sumscore)	94!	79	87	88	70	79	78	79	74	114	114	114	114
% predicted vital capacity	14%	16%	15%	13%	9%	16%$	13%$	13%$	82%	95%	97%	100%	80-120%
PEDI (selfcare)	51	67	ND	ND	49	53	53	53	67	73	73	73	73
PEDI (mobility)	21	24	ND	ND	24	24	24	24	36	59	59	59	59
CK (IU)	1560*	545*	438*	365*	414*	285*	231*	133	592*	471*	242	140	
LDH (IU)	1570*	545*	241*	214*	388	347	333	341	475	461	427	330	
ALAT (IU)	228*	64*	52*	54*	35	35	34	39	70*	32*	26	13	
ASAT (IU)	324*	80*	68*	45*	65*	50*	47*	42*	97*	57*	37*	28	
Wheelchair use	+	+	+	+	+	+	+	+	+	-	-	-	
Walking	10-20 m. parallel bars	parallel bars	parallel bars	parallel bars	-	-	-	-	-	normal	normal	normal	
Cycling (resistance in Watt)	+	+	+	+	+	+	+	+	NP	180	140	180	
Ventilation Use	+	+	+	+	+	+	+	+	-	normal	normal	normal	
Hours without Ventilation	6	14	8	10	0	0.5	0.1	0.5-0.75	24	24	24	24	

$ measured with a different device than during the previous treatment
NA=Not applicable
ND=Not determined
NP=Not possible (at that time)
* = elevated compared to age and gender matched reference values
# Total HHD does not include elbow extension and squeezing for this patient
! Measured by a different person than later values

After dose increase to 30 mg/kg biweekly he was less tired. He regained the ability to pick up things from the floor and could be without ventilation for 30-45 minutes per day. Ten weeks after dose-increase, the patient reported restoration of physical condition. Objective measurements of muscle strength and function did not show substantial differences.

Patient 3 was 11 years old when he started treatment. At that time he was wheelchair bound, but could move his legs. VC was within normal age-limits. During the first three years of therapy with rabbit enzyme, his muscle strength increased dramatically. His muscle function normalized and he abandoned his wheelchair, learned to ride a bike, and play soccer [4].

During the first 6 months after transition to the lower dose of CHO-enzyme he reported loss of stamina. Objectively, his ability to cycle against resistance was decreased (180->140 Watt, Fig. 1A). He reported more fatigue, feelings of discomfort and headaches. In addition, he lost the ability to squat and stand and hop on one leg. The GMFM decreased from 100 to 97.8% (Fig. 1B).

After dose increase to 30 mg/kg biweekly, feelings of discomfort, fatigue, and headaches gradually disappeared. The patient's muscle strength increased, his muscle function normalized (Fig. 1B) and he regained endurance (Fig. 1A). Pulmonary function continued to show an age-related increase.

ALAT, ASAT, LDH and CK showed a continuous significant decrease over the 4.5 years of treatment in all three patients ($p<0.001$, Fig. 1C).
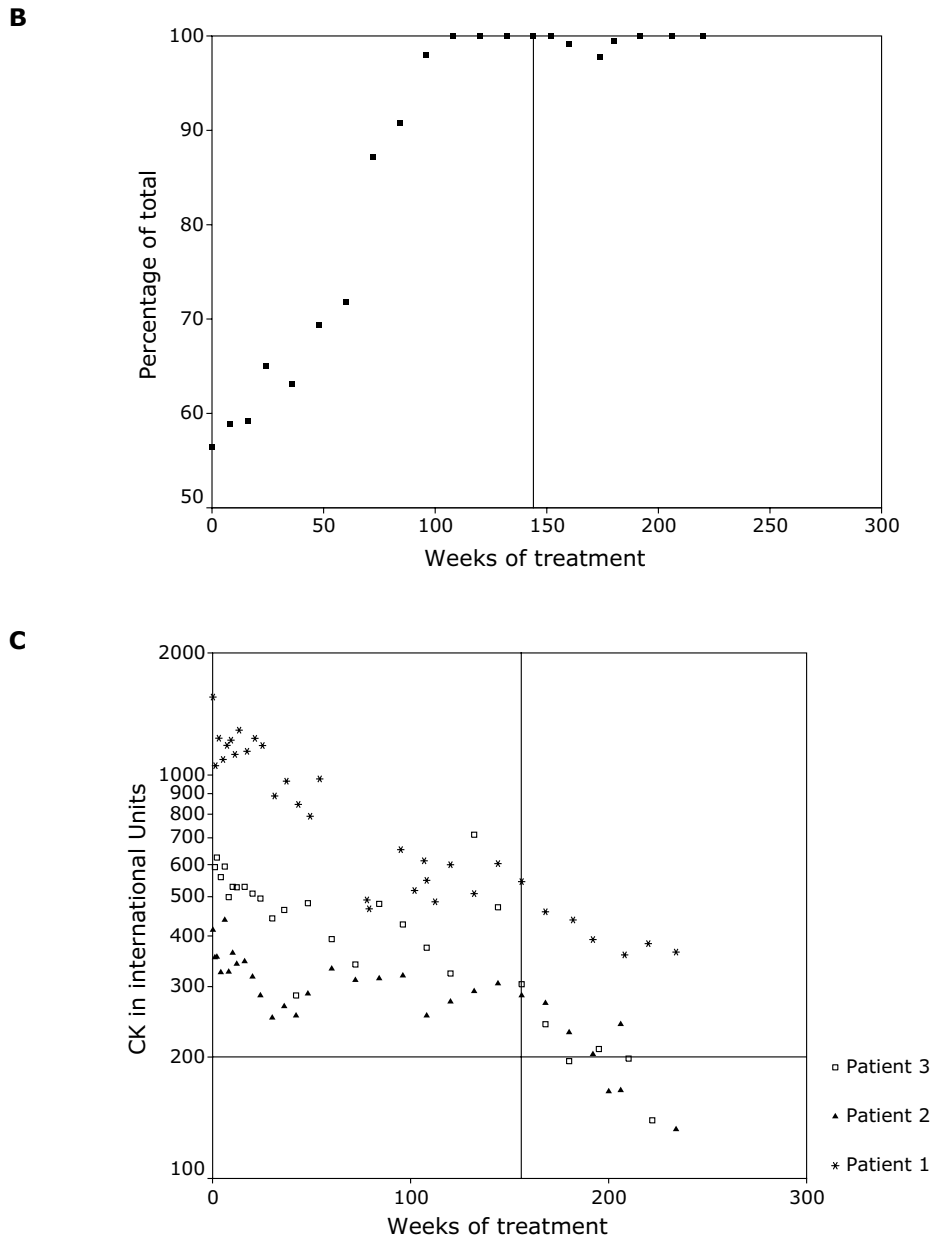
A

B

C
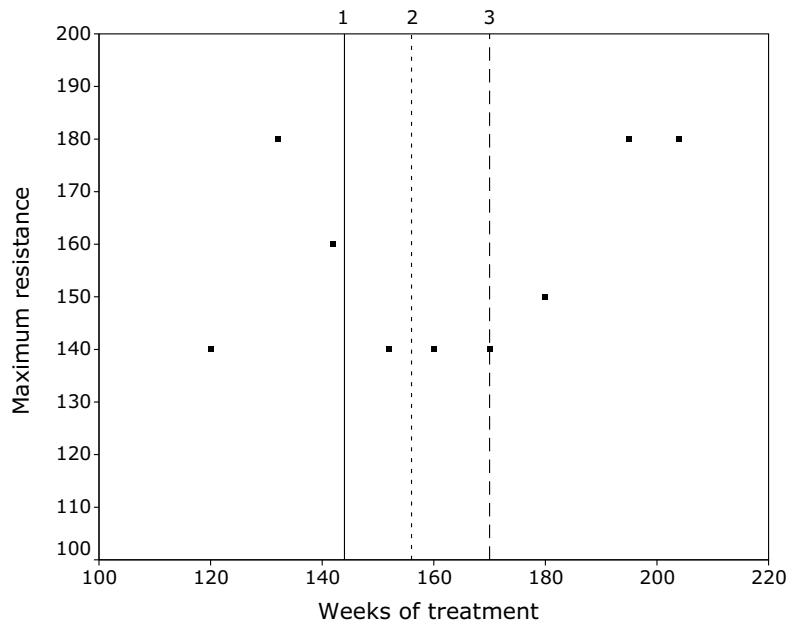
Figure 1; (A) The effect of treatment on 'cycling against resistance' for patient 3. Vertical lines mark the changes in treatment regimen; 1 = start of CHO-enzyme in a dose of 10 mg/kg/week; 2 = switch to 20 mg/kg/biweekly; 3 = increase to 30 mg/kg/biweekly. From week 120 to 132 the patient received 20 mg/kg/week of enzyme from rabbit milk, from week 132 to 144 the patient received 4 times 20 mg/kg of this enzyme biweekly had no infusions in the next 4 weeks before transition. (B) The effect of treatment on the muscle function of patient 3. the vertical line marks the transition to CHO-enzyme. (C) The Creatine Kinase levels in blood, during 4.5 years of treatment. The horizontal line indicates the upper limit of reference values. The vertical line marks the transition to CHO-enzyme.

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Discussion

From the results of our study we conclude that recombinant human α-glucosidase is tolerated well, irrespective of the enzyme source be it from the milk of transgenic rabbits or cultured CHO-cells.

At the time of transition no patient had received the CHO-enzyme in a higher dose than 10 mg/kg, and the two products were impossible to compare due to limited published data. We now know that doses of 30 mg/kg of CHO enzyme can be administered without major side effects. Premedications were not required when we used the following infusion regimen: 0.2-0.3 mg/kg/hr for the first hour, 0.7-0.8 mg/kg/hr for the second hour and 15-17 mg/kg/hr for the rest of the infusion.

The preliminary data obtained in this study show that a dose of 30 mg/kg/biweekly of the CHO-product maintains the effect that was obtained with 20 mg/kg/week of α-glucosidase from rabbit milk. We were surprised to experience that patients started to report more fatigue, loss of muscle function and decreased capability to be without ventilation already in the first few months after transition to the lower dose of 10 mg/kg/week or 20 mg/kg/biweekly. The explanation may be that the patients missed 4 consecutive infusions before transition and were treated subsequently for 3 months with the relatively low dose of 10 mg/kg/week, at sub-optimal (too slow) infusion-rates. The following period of three months treatment with 20 mg/kg biweekly may have been too short to reverse the clinical course. It was remarkable that the patients reported improvement of their clinical condition soon after the dose-increase to 30 mg/kg. The patient's experience was in only one case corroborated by objective measurements. At a dose of 30 mg/kg biweekly the patients currently perform similar or better than with transgenic product.

Four-and-a-half years of enzyme replacement therapy with recombinant human α–glucosidase in Pompe's disease have shown the feasibility of the approach, but further trials will be required to determine the optimal dosing regimen in both classic and late-onset Pompe's disease.

Acknowledgements

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Morphological changes in muscle tissue of patients with infantile Pompe's disease receiving enzyme replacement therapy

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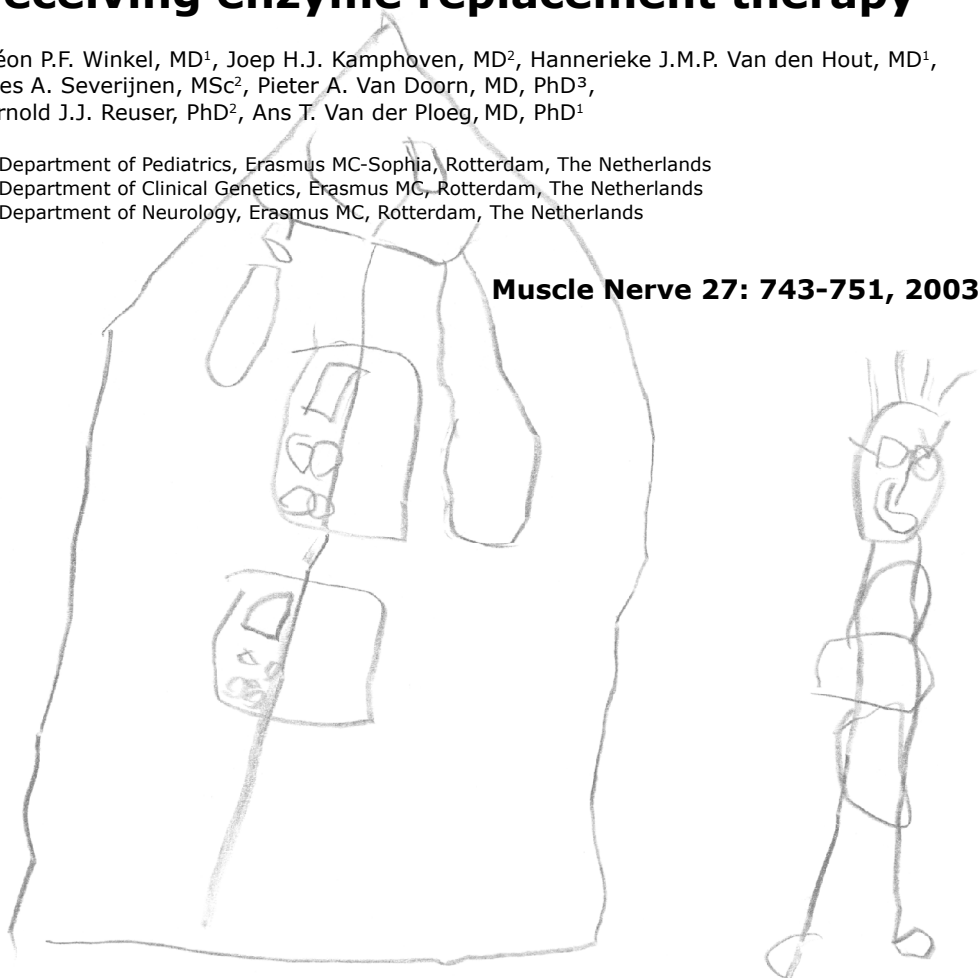
Abstract

Pompe's disease (glycogen storage disease type II; GSDII) is an autosomal recessive myopathy caused by lysosomal α-glucosidase deficiency. Enzyme replacement therapy (ERT) is currently under development for this disease. We evaluated the morphological changes in muscle tissue of four children with infantile Pompe's disease who received recombinant human α-glucosidase (rhAGLU) from rabbit milk for 72 weeks. The patients were aged 2.5 – 8 months at entry. Prior to treatment, all patients showed lysosomal glycogen storage in skeletal and smooth muscle cells, vascular endothelium, Schwann cells, and perineurium. The first response to treatment was noticed in vascular endothelium and in peripheral nerves after 12 weeks of treatment at an enzyme dose of 15-20 mg/kg. Increasing the dose to 40 mg/kg led, after 72 weeks of treatment, to a reduction of glycogen storage and substantial improvement of muscle architecture in the least affected patient. Not all patients responded equally well, possibly due to differences in degree of glycogen storage and concomitant muscle pathology at the start of treatment. We conclude that intravenous administration of rhAGLU from rabbit milk can improve muscle morphology in classic infantile Pompe's disease when treatment is started before irreversible damage has occurred.

Introduction

Pompe's disease (glycogen storage disease type II) is an autosomal recessive lysosomal storage disorder, caused by acid α-glucosidase deficiency [13].

Classic infantile Pompe's disease presents in the first months of life. Affected infants have respiratory and feeding difficulties, severe hypotonia, and a hypertrophic cardiomyopathy. Other frequent symptoms are macroglossia and hepatomegaly. Major milestones are typically not achieved, and most infants die within their first year of life (median survival, 6-8 months) [13, 23].

Enzyme replacement therapy (ERT) is presently under development and appears to be a realistic option for treatment of Pompe's disease [13, 24]. The method was first investigated in vitro and later in animal models before it was tested in patients [2, 10, 14, 26-28]. Seven patients (4 infants, 2 teenagers and 1 adult) started experimental treatment with recombinant human α-glucosidase from rabbit milk in our hospital in 1999. Based on the preliminary findings in the infantile group we concluded that ERT with recombinant human α-glucosidase is safe and effective [24].

During the clinical trial of ERT multiple muscle biopsies were performed at different intervals after the treatment was started. The purpose of this paper is to describe the findings in these biopsies, particularly with regard to how ERT may modify the storage of glycogen in skeletal muscle, intramuscular nerves, and blood vessels.

Methods

Patients

Four patients with classic infantile Pompe's disease were included in our open-label study to assess the safety and efficacy of ERT with recombinant human α-glucosidase from rabbit milk [24, 25]. The diagnosis was based on clinical symptoms and deficiency of acid α-glucosidase activity, and confirmed by mutation analysis. The study was approved by the medical ethics committee of our hospital. At the time of entry to the study, the patients were 2.5 to 8 months old and all four had complete α-glucosidase deficiency [24, 25]. The least affected patient (patient 1) then was a 3-month-old boy in relatively good condition. The other three patients were girls. Patient 3 (2.5 months old) was more affected than patient 1, but less than patients 2 and 4 who were then 7 and 8 months old, respectively, and in an end stage of their disease [24, 25]. Clinically, patient 1 responded well to treatment. At

the time of each consecutive biopsy, he was able to move his legs against gravity and showed close to normal motor development. Patient 3 showed delayed motor development; she could move her legs, but not against gravity. Patients 2 and 4 were unable to move their legs.

Therapeutic regimen
Pharming/Genzyme LLC supplied recombinant human α-glucosidase from rabbit milk [4, 5]. The patients received this drug intravenously on a weekly basis. Figure 1 shows the dose regimen per patient.
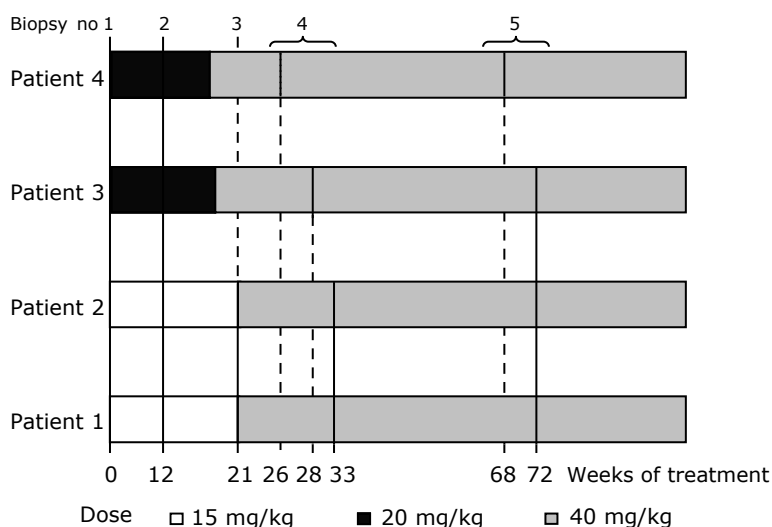

Fig.1
Dose and weeks of treatment at time of biopsy

Processing of biopsies
Muscle biopsies were taken from the quadriceps muscle at preset time points, using a standard open surgical procedure (Fig.1). The surgeons were asked to avoid the sites of previous biopsies. Samples were split for light (LM) and electron microscopy (EM) and fixed overnight in 0.1M cacodylate buffered glutaraldehyde (4%) pH 7.3. After dehydration in a graded series of acetone, LM sections were embedded in glycolmetacrylate (GMA) and stained with periodic acid Schiff reagent (PAS) [1]. Diastase digestion was performed to positively identify glycogen as opposed to acid mucin. EM sections were postfixed with OsO4 and embedded in Eppon [7]. Ultrathin sections (EM) were contrasted with uranyl acetate and lead citrate and examined with a Philips Morgagni Electron Microscope 286D (Philips, Eindhoven, The Netherlands).

Timing of biopsies
For all patients, the first biopsy (Fig.1) was taken before start of treatment and the second biopsy was after 12 weeks of treatment with 15-20 mg/kg. The third biopsy was only performed for patient 1 and 2 after an additional 9 weeks with the same dose. Another biopsy was obtained from all patients after 12 weeks of treatment with 40 mg/kg. The last biopsy was for patient 4 after 68 weeks and for patients 1-3 after 72 weeks of treatment with 40 mg/kg.

Semiquantitative rating

To minimize artificial differences in staining intensity, all tissue sections from the same patient but from different time-points were collected on the same glass slide and stained simultaneously. The sections were then assessed in a blinded fashion by 6-8 observers.

A qualitative rating system was designed to codify the glycogen storage in and its effects on the various tissues in the biopsy and to allow possible differences in extent of glycogen storage between skeletal muscle cells, blood vessels, and intramuscular nerves to be tabulated. The system is intended to allow comparison in sequential biopsies from the same patient and not between patients, because of interpatient variation (Table 1).

The PAS-staining intensity of muscle was rated from 0 (no staining) to 3 (very strong), the cross-striation from 0 (normal) to 3 (absent), and the number of vacuoles from 0 (none) to 3 (many). These three ratings together form the "muscle score". We similarly rated the PAS-staining intensity of endothelial and smooth muscle cells in the wall of blood vessels, (arteries and veins) and the staining intensity of the Schwann cells and the perineurium of peripheral nerves. The "total score" is the sum of all ratings. A high total score reflects severe pathology (maximum = 27) and a low score signifies good morphology (best score = 0 = near normal).

PAS intensity	Score	Striation	Score	Vacuoles	score
No staining	0	None at all	3	None at all	0
Little in some	0	Significant in most	2	Little in some	0
Little in most	1	Significant in all	1	Little in all	1
Little in all	1	Normal	0	Significant in some	1
Significant in most	2			Significant in all	2
Strong in most	2			Many in some	2
Very strong in most	3			Many in all	3
Very strong in all	3				

Table 1. Rating system for pathological changes
Some/all/most refers to fibers when skeletal muscle is involved, to vessels when blood vessels are involved and to peripheral nerves when these are involved.

Results

Pathology before treatment

Before the start of treatment, there was a marked accumulation of glycogen in skeletal muscle, smooth muscle of arteries and veins, vascular endothelium, Schwann cells, and the perineurium of peripheral nerves of all patients. In each case the global structure of the tissues was preserved, and irreversible damage in the form of fibrosis was not observed (Fig. 2). The best performing patient (patient 1) showed the overall best muscle architecture at the time of inclusion. The cross-striation was relatively well preserved, although most fibers were substantially affected and had (lysosomal) glycogen deposits varying in number and size (Fig. 2A). In contrast, the most affected patient (patient 2) had severe muscle damage with virtually complete loss of cross-striation and a sizeable number of relatively large empty spaces (vacuoles). Only an estimated 5% of muscle fibers was relatively unaffected (Fig. 2B). Patients 3 and 4 had an intermediate level of pathology (Fig. 2C and D, respectively) compared to the least and most affected patients.
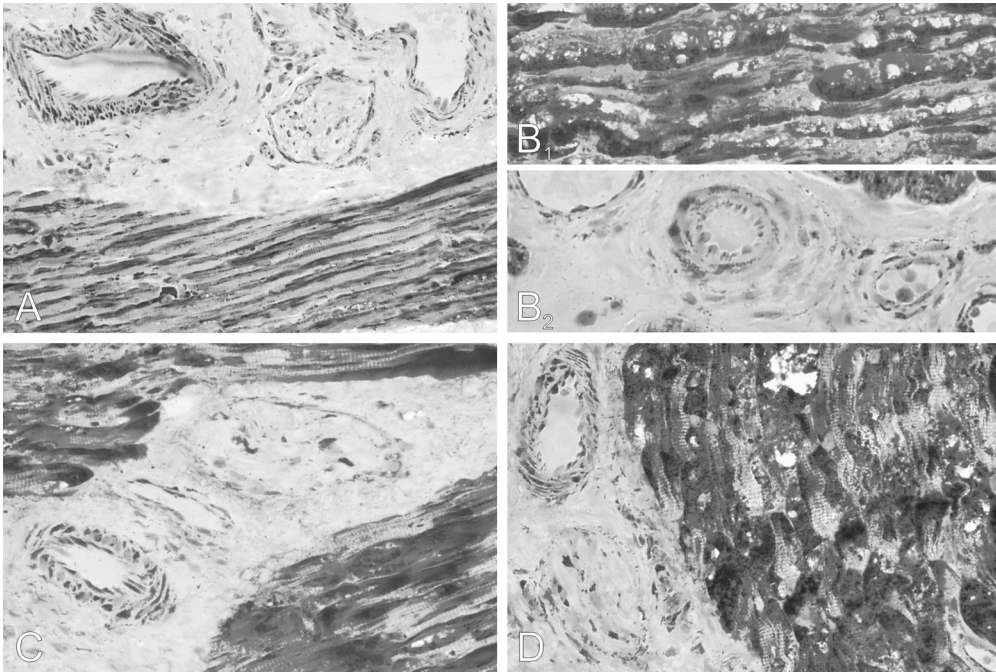
Fig. 2. Pre treatment variation of pathology in muscle sections.
Longitudinal sections of the quadriceps femoris of patients 1-4 (A-D) were stained with periodic acid-Schiff (PAS) to demonstrate glycogen storage. Glycogen storage is seen in cross-striated muscle, in smooth muscle, in vascular endothelium, and in Schwann cells and perineurium of peripheral nerves. Magnification: A, 250x; B-D, 400 x

EM images taken before the start of treatment provided more detailed information about the severity of the glycogen accumulation. The muscle architecture of patient 1 was only mildly disturbed (Fig. 3A). The contractile filaments were still intact, while there were lysosomal and extra-lysosomal glycogen deposits. The most affected patient (patient 2) had muscle fibers with far more and larger lysosomes and cytoplasmic fields of glycogen causing fibril splitting (Fig. 3B). Lysosomal glycogen storage was also seen in vascular endothelial cells, which are recognizable by their pinocytotic vesicles (Fig. 3C). The pinocytotic function of these cells is apparently not disturbed.
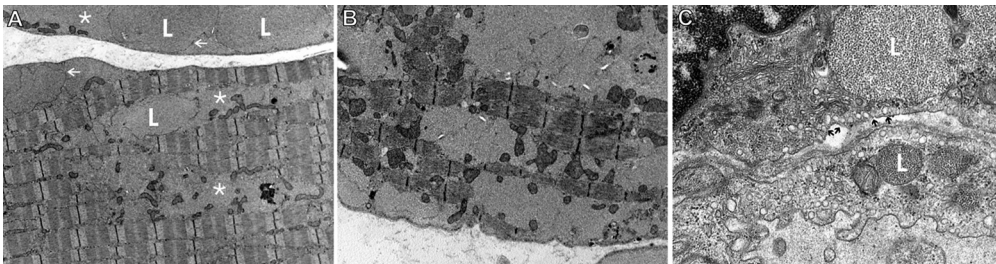

Fig. 3. Electron microscopy of muscle sections at baseline.
Lysosomal (L) and cytoplasmic (V) inclusions in skeletal muscle of patient 1.
The arrows point to the lysosomal membrane (A); Fibrils are split by glycogen deposits in muscle of patient 2 (B); Lysosomal glycogen storage (L) in endothelial cells of patient 3. The arrows point to pinocytotic vesicles(C) Magnification: A, 4400x; B, 5600x; C, 22000x

Glycogen degradation and morphological changes during treatment

The muscle pathology of patient 1 diminished after the start of treatment, mainly during weeks 33 to 72. In contrast, the morphological changes in the biopsies of patients 2, 3 and 4 were much more subtle. In these latter three cases, a clear loss of glycogen occurred from the intramuscular nerves and blood vessels and less so from the skeletal muscle, but this effect was accompanied by increased loss of muscle architecture.

Figure 4 depicts the changes observed in patient 1. Figure 4A was taken prior to treatment and shows PAS-staining in a peripheral nerve, artery, and skeletal muscle fibers. After 12 weeks of treatment at a dose of 15 mg/kg, the first therapeutic effects were observed (Fig. 4C). The overall PAS-staining intensity was still high, but glycogen had disappeared from the peripheral nerves (Schwann cells and perineurium) and from the vascular endothelium. The PAS-staining intensity of the smooth muscle cells of the vascular walls was diminished but some glycogen storage was still present. The least effect was noticed in skeletal muscle. The fibers appeared as affected as before treatment, and the number of vacuoles (empty spaces) seemed slightly increased (Fig. 4A, C). These findings were confirmed by electron microscopy (Fig. 4B, D). Treatment for an additional 9 weeks, with the same dose, did not lead to further morphological improvement (not shown). We then increased the dose to 40 mg/kg for a subsequent 12 weeks period. After this period we observed reduction of PAS-staining intensity and improvement of muscle morphology in patient 1 (Fig. 4E). The vascular smooth muscle cells still contained some glycogen deposits, but these were mostly restricted to the outer layer of the blood vessel wall. The EM pictures showed some vacuoles and fields of glycogen disturbing the normal muscle architecture (Fig. 4F). After 72 weeks of treatment (21 weeks with 15 mg/kg plus 51 weeks with 40 mg/kg), there were large areas with virtually normal muscle morphology (Fig. 4G). All fibers were cross-striated (Figs. 4G and 4H) and the sarcomers were nicely aligned (Fig. 4H), although some fibers still contained small vacuoles. Cytoplasmic and lysosomal glycogen had disappeared, also from the walls of arteries and veins.

The different response to treatment of patient 1 compared to 2 is illustrated in Figure 5. At the start of treatment, patient 2 had a high proportion of affected fibers, more vacuolization and larger fields of cytoplasmic glycogen than patient 1 (Fig. 5C, A). Cross-striated muscle fibers were hardly seen in the biopsy of patient 2. After 72 weeks of treatment the PAS-staining intensity was diminished in the biopsies of both patients. By then, however, the muscle morphology of patient 1 had substantially improved (Fig. 5B), whereas the muscle morphology of patient 2 was still poor due to an increase of the number of vacuoles (Fig. 5D). Comparison at the EM level confirmed these findings. Sections of patients 3 and 4 showed a muscle architecture comparable to that of patient 2 (not shown).

In one of the EM sections obtained after 72 weeks of treatment, we observed lysosomal glycogen deposits in a satellite cell, demonstrating that the storage process continued despite ERT (not shown).

Fig. 4. Effect of ERT on muscle morphology.
All images are derived from patient 1. The left panel is stained with PAS, the right panel are electron micrographs. A and B: skeletal muscle pathology at baseline; C and D: the changes after 12 weeks of treatment with 15 mg/kg weekly. Note the clearance of glycogen in peripheral nerves and vascular endothelium; Muscle morphology has not changed; E and F: the changes after 12 additional weeks of treatment with 40 mg/kg: Note the overall reduction of PAS staining intensity. Muscle morphology has improved; there are still fields of cytoplasmic glycogen and some vacuoles; G and H: after 72 weeks of treatment: hardly any glycogen is left in skeletal muscle and blood vessels. Magnification: A, C, E and G: 400x; B&H: 7100x; D: 5600x; F: 2800x
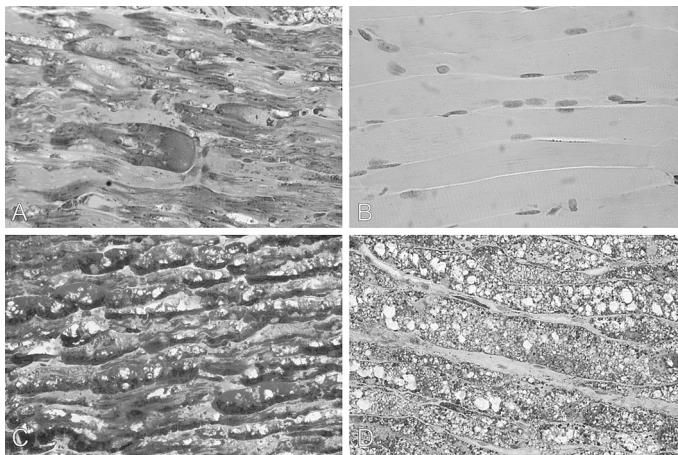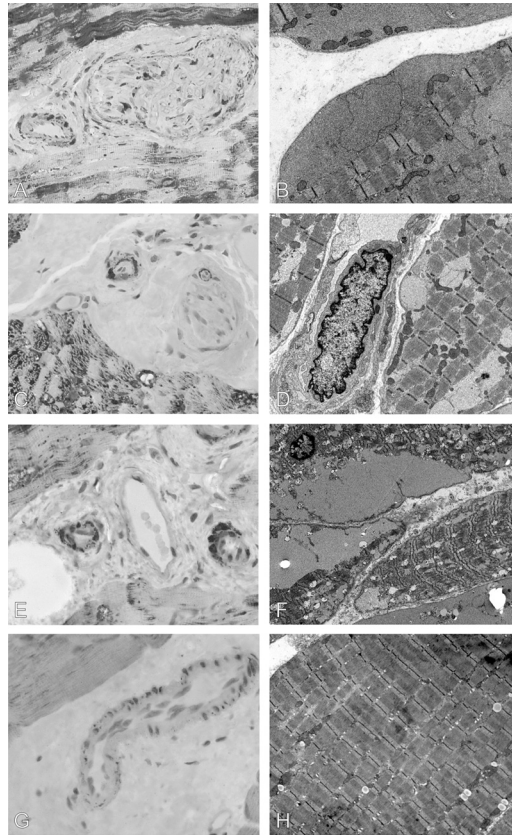
Fig.5. Differences in therapeutic response between patients 1 and 2.
The left panel represents the situation at baseline, the right panel after 72 weeks of treatment. All sections are stained with PAS. A and B are from patient 1, and C and D are from patient 2. Reduction of PAS staining intensity is seen in both patients, with concomitant improvement of muscle morphology in patient 1, whereas muscle morphology in patient 2 shows little improvement. Magnification: A-D, 400x

Clinical response to treatment

After 72 weeks of treatment, all patients had normal muscle α-glucosidase activity. The glycogen concentration in the muscle of patients 2, 3 and 4 had not changed significantly, but was decreased to normal in patient 1 (unpublished results). Patient 1 also showed significant clinical improvement. He achieved normal major milestones of motor development (rolling, sitting, standing and walking) as established by AIMS scores. Patient 2 initially showed some improvement of skeletal muscle function. She learned to move her arms and had improved head balance, but lost these abilities again after a series of pulmonary infections. Patient 4 learned to sit with support, but did not reach the milestones of standing and walking. Her arm function improved significantly. Patient 3 learned to sit without support, could move her legs, but could not stand. All four patients were in a better clinical condition after 72 weeks of treatment than they were at entry into the trial [24, 25].

Semiquantitative rating of muscle pathology

PAS-stained muscle tissue sections were microscopically inspected and ratings were given for PAS-staining intensity, number and size of vacuoles and cross-striation as outlined in the Methods section (Table 1).

The muscle score at inclusion was 8 for the most affected patient (patient 2) and 7 for the others (Table 2). The total score was 23 for patient 2 and 25 for the other three patients. After 12 weeks of treatment none of the patients had an improved muscle score, but the total score had fallen to 13-14. This was mainly due to a lower rating of the PAS-staining intensity of the endothelium, peripheral nerves, and smooth muscle cells of the blood vessels. After the following 12 weeks of treatment with 40 mg/kg, the total score was further diminished by a lower rating of the PAS-staining intensity of the muscle. After 72 weeks of treatment, patient 1 had a near-normal total score. Treatment of the other three patients led to a lowering of the total score but their muscle score stayed the same because the diminished PAS-staining intensity was counterbalanced by an increase of vacuolization.

Item	Patient no	Biopsy number				
		1	2	3	4	5
PAS	1	3	3	3	2	0
	2	3	3	2	1	2
	3	3	3	-	2	1
	4	3	3	-	3	2
Cross-striation	1	2	2	2	2	0
	2	3	3	2	2	3
	3	2	2	-	3	3
	4	2	3	-	3	2
Vacuoles	1	2	2	3	2	1
	2	2	2	3	3	3
	3	2	3	-	2	3
	4	2	3	-	3	3
Muscle score[a]	1	7	7	8	6	1
	2	8	8	7	6	8
	3	7	8	-	7	7
	4	7	9	-	9	7
Vessel score	1	12	5	5	4	0
	2	10	5	3	0	1
	3	12	4	-	0	0
	4	12	5	-	3	0
Nerve score	1	6	2	0	-	0
	2	5	-	0	0	0
	3	6	2	-	2	0
	4	6	-	-	6	0
Total score[b]	1	25	14	13	10	1
	2	23	13	11	6	9
	3	25	14	-	8	7
	4	25	14	-	18	7

[a] The muscle score is the sum of PAS intensity, cross striation, and vacuoles
[b] The total sore is the sum of the nerve , vessel, and muscle scores

Table 2. Patients scores

Discussion

While treating patients with infantile Pompe's disease with recombinant human α-glucosidase from rabbit milk, we had the opportunity to study the muscle pathology at various stages of the disease. At the start of treatment, the four patients were affected to a different degree as judged by clinical and histopathological findings. In all cases, the glycogen storage was not limited to striated muscle, but was also present in peripheral nerves and blood vessel walls. The patient with the best baseline condition responded most favorably to the treatment in terms of clearance of lysosomal glycogen, improvement of tissue morphology, and overall physical performance. Therapeutic effects were first observed in the blood vessels and the peripheral nerves, and only later in the skeletal muscle. The response was dose related and clearly depended on the stage of the disease before treatment.

Pathology and Pathogenesis
The muscle biopsies taken at the start of treatment confirmed the generalized nature of infantile Pompe's disease. Muscle biopsy specimens fixed and stained with routine procedures typically show fibers with vacuoles of different size and

shape, what is referred to as lace-work pattern [9, 13, 16-18]. In our procedure, glycogen is preserved with a strong fixative (glutaraldehyde), after which the sections are embedded in glycolmetacrylate so that the extent of glycogen storage is dramatically exhibited. Before treatment, all four patients had severely affected skeletal muscle fibers. However, not all muscle fibers in the same section of the same patient were equally affected, and there was interpatient variation. Moreover, we noticed that structural damage can vary substantially along the fiber length, as if the storage process spreads from discrete foci.

The biopsies taken prior to and during treatment are informative about the possible course of the pathogenic process, which starts in lysosomes. As the lysosomal compartment expands, it can be envisioned that the contractile machinery of the muscle is hampered by fibril splitting resulting in diminished vectorial strength [12]. Reduced lysosomal function and rupture of the lysosomal membrane by contractile forces will lead to an increase of the cytoplasmic glycogen concentration. Increased autophagy results in even more lysosomal storage and dysfunction. Release of lysosomal enzymes into the cytoplasm contributes to the cascade of damage.

The glycogen storage in smooth muscle cells of the arteries and veins is quite dramatic. It is conceivable that the integrity of the blood vessel wall is lost as the disease progresses. This may explain the occurrence of aneurysms in the basal arteries of patients with Pompe's disease as described in some case reports [6, 15, 19].

We have also seen vascular pathology in the knockout mouse model of Pompe's disease [3], and storage of glycogen in smooth muscle along the digestive tract and in other organs with a smooth muscle component. Storage in Schwann cells and epineurium of peripheral nerves also was noted earlier, but is not known to have functional consequences [17, 18].

Therapeutic response and dosing
The vascular endothelium and the peripheral nerves are the first sites where a response to treatment is noted. After 12 weeks of low-dose treatment (15 or 20 mg/kg), the glycogen had disappeared from the endothelial and Schwann cells, but it was still present in skeletal muscle fibers and vascular smooth muscle cells. This is not unexpected as the endothelial cells are directly exposed to the circulating enzyme, whereas Schwann cells and perineurium are optimally supplied by the anastomosing vascular system that feeds the nerve bundles.

We consider it likely that cell- and tissue-specific differences in accessibility explain in part the much lower dose of α-galactosidase needed for the treatment of Fabry's disease (0.2-1 mg/kg) [8, 20] compared to the dose needed to correct the muscle pathology in Pompe's disease. In Pompe's disease the administered α-glucosidase must pass the endothelial barrier and the endomysium before it reaches the muscle fibers.

Judging from the changes in muscle morphology over time, we believe that the dose of 15-20 mg/kg of α-glucosidase, during the first 21 weeks of treatment, was insufficient to achieve correction of smooth and skeletal muscle. The higher dose of 40 mg/kg seems necessary because the PAS-staining intensity became less, and the vascular smooth muscle started to be cleared after 12 weeks of additional treatment at this dose. The skeletal muscle morphology of the least affected patient (patient 1) had clearly improved after 72 weeks of treatment.

The rating system that we used to evaluate the morphological changes during the course of treatment proved to be helpful in obtaining objective measures. The total scores and muscle scores correlated well with the clinical observations.

Therapeutic mechanism and differential response
Even at the high dose of 40 mg/kg per week, not all patients responded sufficiently well.

This may relate to the different glycogen load and the associated muscle immobility of the patients prior to and during treatment. From both aspects, patient 1 had the best starting point with the least glycogen accumulation and the best muscle function.

The various muscle sections revealed that fibers with solely lysosomal glycogen storage maintained a relatively normal sarcomeric organization and were cross-striated. It is to be expected that normal function is regained after clearance of lysosomal glycogen by ERT. Likewise, it is conceivable that massive accumulation of cytoplasmic glycogen complicates the repair process. An immobile volume of cytoplasmic glycogen mechanically blocks the intracellular/vesicular transport pathways required for lysosomal targeting of the therapeutic enzyme. Moreover, when the lysosomal compartment is emptied, autophagic activity is needed to clear the cytoplasmic glycogen. This process requires a proper intracellular organization, which is completely lost at a severe stage of the disease.

Muscle movement is required for activation, proliferation, and fusion of myogenic satellite cells with existing muscle fibers [11, 21, 22, 29]. Patient 1 had substantial muscle strength in his legs at the start of treatment and he currently walks, whereas patients 2 and 4 could not move their legs, and their muscle function is still extremely compromised. Patient 3 could move her legs, but could not stand.

Conclusion

We have shown that intravenous administration of recombinant human α-glucosidase from rabbit milk can improve muscle morphology in classic infantile Pompe's disease. Preservation of muscle architecture and residual muscle function at start of treatment seems a prerequisite for the successful outcome of treatment. A high dose of enzyme is required to obtain an effect on skeletal muscle.

Acknowledgements

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Pharmacokinetics of three different preparations of recombinant human α-glucosidase in patients with Pompe's disease

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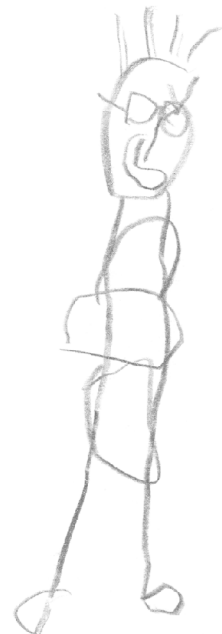
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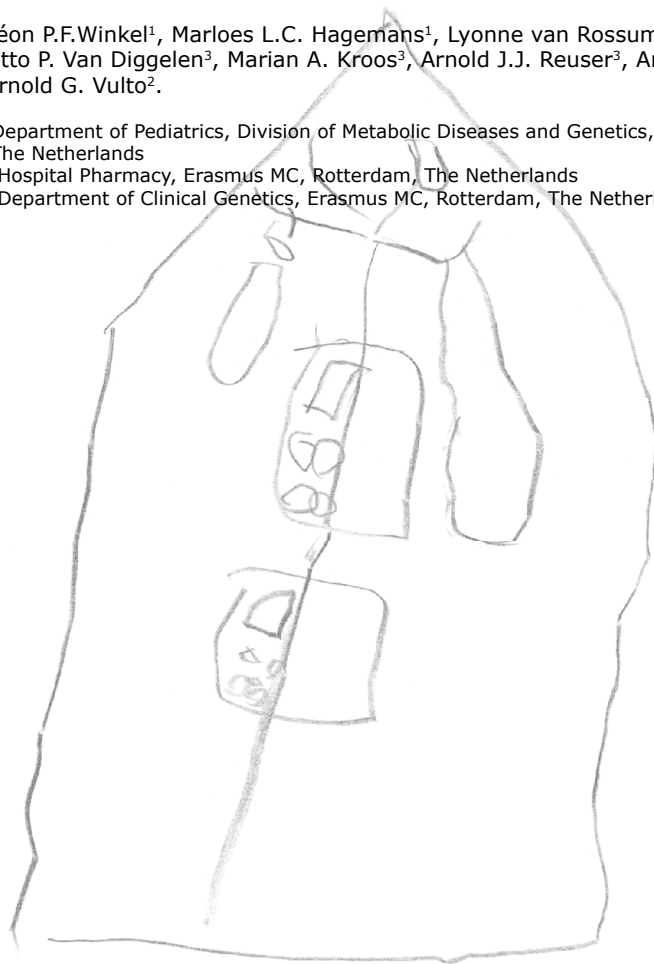
Abstract

Enzyme therapy with recombinant human α-glucosidase for Pompe's disease has been under investigation since 1999. The pharmacokinetic properties of this enzyme have as yet not been described. This information is important for establishing optimal dose, dose-interval and infusion scheme. We studied the pharmacokinetic properties of recombinant human α-glucosidase from rabbit milk and from Chinese hamster ovary cells at different doses (10, 20 & 30 mg/kg bodyweight) and different infusion rates (3.6 and 15-17 mg/kg/hour) in three patients with late-onset Pompe's disease. We found that the plasma C_{max} and AUC were both directly proportional to the dose and infusion rate. Our study suggests that it will be more effective to treat with relatively high doses and a high infusion rate than with lower doses and a low infusion rate. Dosing in mg/kg lean body mass better predicted the C_{max} and AUC than a dose based on crude bodyweight. We therefore advise to calculate the dose on the basis of lean body mass.

Introduction

Pompe's disease is an inherited lysosomal glycogen storage disorder caused by acid α-glucosidase deficiency and characterized by progressive muscle weakness. The disease can be divided into classic infantile and milder subtypes. The classic subtype, as described by J.C. Pompe, comprises rapidly progressive muscle weakness, cardiac hypertrophy, and respiratory insufficiency [1]. Survival is rare beyond 18 months of age [2]. Milder subtypes can present at any age with symptoms of (proximal) muscle weakness, resulting in wheelchair dependency and respiratory insufficiency [3]. Currently no treatment is available. The experimental treatment of Pompe's disease with intravenous administration of recombinant human α-glucosidase from rabbit milk (rabbit AGLU) started in 1999 and has shown promising results. Patients with classic infantile Pompe's disease who receive this treatment survive longer, their heart size decreases and their skeletal muscle shows uptake of the administered enzyme. Patients with milder, late-onset Pompe's disease, treated with rabbit AGLU, show stabilisation or improvement of muscle strength, muscle function and pulmonary function. The response to treatment is variable and seems related to the progression of disease before start of treatment [4-8].

The first recombinant human α-glucosidase was obtained from the milk of transgenic rabbits. This product proved to be safe and effective, but large quantities of enzyme were necessary [4, 7]. The patients with late-onset Pompe's disease who initially received rabbit AGLU were switched to Chinese Hamster Ovary (CHO) cell derived AGLU after three years of treatment. Two different clonal cell lines, with two different purification processes, were used to produce these enzymes. We will refer to these products as Synpac AGLU [9, 10] and Genzyme AGLU, which is the product currently under investigation.

Different aspects of the treatment have been described, such as safety, clinical efficacy and long term effects. The efficacy of the treatment was shown to be dose-dependent in both knock out mice with Pompe's disease [11] and in affected infants [8]. However, the pharmacokinetic properties of α-glucosidase have as yet not been described. These data are important in order to establish the optimal dose and dose interval. We have documented the exposure to the α-glucosidase preparations in plasma and compared the pharmacokinetic properties of the three preparations during changes in dose and infusion rate. We further documented the stability of α-glucosidase in blood and plasma ex vivo. In the past the uptake of α-glucosidase in skeletal muscle was examined as well [8], but the enzyme clearance

from the muscle could not be investigated for obvious ethical reasons. In the present study, leukocytes were used as a model for cellular uptake and clearance of α-glucosidase.

Methods

The present study was part of a single center, open label pilot study to evaluate the safety and efficacy of recombinant human α-glucosidase as treatment for Pompe's disease [4, 7]. The Institutional Review Board of Erasmus MC approved the study and written informed consent was obtained from the patients and/or parents. We studied three different preparations of recombinant human α-glucosidase. Alpha-glucosidase from rabbit milk (rabbit AGLU) was produced by Pharming/Genzyme LLC, Leiden the Netherlands. The other two preparations were derived from two different Chinese Hamster Ovary cell lines, with two different purification processes (Synpac and Genzyme AGLU), and were supplied by Genzyme Corporation, Cambridge, Massachusetts.

Dosing regimen

Pharmacokinetic (PK) properties of recombinant human α-glucosidase were studied in two male and one female patient with late-onset Pompe's disease. The two male patients first received recombinant human α-glucosidase from rabbit milk in a dose of 20 mg/kg/week. The original dose of 10 mg/kg/week was based on preclinical studies in a knockout mouse model of Pompe's disease [11] and was later increased to 20 mg/kg/week to increase clinical effects [7]. The enzyme was administered intravenously as a 2 mg/ml solution. During infusion the infusion rate was increased from 0.2-0.3 mg/kg bodyweight per hour (first hour) to 0.7-0.8 mg/kg bodyweight per hour (second hour), followed by 15-17 mg/kg bodyweight per hour till the end of the infusion.

When the patients were transitioned from the rabbit derived to CHO-derived α-glucosidase, they were first given a dose of 10 mg Synpac AGLU/kg per week, because the safety profile of a dose of more than 10 mg/kg was as yet unknown. Also for safety reasons, we initially applied a lower infusion rate of 3.6 mg/kg/hour for the third and following hours of the infusion. When the CHO-cell derived enzyme appeared to be well tolerated, we increased the infusion rate during the third and following hours of infusion to the previously used 15-17 mg/kg bodyweight per hour. No major side effects occurred. After 12 weeks the dose regimen was changed to 20 mg/kg every second week with the same infusion schedule. When procedure was well tolerated, it was decided to increase the dose to 30 mg/kg every second week. After 1 year the Synpac AGLU was replaced by the same dose of Genzyme AGLU with the same infusion scheme.

The third patient started treatment in a later phase of the study and was treated from the beginning with Genzyme AGLU, starting with a dose of 10 mg/kg/week, and later 20 mg/kg every second week. The type of enzyme preparation, the dosing regimen and the infusion rate at the time of each PK-measurement are listed in Table 1.

Dose	Infusionrate	Enzyme preparation	Patient
10 mg/kg/week	slow	rabbit AGLU	-
		Synpac AGLU	1 & 2
		Genzyme AGLU	3
10 mg/kg/week	quick	rabbit AGLU	-
		Synpac AGLU	1 & 2
		Genzyme AGLU	3
20 mg/kg/2 weeks	quick	rabbit AGLU	1 & 2
		Synpac AGLU	1 & 2
		Genzyme AGLU	3
30 mg/kg/2 weeks	quick	rabbit AGLU	-
		Synpac AGLU	1 & 2
		Genzyme AGLU	1 & 2
slow	3.6 mg/kg/hr		
quick	15-17 mg/kg/hr		

Table 1; Dose, dosing regimen, infusion rate and preparations of α-glucosidase studied per patient

Measurement of acid α-glucosidase activity in plasma and leukocytes in vivo
Blood samples were drawn at the following timepoints: before infusion, directly after infusion and subsequently after 30, 60, 120, 180, 240, 360 minutes and 24 hours. In some instances an additional sample was taken at 48 hours after infusion. The samples were directly put on ice and centrifuged during 6 minutes at 1500g (4 °C). The plasma was collected, frozen in liquid nitrogen and stored at -80 °C until the acid α-glucosidase activity was determined. The blood cell-pellet was washed three times with 10 mM phosphate buffered saline pH 7.0 (PBS). The leukocytes were isolated [12] and stored at -80 °C until analysis. Plasma was assayed for acid α-glucosidase activity by incubating 10 μl diluted plasma with 20 μl 4-methylumbelliferyl-α-D-glucoside (4-MU-α-glc) for 1 hour at 37 °C (pH 4.0). The reaction was terminated by adding 200 μl 0.5 M sodium carbonate buffer pH 10.7, and the fluorescence was read (excitation 360 nm, emission 460 nm). The leukocytes were assayed for acid α-glucosidase activity as described before [13, 14].

Pharmacokinetic analysis
The obtained plasma α-glucosidase activity data were fitted to a non-compartmental model (WinNonLin Professional®, Version 3.1, model 202, Pharsight, Mountain View CA, USA), and the maximum plasma concentration (C_{max}), area under the curve (AUC) and Clearance (Cl) were derived from this model.

Body composition
We performed Bioelectrical Impedance Analysis in order to determine the body composition of each patient. The OMRON BF302 Body Fat Monitor (OMRON Healthcare Europe, Hoofddorp, the Netherlands) was used to determine body fat percentage and calculate the lean body mass [15].
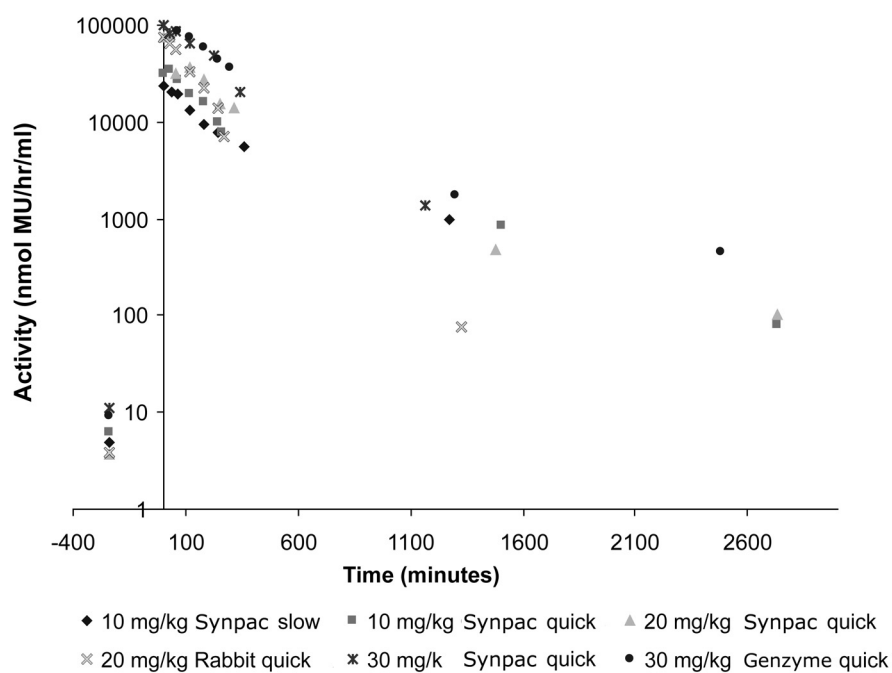
Stability of α-glucosidase in plasma ex vivo
To measure the stability of α-glucosidase in blood and plasma we heparinized blood from a healthy volunteer. Forty μl (200 μg) of the different enzyme preparations

were added to 1.5 ml whole blood and 20 µl (100 µg) was added to 1.5 ml of plasma. These enzyme concentrations were chosen to correspond with an infusion dose of 10 mg/kg bodyweight in vivo. The incubations were performed at 37 °C to mimic the body temperature and stopped by freezing the sample in liquid nitrogen either directly after enzyme addition, or after 30, 60, 120, 180, 240 and 360 minutes and 24 hours. Acid α-glucosidase activity was determined as described above. To investigate possible enzyme breakdown, western blot analysis was performed on these samples. SDS-PAGE (8%) was performed under reducing conditions (by adding mercaptoethanol to the sample buffer) followed by electroblotting.
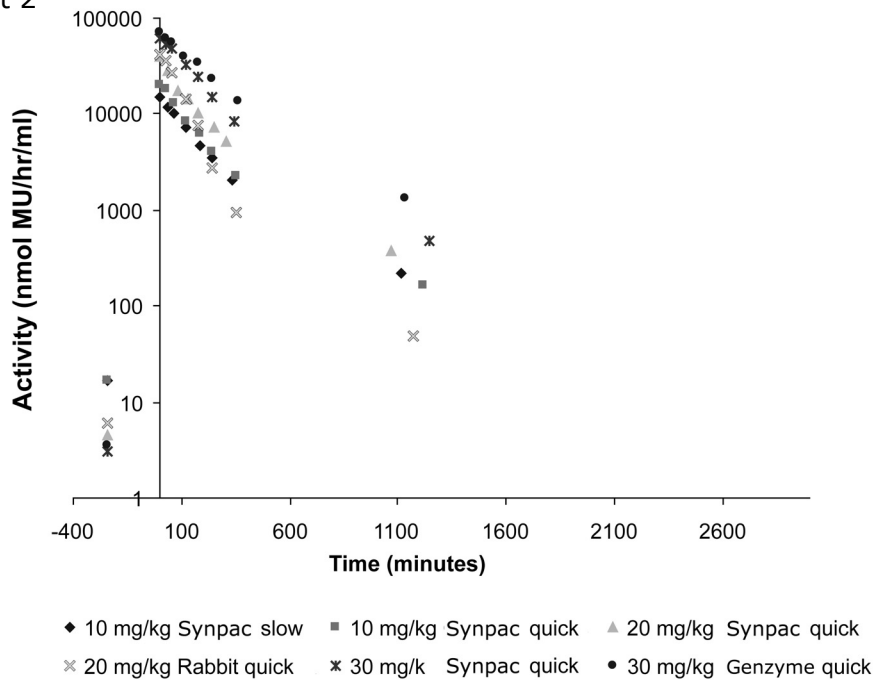
Results

Heparinized blood samples from three patients with Pompe's disease treated with three different enzyme preparations were drawn before, immediately after and at several time-points following the end of the infusion. Figure 1 shows the plasma α-glucosidase-activity in these samples on a logarithmic scale. The activity decreased logarithmically with time. The figure further shows that both a higher dose and a higher infusion rate increased the C_{max}.

Patient 1

● 10 mg/kg Synpac slow   ■ 10 mg/kg Synpac quick   ▲ 20 mg/kg Synpac quick

✕ 20 mg/kg Rabbit quick   ✱ 30 mg/k   Synpac quick   ● 30 mg/kg Genzyme quick

Patient 2

◆ 10 mg/kg Synpac slow   ■ 10 mg/kg Synpac quick   ▲ 20 mg/kg Synpac quick
✕ 20 mg/kg Rabbit quick   ✳ 30 mg/k Synpac quick   ● 30 mg/kg Genzyme quick

Patient 3

◆ 10 mg/kg Genzyme slow   ■ 10 mg/kg Genzyme quick   ▲ 20 mg/kg Genzyme quick
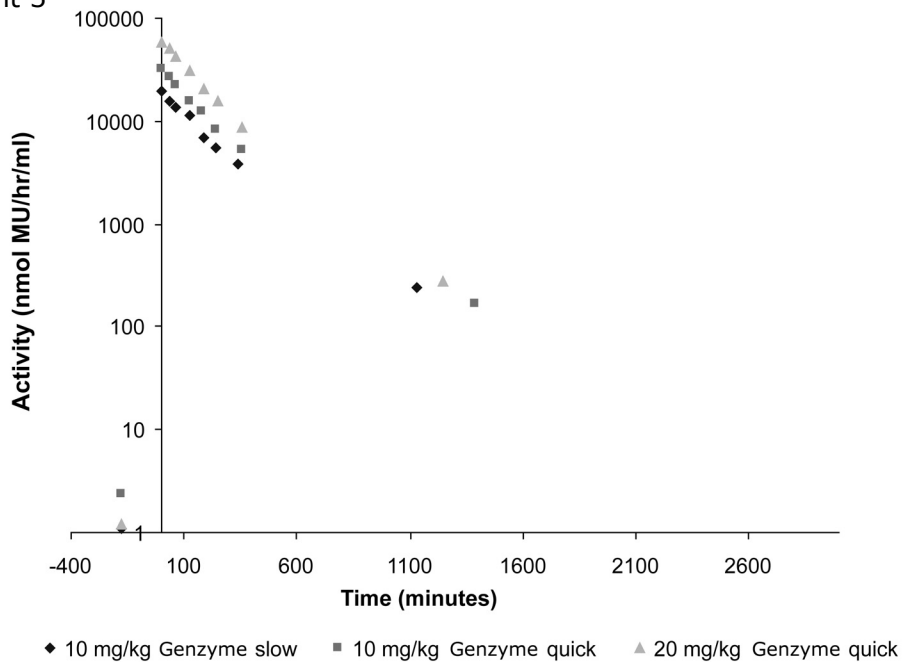
Figure 1; Alpha-glucosidase activity in plasma of three patients with late-onset Pompe's disease (1, 2 and 3) before and after infusion with three preparations of recombinant human α-glucosidase

The area under the curve (AUC) shown in figure 1 was calculated, and showed a linear and positive relationship to the administered dose. The AUC differed between the three enzyme preparations. For both patients 1 and 2, 20 mg Synpac AGLU/kg gave a slightly larger AUC than 20 mg rabbit AGLU/kg. At a dose of 30 mg/kg, Genzyme AGLU resulted in a larger AUC than Synpac AGLU (Table 2).

Patient (characteristics)	Dose / Infusion rate / Enzyme preparation	Cmax (*10E4 nmol 4MU/hr/ml)	AUC (*10E6 min* nmol/hr/ml)	CL(obs) (ml plasma /min)	Peak value leukocytes (nmol glc/hr/mg protein)
1					
male 35 yrs	10 mg/kg slow Synpac AGLU	2.37	9.25	13.0	332
weight 75 kg	10 mg/kg quick Synpac AGLU	3.52	11.97	10.3	486
LBM 52 kg	20 mg/kg quick Synpac AGLU	7.37	21.98	10.9	492
	20 mg/kg quick Rabbit AGLU	7.90	16.29	29.2	169
	30 mg/kg quick Synpac AGLU	9.84	35.05	10.5	544
	30 mg/kg quick Genzyme AGLU	11.00	46.92	9.5	338
2					
male 15 yrs	10 mg/kg slow Synpac AGLU	1.51	4.27	19.6	176
weight 48-50 kg	10 mg/kg quick Synpac AGLU	2.06	4.29	17.8	173
LBM 46-48 kg	20 mg/kg quick Synpac AGLU	3.77	8.32	17.6	276
	20 mg/kg quick Rabbit AGLU	4.40	6.44	34.1	87
	30 mg/kg quick Synpac AGLU	6.37	17.76	13.6	260
	30 mg/kg quick Genzyme AGLU	7.09	22.54	13.2	221
3					
female 32 yrs	10 mg/kg slow Genzyme AGLU	1.91	6.56	14.6	146
weight 47-51kg	10 mg/kg quick Genzyme AGLU	3.19	8.41	9.0	140
LBM 33-35 kg	20 mg/kg quick Genzyme AGLU	5.97	15.64	9.0	153

The variation of duplicate measurements was within 4-6%.

Normal alpha-glucosidase concentration in plasma of healthy volunteers (n=10); Mean 3 nmol 4MU/hr/ml plasma (Column Cmax)
Normal alpha-glucosidase activity in leukocytes of healthy volunteers (n=10); range 80-250 nmol glc/hr/mg protein (Column Peak value leukocytes)

Values were derived from a Non-Compartmental-infusion model

Table 2; Pharmacokinetic properties of three preparations of recombinant human α-glucosidase

Different doses or different infusion rates did not influence the rate of α-glucosidase clearance from the plasma substantially. Nonetheless the clearance of the rabbit AGLU was more rapid than the clearance of both CHO-cell derived α-glucosidases (Table 2).

Body composition
Even at the same dose and the same infusion rate, the C_{max} and AUC values differed between patients. For example, after administration of 30 mg/kg Genzyme AGLU (infusion rate of 15-17 mg/kg/hour) the C_{max} was 11.00 *10^4 nmol MU/hr/ml in patient 1 and 7.09 *10^4 nmol MU/hr/ml in patient 2.

We examined the lean body mass as a possible factor that might explain these differences. For this purpose the percentage of body fat was determined by Bioelectrical Impedance Analysis. Patient 1 had 32.5% body fat, patient 2, 4.3% and patient 3, 25.8%. With these percentages, the calculated lean body mass was 52 kg in patient 1, 46-48 kg in patient 2 and 33-35 kg in patient 3. Figure 2 shows

the C_{max} versus the dose in mg/kg crude bodyweight and versus the calculated dose in mg/kg lean body mass. When the latter method was applied, a similar linear relationship between dose and C_{max} was found for all three patients.
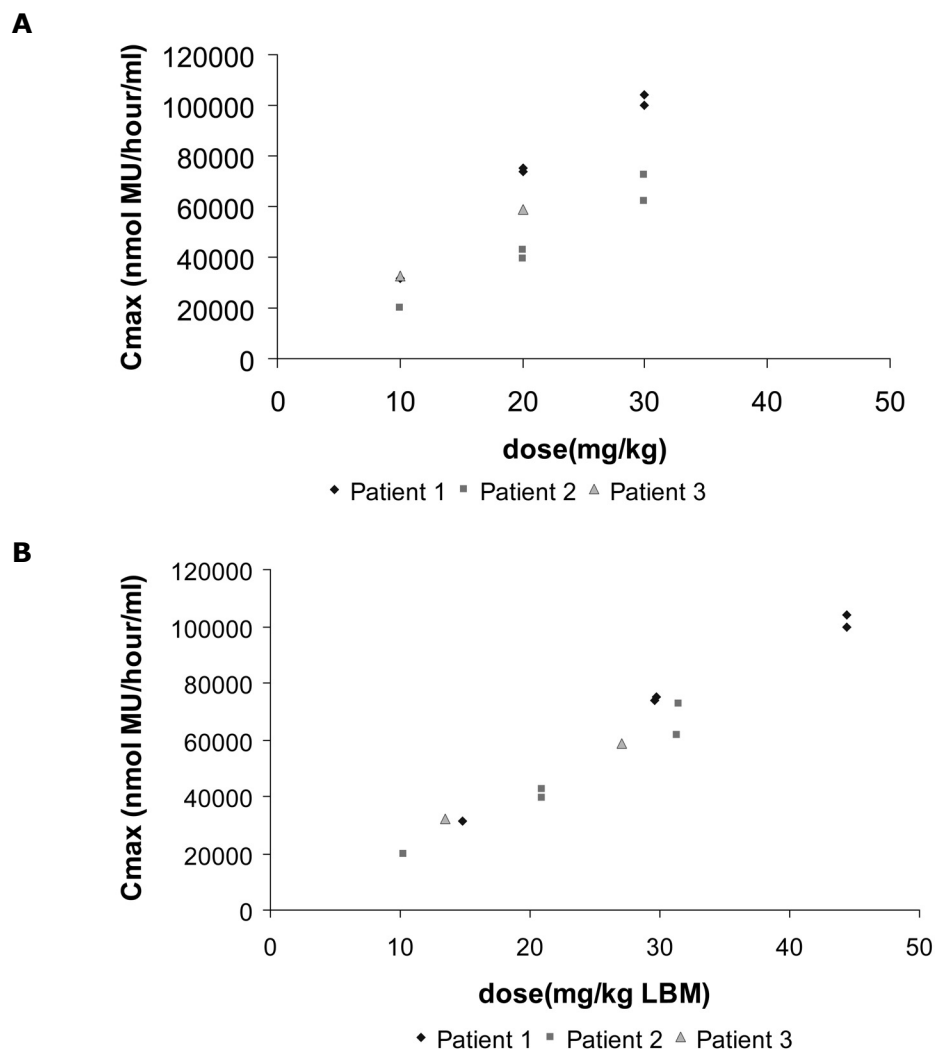
A

B

Figure 2; C_{max} versus dose in mg/kg crude body weight (A), and C_{max} versus dose in mg/kg lean body mass (B).

Leukocytes
In addition to the plasma samples, the uptake of α-glucosidase by white blood cells was studied. The acid α-glucosidase-activity in the leukocytes increased to normal values during the first 4-6 hours after start of infusion and then stabilized during the 24 to 48 hours following infusion. When measured again after 7 or 14

days, the activity was the same as before the infusion. We found a 2-3 fold higher α-glucosidase-activity in the leukocytes of the patients after treatment with either of the two CHO-enzyme preparations, than after treatment with rabbit AGLU. Furthermore we observed higher maximum acid α-glucosidase-activities in the leukocytes of patient 1 than of patient 2 and 3 (Table 2).

Stability of α-glucosidase ex vivo
The clearance of the enzyme in blood and plasma is determined by its stability as well as by its uptake by the organs and tissues. To dissect the clearance into these two components, we added the three enzyme preparations ex vivo to both heparinized plasma and whole blood samples at a concentration similar to a dose of 10 mg enzyme/kg bodyweight in vivo. The enzyme activity decreased logarithmically with time for all three enzyme preparations (Figure 3) both in whole-blood and in plasma. This resulted in a half-life in plasma of 48-59 minutes, and of 95-103 minutes in whole blood. Western blot analysis showed that the three enzyme preparations (110 kD) were equally stable and that no degradation products were formed for at least 6 hours after incubation.
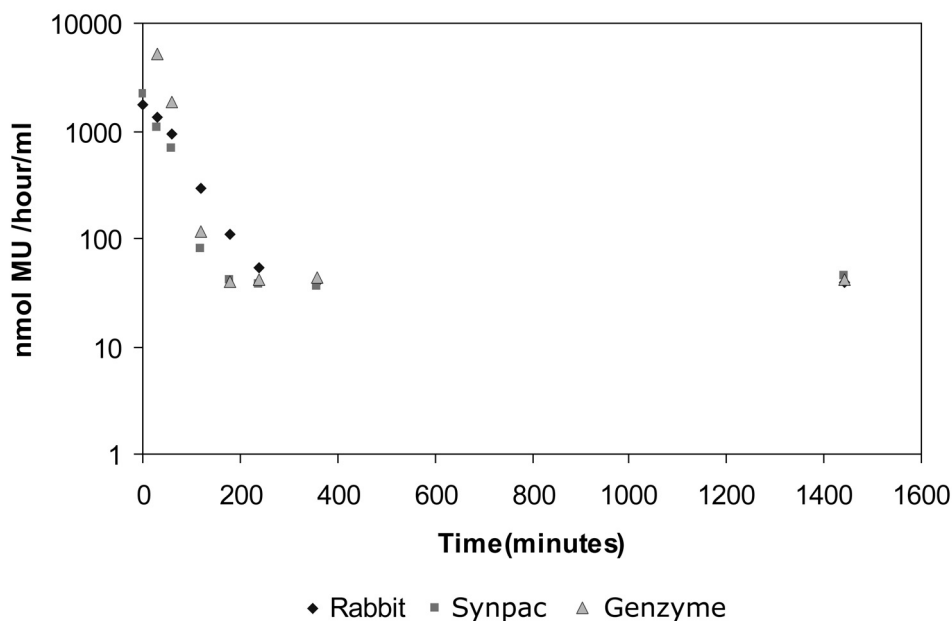
A

◆ Rabbit   ■ Synpac   △ Genzyme
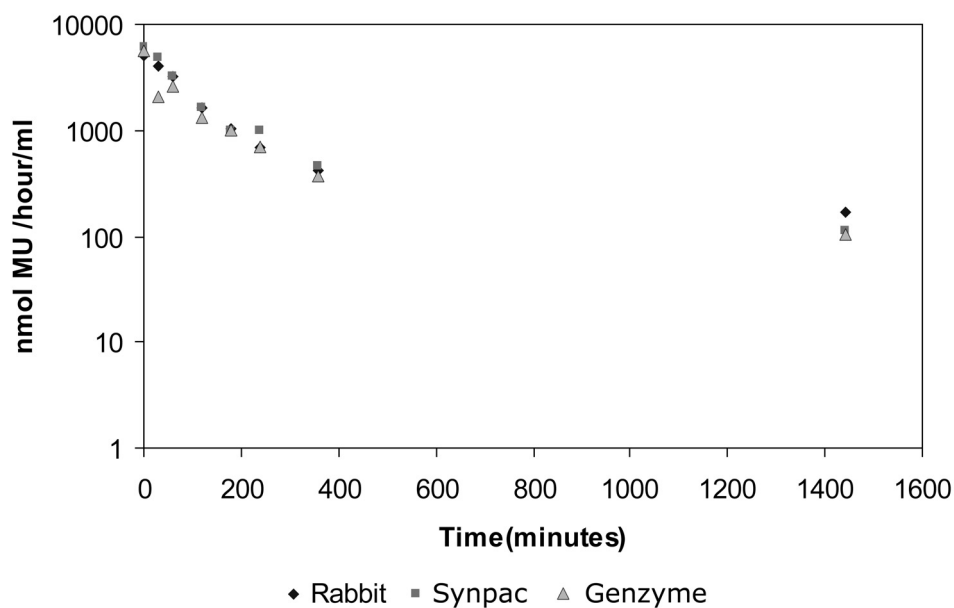
B

Figure 3; Ex vivo stability of α-glucosidase measured in whole blood (A) and in plasma (B).

Discussion

Since 1999 we have experimentally treated patients with Pompe's disease with infusions of recombinant human α-glucosidase. Infusions have appeared safe and were tolerated well [4, 7, 8] (Winkel et al. unpublished results). The present study shows that the C_{max} and the AUC measured in 3 patients with late-onset Pompe's disease correlate linearly with the administered dose and the rate of enzyme infusion. This holds equally for the three preparations of recombinant human α-glucosidase. The most distinct difference between the preparations was the relatively high clearance rate of rabbit AGLU. As the patients in question were first treated for three year with rabbit AGLU before they switched to Synpac and later Genzyme AGLU, we can not exclude that the difference in clearance rate has somehow to do with the length of time or the order in which the preparations were given. On the other hand, it may be an intrinsic feature of the different preparations.

We have experienced that a dose of 10 mg/kg is minimally required to obtain uptake of the enzyme in muscle tissue, and only 40 mg/kg resulted in normal α-glucosidase levels in muscle tissue [7, 8]. The C_{max} in plasma is the driving force that feeds α-glucosidase to the cells. Because both the dose and the infusion rate determine C_{max} it is expected that high doses are more effective than low doses and a higher infusion rate better than a lower infusion rate. We have based our infusion schedule on these principles and apply a high infusion rate of 15-17 mg/kg/hour, but start with a slow infusion rate of 0.2-0.3 mg/kg/hour in the first and 0.7-0.8 mg/kg/hour in the second hour to prevent infusion associated adverse events [8].

The more rapid clearance of rabbit AGLU can have different causes. It may be due to instability of the enzyme species in blood or plasma, or by uptake in organs. A more rapid clearance by the kidney is very unlikely because the molecular mass of 110 kD does not allow the enzyme to pass the filtration barrier. As the three enzyme preparations are equally stable in whole blood and plasma when incubated *ex vivo*, it seems that rabbit AGLU is more rapidly absorbed by the organs.

Our experiments do not allow determining to which organs the enzyme is preferentially targeted. Experiments in mice, however, have shown that the same enzyme activity levels are reached in the various organs (liver, heart and skeletal muscle) independent of the enzyme source [16]. Thus, we do not expect that there are large differences in the therapeutic efficacy of the different forms of AGLU. Our preliminary clinical findings support this view (Winkel et al. unpublished results).

Somewhat unexpectedly, we found substantial differences in the C_{max} and AUC between patients who were treated with the same dose of enzyme per kilogram bodyweight and given at the same infusion rate. We reasoned that a difference in body composition could play a role. When expressed per kg lean body mass, the C_{max} reached in all patients was very similar. The patient with relatively more fat (patient 1) received in practice a higher dose. This also explains the higher enzyme activity in the leukocytes (Table 2). The suggestion arises that dosing per kg lean body mass is more appropriate than dosing per kg crude bodyweight, because it better predicts the C_{max} that will be achieved in plasma.

We have observed in earlier studies that the uptake of α-glucosidase by lymphocytes is largely mannose 6-phosphate receptor dependent (Winkel et al. unpublished results), and that CHO-derived α-glucosidases bind more readily to this receptor than rabbit AGLU. This explains the differences in peak values that were measured in the leukocytes, but has no relevance for predicting the clinical outcome of enzyme replacement therapy [4, 7, 8, 17, 18] (Winkel et al. unpublished results). Leukocytes can be used to study the intra lysosomal half-life of α-glucosidase.

Conclusion

We conclude that the plasma C_{max} and AUC are directly proportional to both the dose and the rate of enzyme infusion. Therefore it will be more effective to treat patients for instance weekly or biweekly with a relatively high dose and a high infusion rate than two or three times per week with a relatively low dose and low infusion rate. Linear dose proportionality between patients is obtained when the dose is based on lean body mass rather than crude body weight. Thus, dosing on the basis of lean body mass seems more appropriate.

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Recombinant human α-glucosidase causes a rapid decrease of PAS-positive vacuoles in lymphocytes of patients with Pompe's disease

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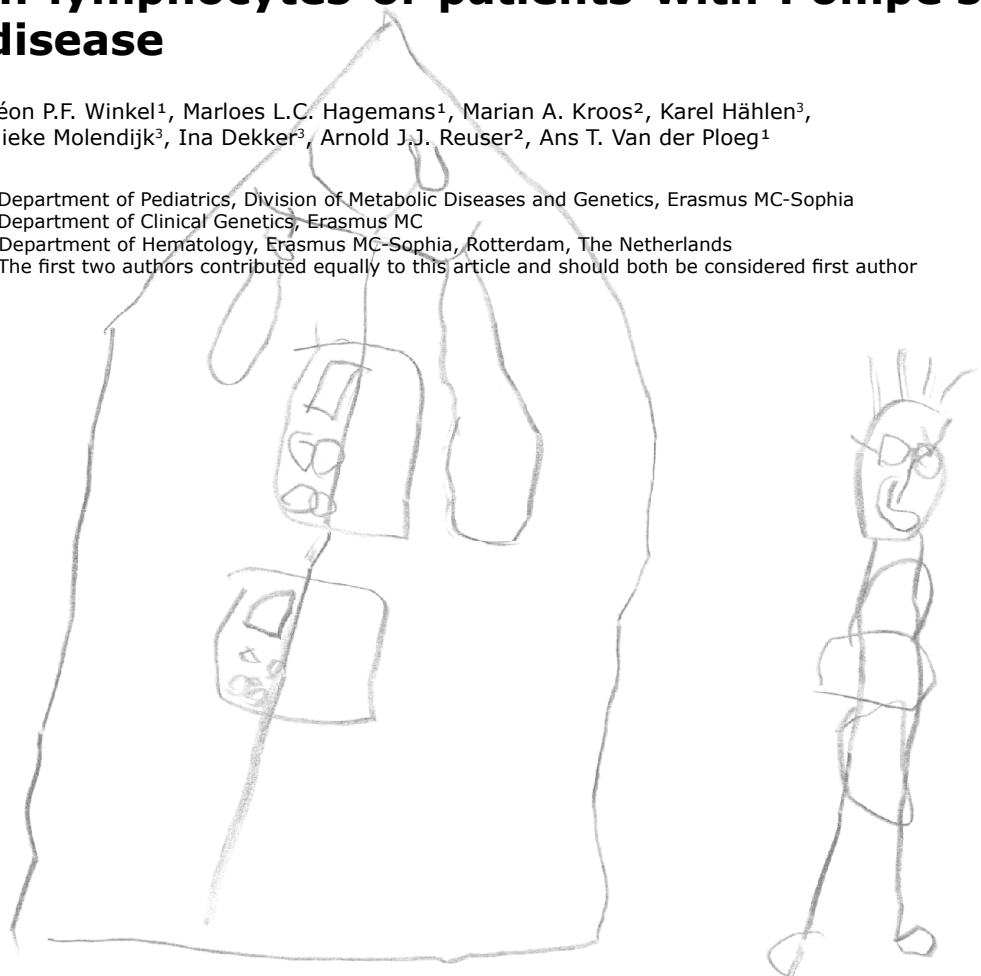
Abstract

In a study on the safety and efficacy of enzyme replacement therapy for Pompe's disease we collected repetitive blood smears to investigate the lysosomal accumulation of glycogen in lymphocytes as a diagnostic tool and as a surrogate marker for therapeutic response. The blood smears were stained with periodic acid-Schiff (PAS) and the number of PAS-positive vacuoles was scored.

Before the start of treatment, the patients with classic infantile Pompe's disease had a significantly higher PAS-score than those with late onset Pompe's disease. The lymphocytic vacuoles disappeared rapidly upon initiation of treatment with recombinant human α-glucosidase. This was long before clinical effects could be measured.

In vitro experiments demonstrate that the uptake of enzyme by the lymphocytes is largely mediated by the mannose 6-phosphate receptor. The fraction of enzyme captured by the lymphocytes is too small to affect the outcome of enzyme replacement therapy in either a positive or negative way.

We conclude that the simple staining of a blood smear with PAS may help to identify Pompe patients among patients with a proximal muscle weakness. The regression of PAS positive lymphocytic inclusions is a very early response to enzyme replacement therapy and the first proof that the administered enzyme is functional.

Introduction

White blood cells express a broad range of lysosomal enzyme activities [1]. For this reason they are often used as starting material for the enzymatic diagnosis of lysosomal storage disorders. In a number of these disorders, the enzyme deficiency results in the formation of lymphocytic vacuoles filled with storage product. Their presence is a diagnostic guide, next to the primarily informative clinical, laboratory, and molecular findings [2].

The occurrence of periodic acid-Schiff (PAS) positive inclusions in lymphocytes was reported in Pompe's disease, a lysosomal glycogen storage disorder caused by acid α-glucosidase deficiency and characterized by generalized muscle weakness [3]. The PAS-reagent reacts with the lysosomal glycogen and produces a typical purple red staining [4-7].

We have applied a semi-quantitative version of the PAS staining procedure on blood smears as one of several methods to evaluate the effects of enzyme replacement therapy in Pompe's disease. Seven patients participated in our study. Four of them had the severe and rapidly progressive classic infantile phenotype and three had a late onset form of Pompe's disease [8-10].

Repetitive blood samples were taken before and during treatment with recombinant human α-glucosidase from the milk of transgenic rabbits. This report describes the rapid clearance of glycogen from the lymphocytic vacuoles after start of treatment, presents the results of investigations into the underlying mechanism, and discusses the diagnostic and therapeutic implications of these findings.

Patients and methods

Clinical studies

The present investigation was part of a single center, open label pilot study to evaluate the safety and efficacy of recombinant human α-glucosidase as treatment for Pompe's disease [8-10]. The Institutional Review Board of the Erasmus MC approved the study and written informed consent was obtained from the patients and/or parents. During the period in which blood smears were collected to determine

PAS-scores, four infantile patients were treated with a weekly dose of 15-20 mg/ kg (bodyweight) recombinant human α-glucosidase from rabbit milk. The three patients with late onset Pompe's disease received a weekly dose of 10 mg/kg.

Blood samples were collected at baseline and at the time points indicated in Figure 1, each time shortly before the start of enzyme infusion. Blood smears were stained with periodic acid-Schiff (PAS) for the presence of glycogen filled vacuoles according to standard procedures. A scoring method was applied that was originally developed to quantify the lymphocytic proliferation in leukemia's [11, 12]. In short, the presence of PAS-positive vacuoles was scored for each lymphocyte on a scale from 0 (no PAS-positive vacuoles) to 4 (a large group of large PAS-positive spots). The sum of the scores of 100 lymphocytes constituted the PAS-score as used in this investigation, and ranged from 0 to 400. Healthy lymphocytes are scored 0 [13].

Laboratory investigations

Lymphocytes were separated from the buffy coat of heparinized blood by centrifugation using lymphocyte separation medium (ICN Biomedicals Inc, Costa Mesa,California). They were cultured in RPMI 1640-media supplemented with 10% fetal calf serum and 0.04% phytohemaglutinine for 4 days and then seeded in 24 well tissue culture plates at a density of 10^6 cells per well in a volume of 1 ml of culture medium. Recombinant human α-glucosidase from rabbit milk (specific activity >250 µmole 4-methylumbelliferone (MU)/h/mg protein) [8, 9] was added in a concentration of either 1.25 or 25 units / ml. One unit is equivalent to 1 µmole 4-MU released per hour from 4-methylumbelliferyl-α-D-glucopyranoside at pH 4.0. The enzyme was provided by Pharming-Genzyme LLC, Leiden, The Netherlands. Uptake of enzyme via the mannose 6-phosphate receptor was inhibited by the addition of mannose 6-phosphate in a final concentration of 10 mM. The lymphocytes were incubated with the enzyme for 16 hours and then harvested by centrifugation. They were washed twice with phosphate buffered saline, homogenized by sonication, whereafter the acid α-glucosidase activity and the protein concentration were measured as described before [9].

Results

PAS positive lymphocytic inclusions

Four infants and three (young) adults with Pompe's disease received infusions of recombinant human acid α-glucosidase from rabbit milk. They participated in a study that aimed to investigate the therapeutic potential of enzyme replacement therapy for Pompe's disease.

Among a long list of other tests, the protocol included the collection of blood samples every fourth week, each time shortly before the start of the enzyme infusion. A blood smear was prepared to evaluate the clearance of glycogen from the PAS-positive vacuoles in the lymphocytes. To this end we used a semi-quantitative method whereby 100 lymphocytes were microscopically inspected. The number of PAS positive inclusions per cell was scored. The sum-score was taken as a measure for the degree of glycogen storage in the lymphocytes.

Before the start of treatment, the mean score of the three patients with late onset Pompe's disease was 56 ± 11 (range 46 to 68). The four infants scored significantly higher (139 ± 20, range 118-163, P = 0.034; Mann Whitney test). Within each group, the older and more severely affected patients had the highest score (Fig. 1AB t=0).

Blood smears of the four infants, who received 15 or 20 mg of enzyme per

kg per week, were prepared every fourth week of treatment (Fig. 1A). We observed that the PAS positive inclusions had virtually disappeared after the first 4 weeks. A similar drastic decline of the PAS-score was also seen in the three older patients who were treated with 10 mg of enzyme per kg per week (Fig. 1B). One of these patients (patient 6) was tested as planned every fourth week of treatment. We failed to obtain a blood smear from patient 5 after the first four weeks of treatment. The third patient with late onset Pompe's disease (patient 7) entered the study when the first results were already available. For this patient we decided to deviate from the original protocol and collected blood samples within the first 4 weeks. Thereby we observed that the PAS-positive inclusions disappeared directly after the start of treatment (Fig. 1B).
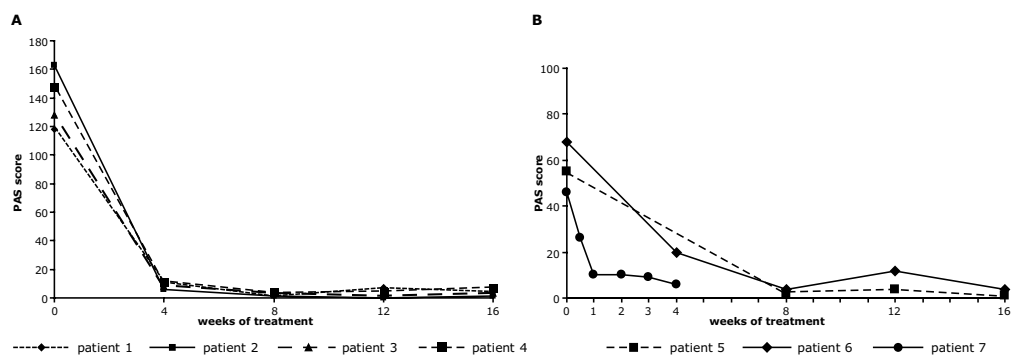

Figure 1.
PAS-scores in lymphocytes vs. weeks of treatment in 4 patients with infantile Pompe's disease (A) and in 3 patients with late onset Pompe's disease (B). All patients were treated with recombinant human α-glucosidase from rabbit milk.

Uptake of α-glucosidase by lymphocytes
To investigate the mechanism of uptake of recombinant human α-glucosidase from rabbit milk by lymphocytes, we separated the lymphocytes from a blood sample of one of the infants and kept them in culture for 4 days. A lymphocyte sample of a healthy person served as control. On these cultured cells we performed uptake studies of recombinant human α-glucosidase in two different concentrations. The high dose mimics the plasma concentration of the recombinant enzyme at the end of the infusion period, when administered in a dose of 10 mg per kg. The results of this experiment are shown in Fig. 2. The uptake of enzyme by cultured lymphocytes was concentration dependent and, at the high dose, more than sufficient to correct the α-glucosidase deficiency of the cells (80 versus 37 nmol MU/hour/mg protein in the control lymphocytes). At both concentrations, the uptake of α-glucosidase was inhibited by 10 mM mannose 6-phosphate. The degree of inhibition was 53% at the high dose.
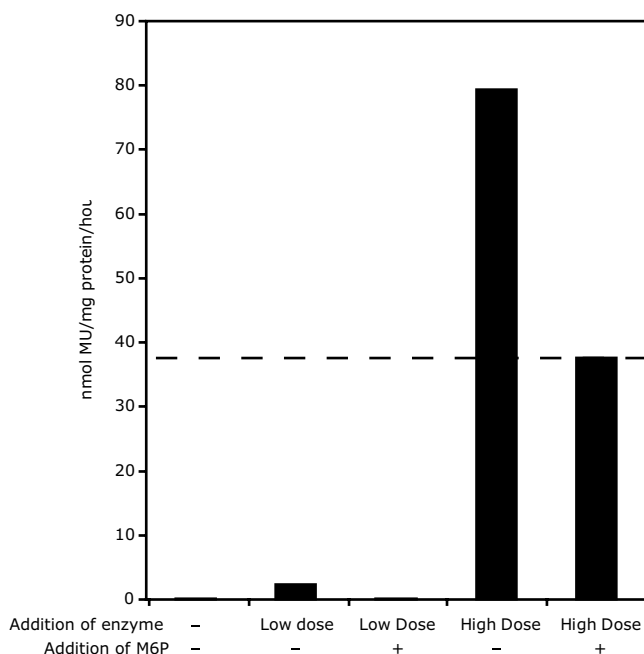
Figure 2.
Uptake of recombinant human α-glucosidase from rabbit milk by cultured acid ɑ-glucosidase deficient lymphocytes. Low Dose = 1.25 Units / ml, High Dose = 25 Units / ml. The incubations were performed in the presence (+) and absence (-) of 10 mM mannose 6-phosphate. The dotted line is the activity of acid α-glucosidase in cultured lymphocytes of a healthy individual. All assays were performed twice in duplicate.

Discussion

All patients in our study had PAS positive lymphocytic inclusions in their peripheral blood due to the lysosomal storage of glycogen. The inclusions disappeared rapidly after the start of enzyme replacement therapy. This was the first response to the administered enzyme that we noted and it preceded the clearance of glycogen from vascular endothelium, smooth muscle cells and peripheral nerves. Correction of skeletal muscle was an even later event [14]. All patients responded with a decrease in the PAS-score within the first few weeks of treatment, but the long term clinical outcome varied substantially among the patients [8-10]. From these data we conclude that the PAS-score has no predictive value for the long-term effects of enzyme replacement therapy, but is a first important indicator that the recombinant enzyme is functional.

The PAS-score can be valuable as a diagnostic tool, because all patients had PAS scores significantly above normal before start of treatment. There even seems to be a correlation between the height of the score and the clinical subtype. In combination with previously published observations [15], our results suggest that a simple PAS-score on blood smears of patients with proximal muscle weakness may be sufficient to include or exclude Pompe's disease in the differential diagnosis. To our knowledge PAS-positive lymphocytic inclusions have not been described in combination with other diseases affecting the proximal muscles. We will test this hypothesis by investigating a larger cohort of patients with Pompe's disease and

patients with clinically similar neuromuscular disorders.

The rapid clearance of glycogen from the lymphocytic vacuoles suggests that the therapeutic enzyme is taken up from the circulation by receptor-mediated endocytosis. The outcome of the in vitro experiments suggests that the bulk of enzyme enters the lymphocytes by mannose-6-phosphate receptor-mediated endocytosis, in a concentration dependent manner. Our results are herewith in line with those of Mononen et al. [16] who performed similar in vitro experiments with recombinant human glycosylasparaginase . When administered to the lymphocytes in the high dose of 25 Units per ml, the α-glucosidase is fully corrective. This dose mimics the enzyme concentration that is reached in the blood of patients at the end of the infusion period with a dose of 10 mg/kg (20-30 µmole/ml; Winkel et al., unpublished results). Under these circumstances the glycogen storage in the lymphocytes is expected to be resolved within 5-10 days based on the rate of glycogen clearance in fibroblasts and cultured muscle cells loaded with α-glucosidase [17, 18]. Thus, our finding that the vacuoles disappear within 1 week fit the expectations.

It can also be explained that the PAS-score remained permanently low after the first week of treatment. With an infusion length of 2-4 hours, an α-glucosidase plasma half-life of more than 2 hours (unpublished results, Winkel et al., unpublished results), and a continuous recirculation of lymphocytes through the peripheral blood [19], a substantial proportion of all lymphocytes will be exposed to the therapeutic enzyme. Once the glycogen has disappeared it will take at least one week before the storage reappears, because the intracellular half-life of α-glucosidase is close to one week [18, 20]. Newly formed lymphocytes may contain PAS positive inclusions, but only 1% of the lymphocyte population is renewed every week [21]. If the recombinant human α-glucosidase corrects the lymphocyte progenitor cells in the bone marrow, the treatment may prevent the formation of PAS positive lymphocytes altogether.

The goal of enzyme replacement therapy is to deliver the enzyme to the target tissues, for instance, macrophages in Gaucher's disease, vascular endothelium, pericytes, renal epithelial cells and cardiomyocytes in Fabry's disease, and cardiac plus skeletal muscle in Pompe's disease. The fraction of enzyme that is taken up by the lymphocytes is in first instance not available for the target organs. Should it be considered as lost for therapeutic purposes or can the lymphocyte act as shuttle for transferring the enzyme to other tissues? Lymphocytes have the advantage that they can cross endothelial barriers and access brain and other tissues that are otherwise inaccessible or difficult to reach such as skeletal muscle [22, 23]. The shuttle function requires exocytosis, a process whereby the lysosomal content is expelled into the extracellular environment [24-26]. It is recognized that exocytosis can occur in many different cell types, either spontaneously or in response to certain stimuli, for instance a high Ca^{2+} concentration [27-30].

Young et al. [31] tested the potential role of T lymphocytes as enzyme shuttle in a mouse model of MPS VII by infusing genetically modified T-cells that over-expressed ß-glucuronidase as a model for bone marrow transplantation. They reported that there was minimal to no cross correction of the tissues of the enzyme deficient recipients. The lack of stimuli to induce exocytosis could be the main reason for the therapeutic failure, and if this were true bone marrow transplantation is not expected to exert a great effect in Pompe's disease [32]. It is even less likely that the lymphocytes that have captured the administered enzyme from the circulation are instrumental in the correction of distant tissues.

However, uptake in the lymphocytic pool does not represent a significant loss of enzyme either, as the lymphocytes consume less than an estimated 0.1% of the administered dose.

We conclude that the simple staining of a blood smear with periodic acid-Schiff reagent may help to identify patients with Pompe's disease among patients with proximal muscle weakness. The decrease of PAS positive lymphocytic inclusions is a very early response to enzyme replacement therapy and proves that the administered enzyme shows appropriate lysosomal targeting. The effect of endocytosis by lymphocytes is neither positive nor negative for the outcome of enzyme replacement therapy as the lymphocytic enzyme pool is insignificant compared to the total administered dose.

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Discussion
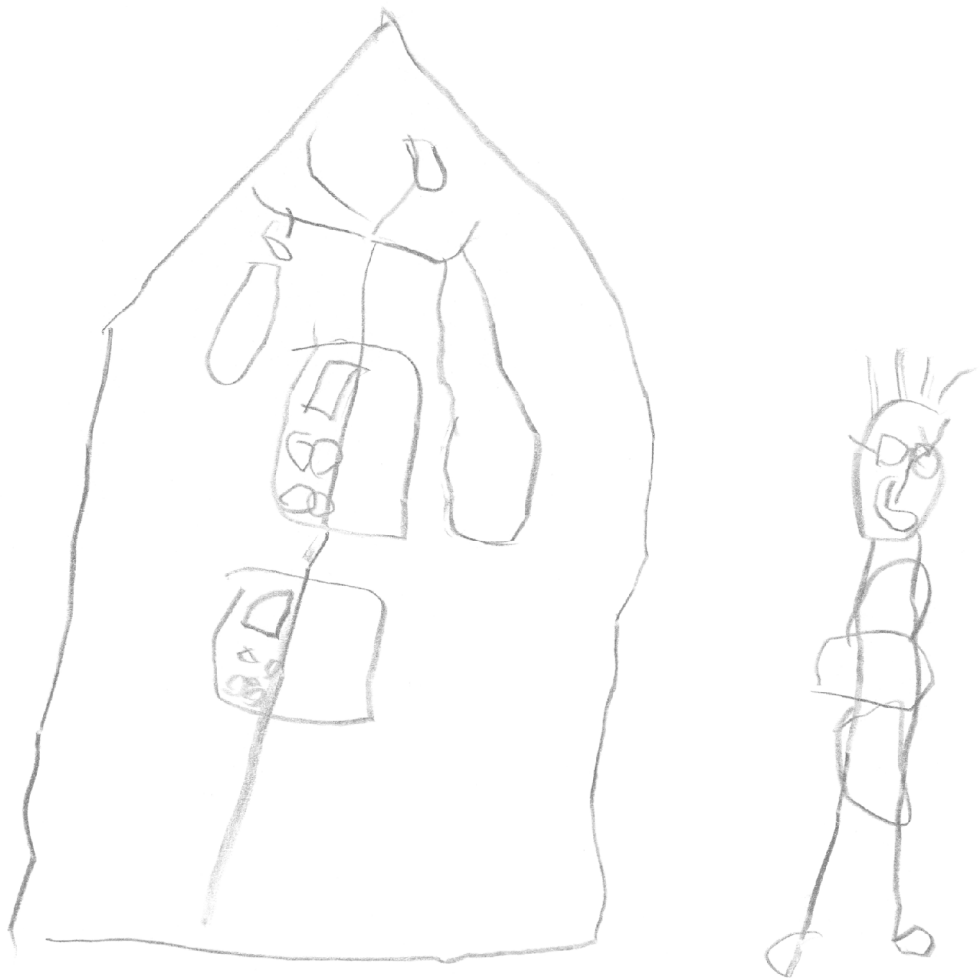
Discussion

The first case of Pompe's disease was described in 1932 [1]. The first attempt at enzyme replacement therapy (ERT) for Pompe's disease was in 1964 with a crude extract of Aspergillus Niger [2]. We started studies on the safety and efficacy of recombinant human α-glucosidase for the treatment of Pompe's disease in 1999. This thesis describes the results of those studies, which are quite novel in that the patients were treated with recombinant human α-glucosidase. The patients initially received infusions with recombinant human enzyme from rabbit milk (rabbit enzyme) for a period of 3 years. Thereafter they were treated with recombinant human α-glucosidase from Chinese Hamster Ovary cells (CHO enzyme). This chapter discusses the outcome of the studies and the implications for future treatment and research.

8.1 Subtyping of Pompe's disease

There is no consensus about the nomenclature used for the different forms of Pompe's disease. In the original publication of Dr. Pompe the syndrome was referred to as idiopathic hypertrophy of the heart. The storage of glycogen was noted as a characteristic molecular feature [1]. As other diseases were characterized by glycogen storage in one or several organs, Dr. Cori introduced in 1954 a classification of the glycogen storage disorders that was widely adopted [3]. The disease phenotype described by Dr. Pompe was listed as glycogen storage disorder type II (GSD II). Soon after, reports were published about patients with variant forms of GSD II. The variation concerned the absence of cardiac involvement, the later onset of symptoms and the longer survival [4-6]. The 'muscular' form of Pompe's disease was used as an alternative name.

When Dr. Hers discovered in 1963 the deficiency of lysosomal α-glucosidase as primary cause of Pompe's disease, he used maltose as a substrate to measure the enzyme activity and Pompe's disease became synonymous with acid maltase deficiency [7]. In fact this is an improper name as glycogen is the natural substrate and storage product.

Albeit, maltose was used as convenient substrate and patients of different ages with skeletal muscle weakness were diagnosed as having GSD II. Depending on the age of the patient and the onset of symptoms these patients were described as having infantile, childhood, juvenile and adult (onset) forms of either GSD II, acid maltase deficiency, or Pompe's disease. More recently, infants with an atypical presentation of infantile Pompe's disease (as described by Dr. Pompe) were classified as having non-classic infantile Pompe's disease [8].

Both for diagnostic and prognostic purposes as well as for evaluating the effects of therapeutic intervention, it is valuable to try to classify the patients. Recently Dr. Van den Hout et al. performed an investigation into the characteristic features of the most severe form of Pompe's disease, resulting in the clear delineation of classic Pompe's disease [9]. In chapter 2 of this thesis the existing literature on cases of Pompe's disease that do not fit the delineation of classic Pompe's disease is summarized. The review of 225 published cases shows that the symptoms and signs are related to muscle weakness, varying from difficulties in walking to respiratory insufficiency due to weakness of the respiratory muscles. No criteria could be identified to delineate sub-types: it is actually a spectrum of disease. Any further subdivision in particular phenotypes is arbitrary, but no subdivision poses a problem too as it makes the indication of disease phenotype non-specific. The names "late-onset" or "delayed-onset" Pompe's disease can be considered for this group of patients, but these names are inappropriate to describe

young children. Taken these observations we propose in chapter 2 to use the term classic Pompe's disease for all infants who develop symptoms in the first year of life, manifest hypertrophic cardiomyopathy, suffer from severe hypotonia and demonstrate developmental delay. These patients typically die within the first year of life [9]. We further propose to use the term non-classic Pompe's disease for all remaining cases and speak about 'infantile', 'childhood', 'juvenile' or 'adult cases' of non-classic Pompe's disease to maintain the possibility to sub-classify the patients by their age. This subdivision correlates to large extent with the level of residual acid α-glucosidase activity, when measured accurately, and with the associated mutations of the acid α-glucosidase gene (www.pompecenter.nl).

8.2 Pathogenesis of Pompe's disease

The accumulation of glycogen in muscle tissue in both subtypes of Pompe's disease causes all sorts of symptoms related to muscle weakness, such as cardiomyopathy (classic Pompe's disease), walking problems, wheelchair dependency and respiratory insufficiency. Why does the acid α-glucosidase deficiency primarily lead to muscle weakness, while the enzyme activity is deficient in all cell types?

Apparently, the lysosomal glycogen storage is more rapid or causes more damage in the skeletal muscle cells than in any other cell type. Rapid accumulation may relate to the relatively high cytoplasmic glycogen content of the muscle cells combined with autophagic activity. The causing of more damage may relate to the architecture of the skeletal muscle cells combined with the contractile activity (chapter 5).

The precise mechanism of muscle wasting due to lysosomal glycogen accumulation in the lysosomes is unknown. It is hypothesized that the gradual loss of muscle mass is caused by a combination of disuse atrophy and lipofuscin mediated apoptosis of myocytes. However, the latter could not be demonstrated in muscle tissue of Pompe knock-out mice [10]. Alternatively it could be that the longitudinal transmission of force is hampered by swollen lysosomes, clustering of non-contractile material and focal regions with degraded proteins, altogether resulting in muscle weakness [11, 12].

In chapter 5 we have examined muscle biopsies of patients with classic Pompe's disease in different stages of disease to find support for these hypotheses. From these biopsies we envisioned that the contractile machinery of the muscle is hampered by fibril splitting when the lysosomal compartment expands, resulting in diminished vectorial strength [10]. Reduced lysosomal function and rupture of the lysosomal membrane by contractile forces will lead to an increase of the cytoplasmic glycogen concentration. The release of lysosomal enzymes into the cytoplasm contributes to the cascade of damage. These findings are more or less in line with the earlier hypotheses. We also found quite dramatic storage of glycogen in smooth muscle cells of arteries and veins. It is conceivable from these findings that the vessel wall loses its integrity as the disease progresses, which may explain the occurrence of aneurysms in the basal arteries of patients with Pompe's disease as described in some case-reports [13]. The storage we have seen in Schwann cells and epineurium of peripheral nerves is not known to have functional consequences.

The findings in patients with non-classic Pompe's disease are similar to those with classic Pompe's disease, but the muscle is less severely and more heterogeneously affected. There are more well preserved parts of muscle tissue and progress of disease is less rapid [14, 15]. The difference undoubtedly relates in part to the level of residual activity in classic compared to non-classic Pompe's disease [11].

Apparently the residual enzyme activity is sufficient to keep the lysosomal glycogen storage below the critical level for some time. Moreover the damaged muscle fibers can either be repaired or replaced by satellite cell proliferation and migration, as long as muscle function is not fully lost [16-19]. One can envisage that over time, the delicate balance between muscle destruction and repair is disturbed, leading to loss of function and manifestation of symptoms.

8.3 The effects of enzyme replacement therapy from rabbit milk

The change of muscle morphology

Chapter 5 describes the changes that were observed in sequential muscle biopsies taken from 4 patients with classic Pompe's disease, who received ERT. These patients were treated first with 15-20 mg rabbit enzyme per kg bodyweight per week, a dose based on preclinical studies in mice and quail [20, 21].

The dose appeared to be sufficiently high to clear the glycogen from vascular endothelial cells and Schwann cells, but was not enough to clear glycogen from skeletal muscle or vascular smooth muscle. This is not unexpected since the endothelial cells are directly exposed to the circulating enzyme. Schwann-cells and perineurium are optimally supplied by the anastomosing vascular system that feeds the nerve bundles. We believe that cell and tissue specific differences in accessibility explain the much lower dose of α-galactosidase needed for the treatment of Fabry's disease (0.2-1 mg/kg) [22, 23] compared to the much higher dose needed to correct the muscle pathology in Pompe's disease. In Pompe's disease the administered α-glucosidase must pass the endothelial barrier and the endomysium before it reaches the muscle fibers.

Because of the clinical deterioration of two patients, we increased the dose to 40 mg per kg bodyweight [24, 25]. We could demonstrate clearance of glycogen in vascular smooth muscle and decrease of Periodic Acid Schiff staining in all patients. Skeletal muscle morphology improved only in the patient whose muscle fibers showed the least glycogen accumulation and who had the best muscle function. This dose led to normalization of α-glucosidase activity in muscle biopsies taken one day after infusion [26].

With these studies we have demonstrated that improvement of muscle morphology through ERT is feasible, when a high dose of enzyme is used and when the patient is still in good clinical condition. Preservation of muscle architecture and residual muscle function at the start of treatment is a prerequisite for the successful outcome of treatment.

The clinical effects and safety of ERT

The morphological findings in muscle biopsies before and during treatment (as described above) in four patients with classic Pompe's disease were in line with the clinical effects of α-glucosidase purified from milk of transgenic rabbits. The cardiac hypertrophy decreased, the survival increased, and motor milestones were achieved in two of the four patients, that are unmet by untreated patients with classic Pompe's disease [24, 26]. It was concluded that the therapeutic effect is largely dependent on the condition of the patient at start of treatment [27].

For this reason we did not only focus on the effects of ERT in classic Pompe's disease, but also included three patients with milder symptoms in our studies. Chapter 3 describes the effects of three years treatment with 20 mg/kg of α-glucosidase from rabbit milk. The pulmonary function stabilized or improved and the patients all reported less fatigue. The youngest and least affected patient

showed impressive improvement of muscle strength and function, leading in his case to wheelchair independence and to normal participation in daily life, sports and school. The response to treatment was less impressive in the two more severely affected patients, but the treatment prevented further loss of muscle strength. From these results we learned that also in non-classic Pompe's disease the efficacy of treatment largely depends on the clinical condition of the patient prior to the start of treatment. But the slower progression of non-classic Pompe's disease enlarges the therapeutic window.

Infusion-related reactions were observed in the 4 patients with classic Pompe's disease, who were included first. The reactions consisted of fever, malaise, erythematous rash, sweating, hypoxia, flushing and tachycardia, and they were managed with an adapted slow infusion rate during the first two hours [27]. Led by this experience, the three older patients in our study were treated with the adapted protocol. Two of them never experienced any infusion-related reactions. The third patient had a 3-month period of mild and transient skin reactions. He sometimes experienced chills during part of the infusion. The mechanistic cause of the reactions remains uncertain, but IgG mediated complement activation is the most likely explanation. The formation of anti α-glucosidase IgG bodies was detected in all patients, but no relation to the clinical effects was found. There was also no relation between the formation of these antibodies and the absence or presence of residual enzyme activity ('CRIM-status') [26].

Approximately 1300 infusions with α-glucosidase from rabbit milk, in doses of 15-40 mg/kg bodyweight, have been administered to these seven patients without serious problems. It demonstrates the purity of the transgenic product in a molecular composition that is well accepted by the human body. It also opens the way to further explore the production of therapeutic proteins in transgenic animals.

8.4 The effects of enzyme replacement therapy from Chinese hamster ovary cells

After three years, the treatment with α-glucosidase from rabbit milk was discontinued and treatment with α-glucosidase from CHO-cells was started in the three older patients. The production in CHO-cells was chosen as preferred method because it was anticipated that this would result in more rapid market approval than transgenically produced enzyme. No transgenically produced proteins have received market approval yet while several recombinant human lysosomal enzymes produced in CHO-cells have received market approval, i.e. Cerezyme (Gaucher), Fabrazyme (Fabry) and Laronidase (MPSI) [23, 28, 29].

Because the patients were very concerned about the switch to a new product their transition was carefully monitored, as described in chapter 4. In order to really compare CHO-enzyme to rabbit enzyme, one should have performed a randomized double blind study, but this was no option.

Initially the dose was decreased from 20 (rabbit milk) to 10 mg/kg/week (CHO). This decision was partially based on a suggestion that the CHO-enzyme would be four times as effective as the rabbit enzyme [30], and partially caused by the limited availability of the new product. Moreover, the highest dose of CHO-enzyme ever given to human subjects was 10 mg/kg/week, at that time. The dose was changed to 20 mg/kg bodyweight biweekly after 12 weeks, because of the theoretical considerations suggesting that it could be more effective to give higher doses less frequent than low dose more often.

After 6 months of treatment, the infusions with the CHO-enzyme appeared equally safe as those with the rabbit enzyme. No serious infusion related reactions were noted. The chills earlier experienced by one patient reoccurred after the switch from transgenic to CHO-enzyme, but disappeared gradually. Pre-medication was never given.

We did not expect rapid changes in clinical condition, but fairly soon after the switch to the lower dose of CHO-enzyme the patients mentioned to be more tired and less able to exercise. The patient who responded best to the transgenic product showed stabilization of muscle strength, but a mild decrease of muscle function and decrease of exercise endurance. No objective changes were recorded in the two other patients who were more severely affected.

Although the response of the patient may in part be ascribed to the psychological effect of transitioning to a lower dose of another product, they reported independently less tiredness and increased stamina after 5-6 infusions with an increased dose of 30 mg/kg/2 weeks. Moreover test results normalized in the best responding patient. During this treatment the decrease of ALAT, ASAT, CK and LDH that had taken place during the first three years of treatment continued in all three patients during treatment with CHO-enzyme. The best responding patient now even has normal values compared to his age-matched controls.

This led to the assumption that a two-week schedule can be used to treat patients with non-classic Pompe's disease, if the dose is high enough: 30 mg CHO-enzyme per kg bodyweight per two weeks seems equally effective as 20 mg rabbit enzyme per kg bodyweight per week. But, the latter can not be taken as a final conclusion. It is merely an hypothesis since there were many changes during the transition period, and the results were obtained in three patients only.

8.5 Experiences and questions

What is the most effective dosing regimen?

The results described in chapter 3, 4 and 5 combined with the earlier studies in Pompe knock-out mice [21, 31] show that 10 mg/kg/week is probably not enough for a significant therapeutic effect on skeletal muscle. What do we think is an effective dose? Chapter 3 shows that a dose of 20 mg/kg/week results in positive effects in three patients with non-classic Pompe's disease, but it took a long time for these effects to become apparent. Experiments in mice showed that linear dose dependent uptake is achieved in the target organs with doses ranging from 4-80 mg/kg [31]. The infantile study showed that 40 mg/kg/week leads to normalization of AGLU-activity in muscle, and to the clearance of glycogen in one patient [32].

In another study three infants with Pompe's disease were treated with 2 times 5 mg/kg/week of CHO-enzyme [30]. The results are difficult to interpret for two reasons. First of all one of the authors recently communicated that the patient who was doing very well did not have classic Pompe's disease (dr. P. Kishnani, personal communication). Secondly, one of the two other patients received doses of enzyme up to 5 times 10 mg/kg/week [33]. New studies with 10 mg/kg per week and 20 or 40 mg/kg/2 weeks have been initiated, but no definite reports have been published to date. Chapter 4 describes the effects of treatment with CHO-enzyme for patients with non-classic Pompe's disease. A stable clinical condition was achieved with 30 mg CHO-enzyme/kg per 2 weeks.

When compiling the limited evidence from both animal and human studies, as described above, we believe that the effective dose lies between 20 and 40 mg/kg. For classic Pompe patients it seems that a dose of 40 mg/kg each week is

preferable because of the severity and rapidly progressive nature of the disease, and the difficulty to reverse advanced muscle pathology. Even the best responding patient in our study does not show complete recovery with this dose (chapter 5). It is at this moment not possible to determine whether or not a lower or less frequent dosing regimen can be effectively applied in these patients.

A dose of 20 mg/kg per week or 30 mg/kg per two weeks seems to work equally well for patients with non-classic Pompe's disease, but this has only been tested in three patients. A lower dose is probably not enough and a higher dose has not been studied yet in these patients, but might result in a more rapid recovery.

What is the best infusion regimen?
Chapter 6 describes the pharmacokinetic studies in the non-classic patients. This has taught us that the initial plasma half-life is 3-6 hours. A higher dose resulted for each patient in higher peak levels in the plasma. An important new finding was that patients with the same dose per kg bodyweight had different peak levels of α-glucosidase in their plasma. It was found that this was related to differences in body fat percentage between these patients. When the dose was recalculated in mg per kg lean body-mass, all patients had the same linear relationship between administered dose and peak levels of α-glucosidase in the plasma. In addition we observed that a higher infusion rate resulted in a higher peak level in the blood, with the possible benefit of a larger gradient as a driving force into the target cells.

To optimally combine the conflicting demand of a slow infusion rate to prevent adverse events and a high infusion rate to optimize the clinical effects we have designed the following infusion regimen. The infusion rate is 0.2-0.3 mg/kg/hour in the first hour, 0.7-0.8 mg/kg/hour the second hour, and the final infusion rate is 15-17 mg/kg bodyweight/hour. With a concentration of 2 mg recombinant α-glucosidase/ml infusion fluid, this leads to an infusion duration of 4 hours for all patients with non-classic Pompe's disease. For the patients with classic Pompe's disease the first two hours are the same, but the final infusion step is increased to 40 mg/kg/hour without problems for infusions with rabbit enzyme. The infusion time is 3 hours and 15 minutes.

For future application it must be decided whether treatment should be calculated on mg/kg lean body-mass basis in stead of mg/kg bodyweight. The latter seems favorable since it results in more comparable doses for each patient.

Residual disease
Even the best responding patient with classic Pompe's disease in our study has residual handicaps after 5 years of treatment. Hearing problems seem to be part of the symptomatology not treatable by ERT [34]. Also weakness of the facial muscles and shortening of the Achilles tendons could not be fully prevented by the therapy. The process of muscle wasting through the disease and the process of repair and regeneration through treatment seem to be in balance, but time will tell whether or not this balance will remain when the patient gets older. We have learned that it is not advisable to start treatment when symptoms have developed beyond the loss of function of extremities [27].

The extent to which the three patients with non-classic Pompe's disease responded to the treatment varied too. Two patients regained energy and social life and the disease progression was halted, however, muscle strength and function showed little or no improvement. Further, we did not see clear improvement in muscle biopsy specimens taken during the course of disease despite glycogen

depletion (unpublished results). The best case scenario was seen in one patient who did show normal muscle strength and function after 4.5 years of treatment. For him residual disease is now limited to visiting the hospital for an infusion every second week. Such a good outcome can only be obtained when treatment is started in time. Long term follow up of these patients and other patients receiving ERT is necessary to determine when treatment needs to be started in order to obtain full benefit.

For patients with classic Pompe's disease it is essential that treatment start immediately after diagnosis.

8.6 Future perspectives

Future clinical studies

The combined results of the studies performed with α-glucosidase from both rabbit milk and CHO-cells [30, 35] (www.clinicaltrials.gov) will be used to file for market approval.

In the time left before the therapy becomes available it is important to gather more information about the natural course of the disease. This can be done by the development of a questionnaire that maps the disease history of a large group of Pompe patients as well as by physical assessment and follow up. The focus should be on minor symptoms that emerge during early childhood, and indicators of disease progression. The available literature is not sufficiently detailed on these aspects (chapter 2).

The best way to determine the natural course of non-classic Pompe's disease is to perform an observational study, or to introduce a placebo group in an ERT trial. A group of untreated patients can be examined regularly (for instance every 6 months during 2 years) for muscle strength and function, pulmonary function, laboratory values, quality of life and development of handicaps.

Based on the studies described in this thesis, the obvious primary endpoint for an enzyme replacement therapy study is stabilization or improvement of muscle strength and function. Another endpoint directly related to muscle function is respiratory condition; pulmonary function should stabilize or improve so that less ventilation will be necessary. Other endpoints for such a study are quality of life and the amount of residual handicap after treatment, since improvement of quality of life with fewer handicaps is ultimately the goal.

Because the optimal dose is still open for discussion the most suitable study design would be a dose finding, placebo controlled study in which one group receives a placebo, one group 20 mg/kg and one group 40 mg per kg lean body-mass, every second week.

The future of ERT for LSD's and for Pompe's disease in particular

At this moment ERT is available for Gaucher's disease, Fabry's disease and MPS I. Similar therapies are being developed for MPS II and VI, and Pompe's disease (this thesis). ERT for other LSD's is tested in preclinical studies.

The longest experience is with the treatment of Gaucher's disease. The biochemical and clinical effects are clear and patients with Gaucher's disease type 1 treated with ERT live longer, with much less burden of disease [36, 37]. ERT for Fabry's disease and MPS I results in clear substrate reduction, but the clinical benefits are less apparent [22, 29, 38]. The dose of enzyme used for treatment of these disease is rather low (0.2-1.0 mg/kg). This thesis describes the positive effects achieved in patients with Pompe's disease, but also shows that a rather high dose (20-40 mg/

kg) is needed and that treatment results in a very variable response.

ERT is an expensive treatment. The costs of treating Fabry's disease with ERT are at present approximately ∈ 175,000 per patient per year and a similar or higher price is expected for Pompe's disease, due to the higher dose. Theoretically the high costs of ERT can be decreased by production of proteins in the milk of transgenic animals such as a goat or a cow. This method is reportedly cheaper than production in CHO-cells ($1 per gram in goat milk compared to $150 per gram in CHO-cells, excluding development costs) [39, 40].

That a product can successfully be extracted from the milk of transgenic animals and subsequently safely administered to patients is shown by the 1300 infusions of recombinant human α-glucosidase from rabbit milk, as described in this thesis. Therefore, protein production in transgenic animals like goats, sheep or cows is an attractive option to respond to the increasing demand of therapeutic proteins to treat lysosomal storage diseases in the future.

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Summary
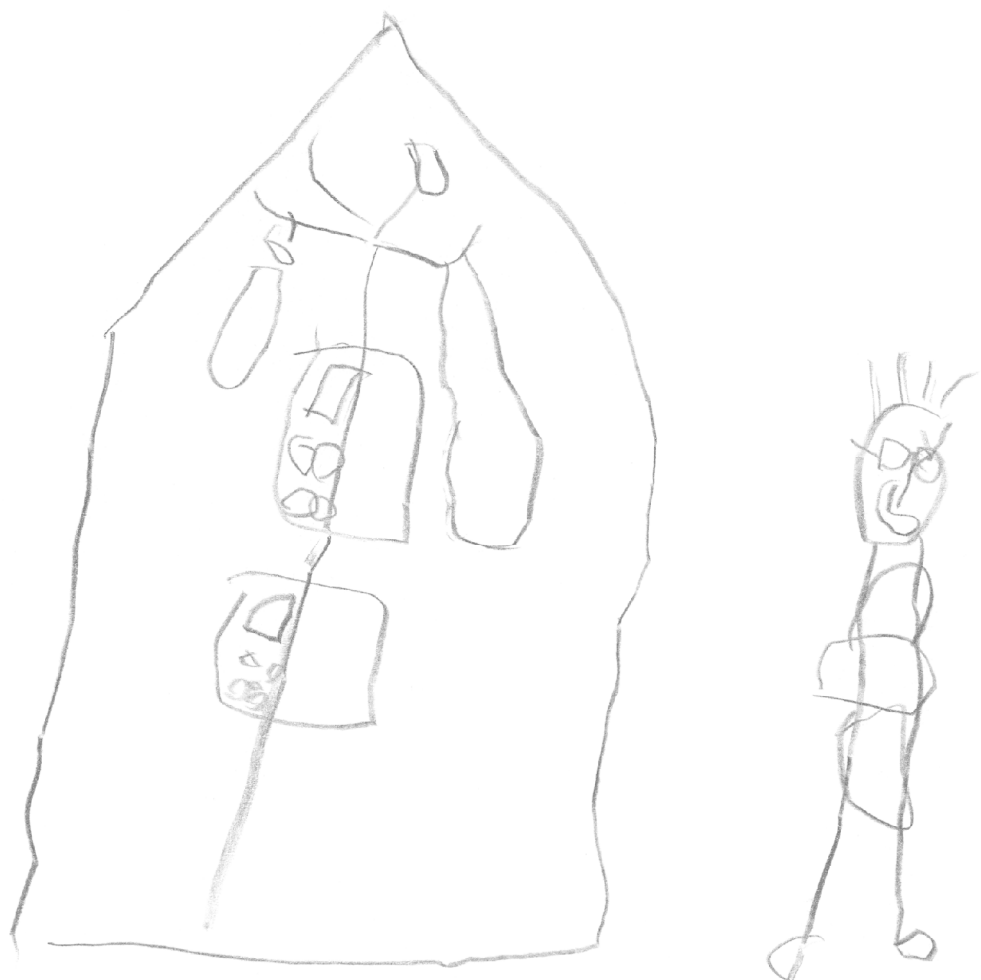
Summary

Pompe's disease is an inherited metabolic illness, caused by an inherited deficiency of an enzyme, called acid α-glucosidase. Acid α-glucosidase is a protein that breaks down glycogen (a chain of glucose molecules) into glucose (a single molecule), in a specific compartment of the cell, the lysosome. The lysosome thus takes care of the removal and recycling of substances in the cell.

The deficiency of acid α-glucosidase results in glycogen accumulation in the lysosome (lysosomal storage). This happens mainly in muscle cells, where under normal circumstances a lot of glycogen is stored and recycled thereafter. The lysosomal storage of glycogen causes gradual destruction of muscle cells, which results in loss of contraction capacity of the muscle cells, and finally in the loss of muscle function(s).

Pompe's disease can become apparent at any age. Complete deficiency of the enzyme results in the severe and quickly progressive classic form of Pompe's disease, characterized by generalized muscle weakness and an enlarged heart. Symptoms present within the first 3 months of life. Loss of muscle strength prevents children of achieving major developmental milestones such as sitting, standing and walking. The patients usually die within the first year of life due to insufficiency of the respiratory muscles and the heart.

A partial deficiency of acid α-glucosidase causes a slowly progressive phenotype, involving mainly skeletal muscle. The first symptoms can occur in early childhood, but can also stay away until late adulthood. The disease presents itself as a proximal myopathy. Fatigue, 'clumsiness' and difficulty climbing stairs are often the first symptoms. Eventually patients may become wheelchair-dependent. The pulmonary function gradually decreases due to weakness of the respiratory muscles. Mechanical ventilation is often necessary, first during the night, sometimes also during daytime.

The studies described in this thesis are focussed on the development of enzyme replacement therapy for this otherwise incurable disease. Treatment of comparable lysosomal storage disorders is currently taking place for Gauchers' disease, Fabry's disease and 3 types of Mucopolysaccharidosis. An overview of these treatments is given in **chapter 1**, which also serves as a general introduction. For Pompe's disease so far there was not enough enzyme, and/or the enzyme was not functioning properly. After the cloning of the human α-glucosidase gene, two methods were developed for the large-scale production of recombinant human acid α-glucosidase. One method uses genetically altered Chinese hamster ovary cells (CHO-cells), the other method uses transgenic animals that produce the therapeutic enzyme in their milk.

Various articles have appeared about the cause of the slowly progressive phenotype in medical literature. **Chapter 2** is a review of 225 cases derived from 107 articles. Herein we find that the 'non-classic' phenotype may present at any age, from 0-71 years. An early onset of symptoms seems to be associated with a faster disease-progression. Respiratory failure is the most frequent cause of death. Symptoms are related to or caused by muscle weakness. CK, LDH, ASAT, ALAT and muscle glycogen levels are frequently elevated, but normal levels do not exclude non-classic Pompe's disease, neither does normal muscle morphology. Measurement of acid α-glucosidase deficiency in fibroblasts or skeletal muscle is required to establish the diagnosis. The use of leukocytes is error prone. No criteria could be identified to delineate sub-types of non-classic Pompe's disease. Clinical

trials in non-classic Pompe's disease should focus on skeletal muscle strength and function, disability, handicap, quality of life and pulmonary function.

Chapter 3 and **4** describe the effects of treatment on these parameters for 3 patients (aged 11, 16 and 32 years). They were all wheelchair bound and two of them were ventilator dependent with a history of deteriorating pulmonary function. After three years of treatment with weekly infusions of α-glucosidase, derived from the milk of transgenic rabbits, the patients had stabilized pulmonary function and reported less fatigue. Weekly infusions were tolerated well, and the youngest and least affected patient showed an impressive improvement of skeletal muscle strength and function. After 72 weeks of treatment he could walk without support and finally abandoned his wheelchair.

After three years the treatment was switched to CHO-cell derived enzyme. The dose of rabbit enzyme was 20 mg per kilogram bodyweight per week (20 mg/kg/week). The patients first received 10 mg/kg/week, thereafter they received 20 mg/kg/2 weeks and finally they were treated with 30 mg/kg/2 weeks. The treatment with the lower dose of CHO-enzyme resulted initially to complaints of fatigue, decreased endurance and loss of (muscle) functions. Increasing the dose to 30 mg/kg/biweekly led to complete recovery. We conclude that patients with Pompe's disease can safely and effectively be treated with α-glucosidase from rabbit milk and CHO-cells. However the efficacy differs from patient to patient and seems to be dependent on the (clinical) condition of the patient at start of treatment. Large differences in efficacy between both products were not found.

The **chapters 5, 6 and 7** describe specific effects of the treatment in more detail. In **chapter 5** we evaluated the morphological changes in muscle tissue of four children with classic Pompe's disease during treatment. After 12 weeks of treatment with 15-20 mg/kg/week, glycogen accumulation decreased in vascular endothelium and in peripheral nerves. Increasing the dose to 40 mg/kg led to a reduction of glycogen storage and substantial improvement of muscle architecture in the least affected patient. We recommend starting treatment as early as possible. **Chapter 6** describes de pharmacokinetic properties of recombinant human α-glucosidase from rabbit milk and from Chinese hamster ovary cells. The results suggest that it will be more effective to treat either weekly or biweekly with relatively high doses and a high infusion rate than more frequently with lower doses and a low infusion rate. Furthermore it shows that dosing in mg/kg lean body mass better predicts the peak plasma-values than a dose based on crude bodyweight. Because high peak values are thought to be positively correlated with efficacy, we advise to base the administered dose on the patients weight in lean body mass. **Chapter 7** describes the possible diagnostic application of lymphocyte vacuoles for Pompe's disease. It was already known that blood smears of patients with Pompe's disease, stained with periodic acid-Schiff, contain glycogen filled vacuoles. It was unknown that the severity of vacuolization is significantly higher in classic than in non-classic Pompe patients. Furthermore this chapter describes the first reaction to treatment which is the disappearance of the vacuoles, long before clinical effects were noted. This is caused by the presence of mannose 6-phosphate receptors on lymphocytes. This receptor mediates the rapid uptake of α-glucosidase. The fraction of enzyme captured by the lymphocytes is too small to affect the outcome of enzyme replacement therapy in either a positive or negative way.

Chapter 8 discusses the results described in this thesis and the remaining questions together with the prospects of the future. The results demonstrated the safety and efficacy of enzyme replacement therapy, and encourage the further development of this treatment. Furthermore they show the possibility of production of therapeutic medicine in the milk of transgenic animals.

Samenvatting

De ziekte van Pompe is een erfelijke stofwisselingsziekte, die wordt veroorzaakt door een erfelijk tekort van een enzym, genaamd zure α-glucosidase. Zure α-glucosidase is een eiwit dat ervoor zorgt dat glycogeen (een keten van glucose moleculen) wordt omgezet tot glucose (één molecuul) in een specifiek gedeelte van de cel, het lysosoom. Het lysosoom speelt een belangrijke rol bij het opruimen en eventueel hergebruik van stoffen in de cel.

Door het tekort aan zure α-glucosidase wordt het glycogeen in het lysosoom niet opgeruimd, waardoor het zich daar ophoopt (lysosomale stapeling). Dit gebeurt met name in de spiercellen, waar normaal gesproken veel (tijdelijke) opslag van glycogeen en hergebruik plaatsvindt. De stapeling van glycogeen veroorzaakt op den duur schade van de spiercellen, waardoor de samentrekkingskracht van de spier geleidelijk vermindert en er uiteindelijk spierfunctie(s) verloren gaan.

De ziekte van Pompe kan op elke leeftijd zichtbaar worden. Een volledig tekort aan het enzym leidt tot de zeer ernstige en snel voortschrijdende, klassieke vorm van de ziekte van Pompe, die wordt gekarakteriseerd door algemene spierzwakte en vergroot hart. 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. Hartfalen en respiratoire insufficiëntie leiden uiteindelijk tot de dood, vaak in het eerste levensjaar.

Een gedeeltelijk tekort aan zure α-glucosidase veroorzaakt een langzaam voortschrijdend ziektebeeld (fenotype), waarbij vooral de skeletspieren zijn betrokken. De eerste symptomen kunnen zich voordoen op de vroege kinderleeftijd, maar kunnen ook uitblijven tot laat volwassen leeftijd. De ziekte presenteert zich als een proximale spierziekte, wat wil zeggen dat de spieren het dichtst bij de romp het meest zijn aangedaan. Vermoeidheidsklachten en moeite met traplopen zijn vaak de eerste symptomen. Uiteindelijk kunnen patiënten rolstoelgebonden raken. De longfunctie loopt geleidelijk terug door zwakte van de ademhalingsspieren, waaronder het middenrif. Nachtelijke (neuskap-)beademing is uiteindelijk vaak nodig en soms ook beademing gedurende de dag.

De studies beschreven in dit proefschrift zijn gericht op de ontwikkeling van enzymvervangingstherapie voor deze anderszins ongeneeslijke ziekte. Behandeling van vergelijkbare lysosomale stapelingsziekten met enzymvervangingstherapie vindt op dit moment plaats voor de ziektes van Gaucher en Fabry en 3 typen Mucopolysaccharidose. Een overzicht hiervan wordt gegeven in **Hoofdstuk 1**, dat tevens dient als een algemene inleiding. Voor de behandeling van de ziekte van Pompe was tot nu toe onvoldoende goed werkzaam enzym beschikbaar. Na de klonering van het menselijk α-glucosidase gen werden twee methodes ontwikkeld voor de productie van recombinant humaan α-glucosidase op grote schaal. De ene methode gebruikt genetisch gemanipuleerde ovarium cellen van de Chinese hamster (CHO-cellen), de andere methode transgene dieren die het therapeutische enzym produceren in hun melk.

Over het beloop van het langzaam voortschrijdende fenotype van de ziekte van Pompe zijn diverse artikelen verschenen in de medische literatuur. **Hoofdstuk 2** is een review van 225 casussen uit 107 artikelen. We vinden daarin dat dit 'niet klassieke' fenotype zich kan presenteren op elke leeftijd, waarbij vroeg optredende symptomen gerelateerd lijken aan een sneller verloop van de ziekte. De symptomen die optreden zijn gerelateerd of worden veroorzaakt door spierzwakte en de meest

frequente doodsoorzaak is respiratoir falen. CK, LDH, ASAT, ALAT en glycogeen in de spieren zijn vaak verhoogd, maar kunnen ook normaal zijn, wat ook geldt voor spiermorfologie. De diagnose moet worden gesteld op basis van het tekort aan zure α-glucosidase activiteit in een spierbiopt of in een kweek van huidcellen (fibroblasten). Er konden geen criteria worden onderscheiden voor verdere subtypering van dit fenotype. Klinische trials voor enzymvervangingstherapie voor de niet-klassieke vorm van de ziekte van Pompe moeten zich richten op spierkracht en –functie, de mate van handicap, kwaliteit van leven en longfunctie.

Hoofdstuk 3 en 4 beschrijven de effecten van therapie op deze parameters bij 3 patiënten (11, 16 en 32 jaar oud bij start van de behandeling). De patiënten waren rolstoelgebonden en twee waren (deels) afhankelijk van kunstmatige ademhalingsondersteuning. Drie jaar behandeling van deze patiënten met zure α-glucosidase, geproduceerd in melk van transgene konijnen, resulteerde in stabilisering van de longfunctie en in minder vermoeidheid. De wekelijkse infusen werden goed verdragen en de jongste en minst aangedane patiënt vertoonde een enorme vooruitgang in spierkracht en –functie. Na 72 weken kon hij zonder steun lopen en uiteindelijk gebruikte hij geen rolstoel meer. Na 3 jaar werd er overgegaan op behandeling met enzym uit CHO-cellen. De dosering van konijnenmelk enzym was 20 mg per kilogram lichaamsgewicht per week (20 mg/kg/week). De patiënten werden 3 maanden behandeld met 10 mg/kg/week, daarna met 20 mg/kg/2 weken en uiteindelijk met 30 mg/kg/2 weken. De behandeling met de lagere dosis CHO-enzym leidde aanvankelijk tot klachten van meer vermoeidheid, verminderd uithoudingsvermogen en verlies van (spier)functie. Het verhogen van de dosis naar 30 mg/kg/2 weken leidde echter tot een volledig herstel. Na 3 jaar behandeling met konijnenmelk enzym en 1.5 jaar behandeling met CHO-enzym concluderen we dat patiënten met de ziekte van Pompe veilig en effectief kunnen worden behandeld met beide producten. Echter de mate van effectiviteit verschilt per persoon en lijkt in hoge mate afhankelijk van de conditie van de patiënt bij aanvang van therapie. Grote verschillen in effectiviteit tussen beide producten werden niet gevonden.

De **hoofdstukken 5, 6 en 7** beschrijven de specifieke effecten van therapie meer gedetailleerd. In **hoofdstuk 5** werden de effecten op de spieren van 4 klassieke Pompe patiënten onderzocht. Na 12 weken behandeling met 15-20 mg/kg/week nam de glycogeenstapeling in de perifere zenuwen en de endotheelcellen van de bloedvaten af. Na verhoging van de dosering naar 40 mg/kg/week werd ook vermindering gezien van glycogeenstapeling in de spieren. Daarnaast was er verbetering van de spierarchitectuur bij de best reagerende patiënt. Het is aan te bevelen zo vroeg mogelijk met behandeling te starten. **Hoofdstuk 6** beschrijft de pharmacokinetische eigenschappen van zure α-glucosidase geproduceerd in CHO-cellen en in transgene konijnen. Het laat zien dat het beter is minder frequent hoog te doseren dan frequent laag en het lijkt ook beter te zijn het infuus snel te laten inlopen dan langzaam. Voorts laat het zien dat dosering per kg vetvrije massa beter de piekwaarde in het bloed voorspelt dan dosering per kg lichaamsgewicht. We achten het beter om de dosis te baseren op de vetvrije massa van de patient, omdat de piekwaarde in het bloed gerelateerd lijkt te zijn aan de mate van effectiviteit. **Hoofdstuk 7** beschrijft de mogelijke diagnostische toepassing van lymfocytenvacuoles bij de ziekte van Pompe. Het was reeds bekend dat bloeduitstrijkjes van patiënten met de ziekte van Pompe, gekleurd met periodic acid-Schiff, vacuoles (glycogeenbolletjes) laten zien in de lymfocyten. Onbekend was dat de mate van vacuolisatie significant hoger is bij klassieke Pompe patiënten dan bij niet-klassieke Pompe patiënten. Voorts beschrijft dit hoofdstuk de eerste

reactie op therapie, namelijk het verdwijnen van deze vacuoles, lang voordat klinische effecten merkbaar waren. Dit verdwijnen is toe te schrijven aan de aanwezigheid van mannose 6-fosfaat receptoren op de lymfocyten. Deze receptor zorgt voor een zeer snelle opname in deze cellen, maar de hoeveelheid enzym die wordt opgenomen in de lymfocyten speelt geen rol op de totale toegediende hoeveelheid enzym.

Hoofdstuk 8 bediscussieert de resultaten die beschreven zijn in dit onderzoek en bespreekt de overblijvende vraagstukken tezamen met de vooruitzichten voor de toekomst. De resultaten demonstreren de veiligheid en de effectiviteit van enzymvervangingstherapie. Bovendien laten zij de mogelijkheid zien van de productie van medicijnen in de melk van transgene dieren. De resultaten van deze studies moedigen de verdere ontwikkeling van enzymvervangingstherapie voor de ziekte van Pompe aan.

Curriculum Vitea

The author of this thesis was born on March 22nd, 1973, in Waarland (a small town in the Northwest of Holland). He graduated from highschool (Han Fortmann College in Heerhugowaard) in 1991. After drawing the wrong number in the numerus fixus lottery in September 1991, he finished his propedeuse in medical biology in September 1992, at the University of Amsterdam. The next year he draw the right number to start medicine at the same university. During the study he studied the relationship between pulmonary function, blood parameters and a patient questionnaire. During his internships he spent 2 months at the department of pediatric pulmonology of the Dutch asthma center in Davos, Switzerland. He finished his internships in December 1998. From January to June 1999 he traveled through parts of Asia with his wife, learning much about different cultures and religions. Two months of research at Kendle, Utrecht was followed by a job as a resident at the department of pediatrics, at the IJsselmeerziekenhuizen in Lelystad. In May 2000 he joined the team of dr. A.T. van der Ploeg and dr. A.J.J. Reuser at the Erasmus MC in Rotterdam, which was working on the development of enzyme replacement therapy for Pompe's disease. Very recently (October 2004) he started working as a resident at the department of Pediatrics of the Erasmus MC-Sophia (head: prof.dr. H.A.Büller; prof.dr. A.J. van der Heijden). He is currently living in Delft with his wife Roos and his two sons Jonathan and Raphaël.

List of Publications

The natural course of non-classic Pompe's disease; a review of 225 published cases
L.P. F. Winkel, M.L.C. Hagemans, P. A. van Doorn, M.C.B. Loonen, W.J.C. Hop, A.J.J. Reuser, A.T. van der Ploeg
-submitted-

Enzyme replacement therapy in late-onset Pompe's disease: a three-year follow up
L.P.F. Winkel, J.M.P. Van den Hout, J.H.J. Kamphoven, J.A.M. Disseldorp, M. Remmerswaal, W.F.M. Arts, M.C.B. Loonen, A.G. Vulto, P.A. Van Doorn, G. De Jong, W. Hop, G.P.A. Smit, S.K. Shapira, M.A. Boer, O.P. Van Diggelen, A.J.J. Reuser, A.T. Van der Ploeg
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Dankwoord

Op het moment dat een dankwoord van een proefschrift geschreven wordt, betekent dit het einde van een periode, het laatste stuk van het 'boekje' maar ook een van de meest gelezen delen. Nog belangrijker is misschien wel dat dit deel het enige is dat ik helemaal zelf heb geschreven, in een keer, zonder revisie, andere versie(s) of andere perikelen. Dat houdt als vanzelf in dat het overige deel van dit werk nooit door mij alleen gedaan en geschreven had kunnen worden. Daarom vind ik het belangrijk iedereen te bedanken voor de afgelopen 4 jaar, waarin ik veel heb geleerd. Alvast voor ik iemand vergeet in het stuk hieronder, iedereen heel hartelijk bedankt voor een fijne en leerzame periode.

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Dit proefschrift was er niet in deze staat geweest zonder figuren, tabellen en plaatjes. Daarbij was de steun en inbreng van Tom de Vries Lentsch en Ruud Koppenol onontbeerlijk. Daarnaast heeft Tom ook de lay-out verzorgd van dit proefschrift en de kaft ontworpen. Serieuze wetenschap kan niet zonder goede vormgeving, daarvoor hebben jullie zorg gedragen!

Een proefschrift moet ook worden gedrukt en zoals elke (ex)promovendus weet is dat geen goedkope geschiedenis. Gelukkig had ik daarvoor goede contacten met mijn oom en AJAX-Arena buurman Frans Oorthuis, die zorgde voor een goede regeling bij HUIG-printing in Zaandam. Bedankt Frans en dat we nog maar vele overwinningen en kampioenschappen mogen meemaken!

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