CLASSIC INFANTILE POMPE DISEASE:

EFFECTS OF DOSING AND IMMUNOMODULATION ON LONG-TERM OUTCOME

ESTHER POELMAN

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De klassiek infantiele vorm van de ziekte van Pompe:
Effecten van dosering en immunomodulatie op de lange termijn uitkomsten

Proefschrift

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Voor Sas, Meetje en Axel

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Part 1

Chapter 1

General Introduction
and scope of this thesis

GENERAL INTRODUCTION AND SCOPE OF THIS THESIS

Pompe disease, glycogen storage disease type II (GSD II), glycogenosis type II, and acid maltase deficiency (OMIM #232300) are all names used for the same rare autosomal recessive disease, which is the subject of this thesis. Until recently this was a deadly disease in infants and severely invalidating in children and adults. The development of enzyme replacement therapy (ERT) has brought new prospects for patients and their families. This first chapter will provide information on the historical background of Pompe disease, genetics and inheritance, diagnosis and treatment. In the final paragraphs, the current treatment options will be discussed with a specific focus on the effects of enzyme replacement therapy dosing and immunomodulation, in patients and how these developments have led to the publications in this thesis.

POMPE DISEASE, A HISTORY

In 1930, the Dutch pathologist J.C. Pompe performed a post-mortem examination on a 7-month-old girl. She was thought to have died from pneumonia, but during the post-mortem examination she was found to have a hypertrophy of the heart. In 1932 Pompe wrote a report on the findings and described that she had glycogen accumulation with 'vacuoles' on microscopic examination of the heart and in other tissue cells [1]. He could not explain the glycogen accumulation but postulated that is was part of a metabolic disorder. The same disease was later that year described by Putschar and Bischoff [2, 3]. The underlying cause of the disease was still unknown and it remained that way for 30 years.

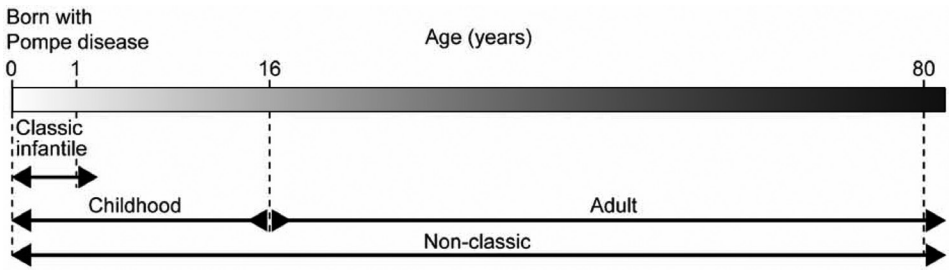
Many discoveries followed that confirmed the observations made by dr. Pompe. In the 1950s drs. Gerty and dr. Cori unraveled the normal pathway of glycogen metabolism while focusing on what they called 'glycogen storage disorders [4]. Another very important hallmark was the discovery of the lysosome in 1955 by De Duve [5]. Lysosomes are organelles responsible for the degradation of larger macro-molecules such as glycogen, but most importantly they can degrade intra-cellular molecules and molecules that enter the cell via endocytosis. Some of the products formed by degradation can be re-used in metabolic pathways and for cell renewal [5]. In 1974 De Duve received the Nobel Prize for his work on the discovery and understanding of the function of the lysosomes. In 1963 Hers et al. discovered that Pompe disease is caused by the (partial) absence of the enzyme acid α-glucosidase [6] and later that year Lejeune et al. demonstrated that the acid α-glucosidase was deficient in the lysosome [7]. With these last two discoveries, Pompe disease became the first ever proven lysosomal storage disorder. Over the years many other lysosomal storages disorders (LSDs) and their missing or defective enzymes were discovered. Today around 70 different lysosomal disorders have been described, most of them are caused by a deficiency of a single lysosomal enzyme [8].

The properties of the lysosome, as discovered by De Duve, that lysosomes can degrade macro-molecules that are transported to the lysosomes via autophagy, but can also degrade extracellular macro-molecules that reach the lysosomes by uptake of these molecules from the extra cellular space (though endocytosis), make lysosomal enzyme deficiencies amenable to enzyme replacement therapy.

NOMENCLATURE IN POMPE DISEASE

There is no real consensus between experts on the nomenclature of the various subtypes of Pompe disease, but a proposition was done by Güngör and Reuser in 2013 to improve uniformity in the nomenclature (figure 1) [9].

Figure 1. The spectrum of Pompe disease



As published in Güngör and Reuser, Am J Med Genet Part A 161A:399–400.[9]

Patients with the classic infantile phenotype have two severe variants in the alpha-glucosidase gene (*GAA* gene) resulting in virtual no residual acid α-glucosidase activity. The patients are on the severe end of the clinical spectrum. They present within the first months of life with generalized muscle weakness, hypertrophic cardiomyopathy, respiratory problems and feedings difficulties [10-14]. Without treatment, these infants die within the first year of life due to cardiorespiratory failure [10, 11].

Patients with childhood or adult onset phenotype have at least one, less severe, variant in the *GAA* gene and up to 25% residual activity can be measured. Their disease course is characterized by slowly progressive limb-girdle muscle weakness and weakness of the respiratory muscles [15-17]. The initial symptoms involve difficulties in running, walking, fatigue, pain and shortness of breath [12, 15-18]. The symptoms and disease course vary, even between affected family members [19-21]. Patients with all forms of Pompe disease (including adults) have a lower life expectancy than the general population [22].

The classic infantile Pompe patients are often called infantile onset Pompe disease (IOPD) patients in literature. However, IOPD patients can include the classic infantile Pompe patients and 'atypical infantile' Pompe patients. These 'atypical infantile' Pompe patients were first described by Slonim et al. [23]. The 'atypical infantile' patients present with generalized muscle weakness within the first year of life with or without cardiac hypertrophy, but

when hypertrophy is present it is less severe than in the classic infantile Pompe patients [23]. In contrast to their classic infantile counterparts, these patients do survive beyond the first year of life and do not die from cardiorespiratory failure.

The focus of this thesis is to describe the effects of treatment (ERT and additional treatments) in the classic infantile Pompe patients. Studies on the effects of ERT in the 'atypical infantile' Pompe patients are not subject of this thesis.

PATHOGENESIS

Genetic Background

Pompe disease manifests itself when two pathogenic variants in the gene that codes for the lysosomal enzyme acid α-glucosidase (*GAA*), localized on chromosome 17q25.2-25.3, are present [24-28]. The variants in the GAA gene found in Pompe disease are collected together in the Pompe Disease Mutation Database (to be found on www.pompecenter.nl). At present around 500 (potentially) pathogenic variants have been identified. Normally, acid α-glucosidase is synthesized as a 110-kDA precursor and then processed into a 95kD intermediate and 76kD and 70kD mature α-glucosidase through the Golgi complex [29, 30]. The pathogenic variants disrupt the normal production, processing and/or routing of acid α-glucosidase to the lysosome and as a consequence glycogen accumulates in the lysosome.

Some variants occur in higher frequencies in certain ethnic groups and some occur more often in certain subtypes of Pompe disease. The variants c.525delT, c.2481+102_2646+31del (del exon 18), c.925G>A are commonly found in Caucasians [31, 32], while the c.2560C>T often occurs in patients from African, African-American and Brazilians of African descent [33]. The c.1935C>A variant is found in patients from Asian descent [34], the c.377G>A in patients from Argentinean descent [35]. The c.1905C>A, c.-32-3C>A, and c.2560C>T variants are found in patients from Brazilian descent [36]. The splice-site mutation c.-32-13T>G (also known as the IVS1 mutation) is commonly found in Caucasians with childhood and adult onset Pompe disease [19, 21, 37, 38] and leads to the formation of 10-25% of normally processed and active alpha-glucosidase. The IVS1 mutation never occurs in classic infantile patients.

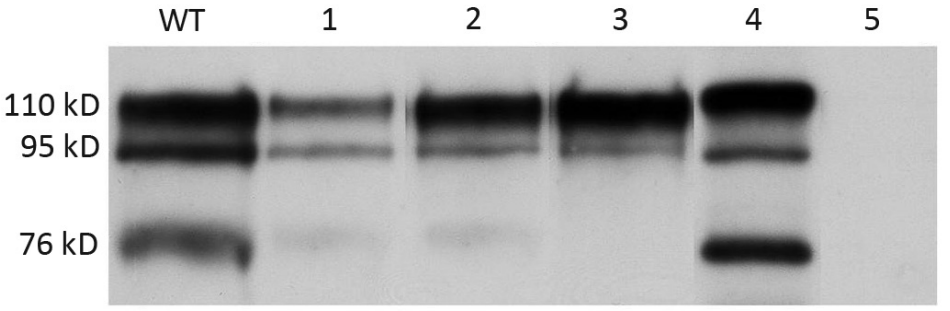
Depending on the severity of the pathogenic variants found in the *GAA* gene, patients have either a total or partial absence of the lysosomal enzyme acid α-glucosidase. Classic infantile Pompe patients have virtually no endogenous acid α-glucosidase activity (less than 1% of normal), whereas childhood and adult onset patients have residual enzyme activity (up to 25% of normal). The presence of residual GAA protein, which can be visualized by Western Blot analysis (immunoblotting) and which procedure has been described by Hermans et al. [39], plays an important role in Pompe disease. Patients who do not produce any GAA protein are called cross-reactive immunological material (CRIM)-negative. Patients who produce a (small) detectable amount of enzyme protein are CRIM-positive. Childhood and adult onset patients are per definition CRIM-positive due to their residual enzyme activity. Classic infantile Pompe patients can either be CRIM-positive or CRIM-negative (Figure 2). About two-third of classic infantile Pompe patients are CRIM-positive, one-third are CRIM-negative. CRIM-negative patients generally have a poorer clinical outcome despite treatment with ERT, and as reported generally die before they reach the age of three years. [40-45]

Incidence and Diagnosis

The incidence of classic infantile Pompe disease in The Netherlands is reported to be 1 in 138,000 in classic infantile patients and 1 in 57,000 in childhood/adult onset patients [46]. The overall incidence is 1:40.000. In other countries and other ethnic groups incidences may vary between 1 in 14,000 to 1 in 600,000 [47]. These numbers are calculated using estimates of current diagnoses.

Better estimates of the incidence might soon be available as newborn screening (NBS) is underway in Taiwan and in the United States (US). In Taiwan, NBS for Pompe disease was introduced in 1981 [48]. In the US, NBS is underway in several US states since 2012. Their initial bid to include Pompe disease in the NBS was denied in 2007 because of low specificity (leading to many false-positive results, leading to an overestimation of Pompe disease) and no second tier to rule out late onset Pompe disease [47]. NBS is usually performed on dry blood spots (Guthrie cards). Dry blood spots are adequate for screening, but not to confirm the diagnosis. To confirm Pompe disease patients must be referred to hospital for additional analysis.

Figure 2. Immunoblot of synthesis and maturation of acid α-glucosidase



WT: wild type, normal synthesis of α-glucosidase with the presence of the 110 kD precursor, 95 kD intermediate and 76kD mature α-glucosidase. **1**: Late onset patient with reduced synthesis but normal maturation. **2**: Late onset patient with normal synthesis, but reduced maturation. **3**: Classic infantile patient with normal synthesis, but no maturation. **4**: Classic infantile patient with normal synthesis and maturation, but no activity. **5**: No synthesis. Lanes 1 through 4 represent CRIM positive patients, lane 5 a CRIM negative patient. Adapted from C.M. van Gelder, *Enzyme-replacement Therapy in Classic Infantile Pompe Disease: Long-term outcome, dosing and the role of antibodies (PhD-Thesis)*. 2013, Erasmus University Rotterdam [49].

Due to the rarity of Pompe disease and the clinical heterogeneity, delays in diagnosing Pompe disease are common. Classic infantile patients present with generalized muscle weakness, hypertrophic cardiomyopathy, respiratory problems and feedings difficulties within the first months of life [10-14]. Standard/routine laboratory testing of aspartate

transaminase (AST), alanine transaminase (ALT) and creatine kinase (CK), and lactate dehydrogenase (LDH) will often reveal high serum concentrations [10]. These high levels in combination with clinical symptoms often lead to a fairly quick diagnosis once patients are hospitalized. But also in these patients there are delays in the diagnosis. For childhood and adult patients, with slowly progressive limb-girdle muscle weakness and weakness of the respiratory muscles [15-17], disease onset and progression vary greatly. This may lead to years of delay in diagnosis and thus delayed start of treatment for many/most patients [50-53]

When a patient is suspected of having Pompe disease, the diagnosis can be made by measuring enzyme activity in leukocytes, cultured fibroblasts and by performing mutation analysis. In practice, initial diagnosis is made by measuring enzyme activity in leucocytes. Enzyme activity is then measured in cultured fibroblasts, as they are also the most optimal material to determine CRIM-status via immunoblotting. However, with increased accuracy of enzyme diagnosis on leukocytes due to improved methods, fibroblast cultures are less frequently taken in recent years. Mutation analysis then confirms Pompe disease.

ENZYME REPLACEMENT THERAPY (ERT)

Pompe disease was the first LSD in which ERT was ever attempted. In the mid-1960s and early 1970s attempts to correct intracellular glycogen accumulation failed as they were using, in retrospect, too low a dose of impure and highly immunogenic enzyme from either *Aspergillus niger* or human placenta [54-57]. The combined work of Kaplan et al., Neufeld et al and Sly et al. on the function of the mannose-6-phosphate (M6P) receptor revealed that this receptor plays an important role in the uptake of enzymes by the lysosome in vitro [58-61]. In 1978 the first attempts were made to develop a receptor-mediated ERT to correct the lysosomal deficiencies [62].

For over 40 years the Erasmus MC has invested in researching Pompe disease and finding a treatment. Reuser et al. revealed that there is no uptake of alpha-glucosidase in fibroblasts and muscle cells when acid alpha-glucosidase from human placenta is used, if it is not bound to M6P [63]. Acid alpha-glucosidase from bovine testis is bound to M6P and efficiently taken up by cells. Van der Ploeg et al. demonstrated that the M6P containing alpha-glucosidase purified form human source (urine) was transported to the lysosomes after uptake and degraded stored lysosomal glycogen of cultured muscle cells of an infantile patient. As cultured cells are an easy target for exogenous added enzyme, further research into the transport of enzyme over the capillary wall was needed to elucidate whether transport of enzyme was possible over endothelial cells. Van der Ploeg et al. confirmed in 1990 by specific immunoprecipitation and immunoblotting that when rat hearts were infused with bovine testis derived acid alfa-glucosidase, the enzyme was taken up by the lysosomes [64]. A year later, van der Ploeg et al. found that intravenous administration of bovine testis derived acid alfa-glucosidase led to uptake of the enzyme in liver, spleen, lung, heart and skeletal muscle of mice, but it did not cross the placenta nor the blood-brain-barrier [65]. During the same time, Hoefsloot et al. described the cDNA sequence of alpha-glucosidase in 1990 [27].

Alglucerase became the first ever registered receptor-mediated ERT in 1991 [66], a treatment for Gaucher disease. Instead of the mannose 6-phophate receptor, the mannose receptor on Kupffer cells and macrophages was used as a target and the enzyme purified form human placenta. Over the years many other treatments for LSDs followed and for many LSDs there is now a registered treatment. The work of Van der Ploeg et al. from 1991 combined with work from Fuller et al. and Bijvoet et al. on recombinant enzyme production were the foundation that led to the treatment for Pompe disease and to the manufacturing of recombinant human alpha-glucosidase (rhGAA) from the milk of transgenic mice [67-69].

In 1999, the Erasmus MC pioneered the first clinical trial in classic infantile patients with recombinant human alpha-glucosidase (rhGAA) from the milk of transgenic rabbits [13, 70, 71]. During the time that rhGAA from rabbits was being tested, a clinical test with recombinant human alpha-glucosidase (rhGAA) from Chinese Hamster Ovary (CHO) cells also commenced [14, 72]. In 2006, alglucosidase alfa (Myozyme) was approved for all patients with Pompe disease by the American and European regulatory authorities (FDA and EMA) after it proved to be effective in classic infantile patients [73-75] and later childhood/adult onset patients [38, 76]. From here onwards, the effects of ERT in classic infantile Pompe patients are discussed.

INITIAL EFFECTS OF ERT: SURVIVAL, MUSCLE RESPONSE AND ANTIBODIES

Between 2000 and 2005 the results from the first three clinical trial were published [13, 70, 73, 75]. In Table 1 the results from the clinical trials are summarized. Patients from three publications (Klinge et al. and van den Hout et al.) received rhGAA from the milk of transgenic rabbits [13, 73].

Van den Hout et al. was the first to publish the effect of ERT in four classic infantile Pompe patients (one patient was CRIM-negative) after 36 weeks of treatment in 2000 [70]. Their four patients received ERT dosed at 15 or 20 mg/kg/week before the dose was increased to 40 mg/kg/week after 14 weeks (patient 3 and 4) and 21 weeks (patients 1 and 2). Even though two patients required invasive ventilation (one already before the treatment was initiated), the LVMI decreased and all four patients survived beyond the age of 12 months and started to develop motor milestones that was not seen in untreated patients. Alpha-glucosidase activity in muscle cells increased on the lower dose, but was still below normal (2.1 to 4.9 nmol/mg per hour was reached, normal alpha-glucosidase activity is 8–40 nmol/mg per hour). After dose increase, alpha-glucosidase activity was within the normal range in all four patients and histological assessment revealed that lysosomal glycogen storage had decreased and muscle morphology improved. This was later published by Winkel et al. in more detail [77]. In 2001 and 2004 Van den Hout et al. published the long-term results (follow-up period of 4 years); all were alive at study end, but only one survived ventilator-free and learned to walk. Cardiac hypertrophy normalized in this one patient and decreased in the other three. One patient died at the age of 4.3 years just after the end of the follow-up period; she was CRIM-negative[13, 71].

Amalfitano et al. described the effects of ERT derived from CHO cells (patients receiving 5 mg/kg twice weekly) in three patients (patients 1 and 2 were CRIM-negative, patient 3 was CRIM-positive) [75]. Follow-up ranged from 14 to 17 months; all patients were alive at study end, but only one survived ventilator-free (patients 3). Cardiac hypertrophy - assessed by left ventricular mass index (LVMI) – which was present in all three patients, decreased in patients 1 and 2 (it was still above normal values at last assessment) and normalized in patient 3. Patient 3 learned to walk and remained ambulant at study end (age 14 months). This led to the conclusion that all CRIM-negative patients performed poorly on 5 mg/kg twice weekly as they required invasive ventilation and had minimal motor gains and that the CRIM positive patient did well on the low dose of 5 mg/kg twice weekly. Patient 1 from the Amalfitano study was later also described by Hunley et al. and Banugaria et al [78, 79]. From these publications it became clear that patient 1 had a change in dose after 20 weeks of treatment from 5 mg/kg twice weekly to 10mg/kg twice weekly that was again increased to 10 mg/kg five times per week at week 56 of follow-up. Furthermore, patient 1 also started on what authors

called immunomodulation, a treatment regimen to eliminate anti-rhGAA antibodies. During treatment, patient 1 developed a nephrotic syndrome with subepithelial immune complex depositions. Hunley et al. stated that this was due to the high and frequent rhGAA infusions, they did not mention the immunomodulation at that time. After the publication of Banugaria et al. it is likely that not only the rhGAA infusions played a role in the nephrotic syndrome, but also the immunomodulation regimen with Cyclophosphamide, plasmapheresis, intravenous immunoglobulins (IVIG and Rituximab (RTX). The conclusion of the authors that the rhGAA dose from CHO cells of 5 mg/kg twice weekly is capable of improving cardiac and skeletal muscle function is not the whole story here.

Klinge et al., who studied the effects of rhGAA from the milk of transgenic rabbits, described in 2005 the 12-month follow-up of two CRIM-positive patients receiving 40 mg/kg/week. Both were alive at study end and did not require invasive ventilation. The cardiac hypertrophy that was observed at diagnosis decreased but remained above normal values. One patient learned to sit unsupported, the other with support. Neither learned to walk. Anti-rhGAA antibodies were found in both patients, only patient 1 had a high titer (titer of 1:100.000 at week 48). All the nine patients from these first trials developed antibodies against the ERT (peak anti-rhGAA antibodies ranged from 1:200 to 1:250,000). The results from these studies showed that ERT leads to an improved survival beyond the first year of life, but that motor development varies in these nine patients receiving different products and dosages.

In 2006, Kishnani et al published results from a 52-week follow-up period of eight classic infantile Pompe patients (two were CRIM-negative) receiving ERT derived from CHO cells (CHO-1) dosed at 10 mg/kg/week [14]. Survival for the first 52 weeks was 75%. Two of the eight patients died, one was CRIM-negative and the other CRIM-positive after 16 and 43 weeks of follow-up. Cardiac size decreased in all patients, in two patients it had normalized at week 52 and three patients learned to walk in the initial 52-week phase. All patients developed anti-rhGAA antibodies, three patients developed a titer of around 1:100,000. All surviving six patients were enrolled in the extension phase of the study and were transitioned to ERT derived from a more robust manufacturing process (CHO-2). Follow-up ranged from 4 months to 3 years. At the end of the extension phase only two CRIM-positive patients were alive (33% of the patients enrolled in the extension study). Of the patients that died, one was a CRIM-negative patient and three were CRIM-positive patients, age at death ranging from 14.7-33.8 months. During the extension study three patients learned to walk, two were persistent walkers. During the extension phase no results of anti-rhGAA antibodies were given. The authors stated that the rhGAA dose ranged from 10 to 20 mg/kg per week or 20 mg/kg every 2 weeks (not further specified per patient).

Table 1. Outcome values of initial clinical trials, AGLU 1602 trial and long-term follow-up studies

	Year	Follow-up duration	N	CRIM -	Starting dose
Van den Hout et al.[70]	2000	36 weeks	4	1	15-20 mg/kg/week
Amalfitano et al.[75, 78, 79]	2001	14-17 months	3	2	5mg/kg twice a week
Van den Hout et al.[13, 71]	2004	4 years	4	1	15-20 mg/kg/week
Klinge et al.[73]	2005	12 months	2	0	40mg/kg/week
Kishnani et al.[14]	2006	52 weeks	8	2	10 mg/kg/week
Kishnani et al.[72]	2007	52 weeks	18	4	9 on 20mg/kg eow 9 on 40mg/kg eow
Kishnani et al.[80]	2009	1.1-3.0 years	16	4[C]	8 on 20mg/kg eow 8 on 40mg/kg eow
Broomfield et al.[45]	2015	0.5-13.7 years	33	12	20 mg/kg eow
Hahn et al.[44]	2015	0.7-10 years	23	2	20 mg/kg eow
Parini et al.[81]	2018	0.5-11.5 years	28	7	20 mg/kg eow

Eow: every other week; N.A.: not applicable; AB: antibody.

[A] See Hunley et al. and Banugaria et al. [78, 79] ,there were many dose changes in patient 1.

[B] Dose was increased during the extension study, unknown when the dose was increased in the individual patient (follow-up duration ranged from 4 months to 3 years duration).

The earlier studies all used different ERT dosages and these studies all had great variability in clinical outcome. To determine the effect of dosing on outcome, the AGLU-1602 trial compared patients receiving 20 mg/kg every other week with patients receiving 40 mg/kg every other week [72]. Eighteen patients were included, nine in each dose group (randomization 1:1). Four patients were CRIM-negative, three were enrolled in the 40 mg/kg eow group. Follow-up duration was 52-weeks. All 18 patients were alive after 52 weeks, three patients required invasive ventilation and seven learned to walk. No differences in clinical outcomes were found between the two dose groups after 52 weeks. Sixteen patients were then enrolled into the 2009 extension study and received ERT dosed at either 20 mg/kg or 40 mg/kg every other week [80]. Two patients did not enroll in the extension study; one had died just after the 52-week study period (age at death was 20 months, CRIM-positive patient from the 20mg group) and the other withdrew from the study and died at the age of 32 months (CRIM-negative from the 40mg group).

Of the 16 enrolled patients, the median treatment duration was 2.3 years (range 1.1-3.0 years). At study end 13 patient (81%) were alive. The patients that died were all from the 40mg group, two were CRIM-positive and one was CRIM-negative. Another CRIM-negative patient died one year after the study ended at the age of 44 months. Nine (56%) required no ventilation and seven (44%) learned to walk of whom 6 (38%) could still walk at study

Increase dose	Survival	Vent. Free survival	Normal LVMI	Walking	Persistent walker	Peak AB >1:31,250
40mg/kg/week at 14 and 21 weeks	100%	50%	Decreased in all	N.A.	N.A.	AB present
Various doses[A]	100%	33%	33%	33%	N.A.	33%
40mg/kg/week at 14 and 21 weeks	75%	25%	25%	25%	N.A.	75%
No	100%	100%	Decreased in all	0	N.A.	50%
20 mg/kg eow or weekly [B]	75%	63%	25%	38%	25%	38%
No	100%	66%	Decreased in all	39%	N.A.	16%
No	81%	56%	44%	N.A.	44%[D]	38%
40 mg/kg eow or weekly	61%	39%	48%	36%	33%	10%
30 mg/kg eow to 40 mg/kg/week	57%	43%	96%	39%	22%	8%
20 mg/kg/week to 40mg/kg/week	61%	29%	54%	25%	19%	11%

[C] 3 of the 4 CRIM negative patients were included in the 40mg group and developed high anti-rhGAA antibody titers.
[D] 7 learned to walk of the 16 patients in the extension trial. The two patients would did not enroll in the extension trial both did not learn to walk.

end. Anti-rhGAA antibody titers were found in 14 (88%) patients, with six (38%) patients having high maximum and persistent titers (titer ≥1:31,250); five of these patients were in the 40 mg/kg every other week group. There was however also an overrepresentation of CRIM-negative patients in the 40 mg group as three of the four CRIM-negative patients were in the 40 mg/kg every other week group. Based on these studies (and studies in childhood and adult onset patients), the recommended treatment dose for Pompe disease was set on 20 mg/mg every other week. And while these studies all concluded that ERT improves survival, there were also limitations: Various patients did not survive ventilator-free or did not learn to walk, and most patients had residual muscle weakness. In addition, most patients developed anti-rhGAA antibodies. Moreover, the outcome of the CRIM-negative patients in these studies were worse than of their CRIM-positive counterparts as none of the CRIM-negative patients in these studies survived ventilator-free and had minimal motor gains.

The effects of ERT in CRIM-negative and CRIM-positive patients were studies separately, focusing on the outcome of the CRIM-negative patients who are known to perform poorly [42, 43]. In 2010 Kishnani et al. published the results on clinical outcome in 10 CRIM-negative patients [43]. All 10 patients died; the median survival was 28.8 months, range 14.7-50.2 months. All CRIM-negative patients initially showed an initial decline in LVMI, despite 26

weeks of ERT the LVMI started to increase again. All had minimal motor gains and developed high anti-rhGAA antibody titers with peak anti-rhGAA antibody titers ranging from 1:25,600 to 1:1,638,400. In 2011 Banugaria et al. reported that the development of high anti-rhGAA antibody titers is not limited to the CRIM-negative patients; a group of CRIM positive patients also developed high anti-rhGAA antibody titers and had the same poor clinical outcome [42]. This was confirmed by Van Gelder et al. in 2015, who found high titers in both CRIM-negative and CRIM-positive patients [40].

The results of the clinical trials by van den Hout et al. and Klinge et al. Amalfitano et al. and the AGLU-1602 trial showed that there was a great effect on the survival of patients. Finally, the results of the pivotal trial AGLU 1602 to the registration of rhGAA (alglucosidase) for all Pompe patients (including adult and a childhood patient. This study was later followed by a placebo-controlled trial in 90 childhood and adult patients [38]. The registered dose was 20 mg/kg eow, lower than the dose suggested by van den Hout et al. The studies performed for this thesis (to study the effects of a higher dose on clinical outcome) are a direct consequence of this registration because despite the positive results on survival there was still room for improvement in terms of ventilator free survival and motor outcome

LONG-TERM EFFECTS OF ERT ON CLINICAL OUTCOME AND ANTIBODY FORMATION

The first trials commenced almost 20 years ago, but larger groups of patients were only treated since ERT was registered in 2006. Since the classic infantile form is rare, studies on long-term results (longer than 3 years) have just become available. In 2015 Broomfield et al. and Hahn et al. published results of patients receiving ERT with duration ranging from 0.7-13.7 years [44, 45]. In 2018 Parini et al. published their long-term follow-up study [81]. In Table 1 the results from these first long-term outcome studies are also summarized.

Most of the patients started ERT at the registered dose of 20 mg/kg every other week, but in many changes in their ERT dose were made later during treatment. In the latter patients the treatment regimen was changed from once per 2 weeks to weekly infusions or patients started to receive a higher dose of for various reasons. The most common reason was clinical decline of the patient.

Broomfield et al. reported on a median follow-up duration of 3.8 years (range 0.5-13.7 years). Overall survival was 61% (20/33), ventilator-free survival 40% and 11 of 33 patients (33%) were persistent walkers at study end. LVMI normalized in 48% of patients. Three patients had a high anti-rhGAA antibody titer defined as >1:31,250 (CRIM status was unknown in these three patients); two of these patients died.

Hahn et al. reported on a maximum follow-up of 10 years (range 0.7-10 years). Overall survival rate was 57% (13/27), ventilator-free survival 39% and 5 of 23 patients (22%) persistent walkers. LVMI normalized in 96% of patients. Two patients had a high anti-rhGAA antibody titer (one CRIM-negative), both patients died.

Parini et al. reported on a median follow-up duration was 6 years (range 0.5-11 years). Overall survival was 61% (17/28), ventilator-free survival was 29% and had 5 of 28 patients (18%) were persistent walkers at study end. LVMI normalized in 54% of patients. Three patients had a high anti-rhGAA antibody titer (all CRIM-negative), one patient died.

In total of 34 (40%) of the 84 patients died in these three studies. Fifteen patients were CRIM-negative, 14 were CRIM-positive and in five the CRIM status was unknown or not available. The median age at death (this includes both CRIM-negative and CRIM-positive patients) was 12 months in Broomfield et al; 21 months in Hahn et al.; and 15 months in Parini et al. Focusing on only the CRIM-negative patients in these studies, 22 (26%) of the 84 patients were CRIM-negative. Only eight (36%) of the 22 CRIM-negative patients were alive at study end.

RESIDUAL MUSCLE WEAKNESS AND OTHER RESIDUAL PROBLEMS

In 2011 and 2012 Slingerland et al. and Van Gelder et al. reported that facial-muscle weakness, speech disorders and dysphagia were common in classic infantile Pompe patients who had survived because of ERT [82, 83]. Case et al. published on the residual muscle weakness in classic infantile Pompe patients, with many patients having weakness of the tibialis anterior muscle (difficulty flexing the foot), of the hip flexor muscles and neck flexor muscles among other problems [84]. Prater et al. also found residual muscle weakness in long-term surviving CRIM-positive patients older than 5 years of the neck muscles and hip extensors, but also reported facial-muscle weakens, ptosis and dysphagia [85]. Hearing loss is also a frequent finding in classic infantile Pompe patients. Van Capelle et al. reported on 11 patients treated with ERT and found that 10 had sensorineural hearing defect and that five patients showed evidence of mild retro cochlear pathology, possibly due to glycogen accumulation in the central nervous system [86]. The hearing loss persisted despite therapy.

Additionally, when looking at the brain, Ebbink et al. found normal to mild developmental delays on neuropsychological tests and periventricular white matter abnormalities in 4 children [87]. Spiridigliozzi et al. found similar results with regard to the cognitive abilities [88, 89]. Other groups also found white-matter abnormalities in brain MRI in classic infantile Pompe patients [90-94]. It is known that ERT cannot pass the blood-brain barrier, so these white-matter abnormalities are something that warrants further investigation.

IMPROVING TREATMENT OUTCOME

One possible way to improve clinical outcome could be to increase the dose to 40 mg/kg/week, as studies have shown that motor outcome can be better in patients receiving a higher ERT dose from start.

The rational for a higher dose can be found in the preclinical studies in mice have shown that uptake of alglucosidase alfa is dose dependent between the dosages of 10 to 100 mg/kg [95-98]. Our first ever treated patients, who received recombinant human alpha-glucosidase from rabbit milk, started treatment on 15 to 20 mg/kg/week before we increased the dose to 40 mg/kg/week [70]. On the original dose, alpha-glucosidase activity remained below normal values in muscle cells and muscle morphology showed hardly any improvement [70, 77]. After twelve weeks of treatment with the higher dose normal alpha-glucosidase activity in muscle cells was observed, and improvement of muscle morphology was observed in three of the four patients [13, 77]. In one patient the muscle morphology completely normalized, this patient also had the best motor outcome as he learnt to walk. In the other patients, muscle fibers were still vacuolated, contained huge amounts of PAS positive material and cross-striation of muscle fibers had largely disappeared. Clinically; one patient became ventilator dependent just before the start of treatment, one a few weeks after start. A third patient died just after the 4-year follow-up visit. Because we now know that muscle cells are hard to treat since only a small fraction of infused enzyme reaches muscle cells and lost muscle function is difficult to repair, all newly diagnosed classic infantile patients are currently started on 40 mg/kg/week.

The differences in clinical outcome of patients starting on 20 mg/kg every other week compared to those receiving 40 mg/kg/week is studied in this thesis.

Another way to improve clinical outcome, is to start treatment at a younger age. Previous studies have shown that the youngest patients at start of ERT have a better overall clinical outcome and lower anti-rhGAA antibodies titer [40, 99]. Newborn screening (NBS) for Pompe disease, as is performed in Taiwan, has led to an earlier diagnosis and start of treatment in patients. Yang et al. described thirteen patients starting treatment before the age of 23 days, receiving 20 mg/kg every other week. They were all alive at study end (mean follow-up 32.7 months, range 13 to 61 months) and showed a good cardiac response. All learned to walk between the ages of 10 and 13 months. Anti-rhGAA antibody titers were low [100]. Chien et al. described that in ten other patients diagnosed by NBS, muscle weakness became prominent beyond the age of two years and that facial muscle weakness and speech disorder were common [90]. They also found white-matter abnormalities on brain MRIs in five patients between the ages of 3-6 years. Cognitive development was normal in these

patients. Combining a higher dose with an earlier start of treatment could be beneficial for classic infantile Pompe patients.

The white-matter abnormalities that are currently being found in classic infantile Pompe patients is worrisome. We know that ERT cannot pass the blood-brain-barrier. Autopsy studies of patients with classic infantile Pompe disease, are from untreated patients before the ERT became available. In these young patients glycogen storage was seen in many tissue cells such as the anterior horn cells of the spinal cord, the brain stem, thalamus, cerebellum and to some extent in the cerebral cortex [101-108]. Further research is needed to investigate the possible relationship between white-matter abnormalities and the cognitive function in classic infantile Pompe patients [87]. If there is a relationship between the white-matter abnormalities and the cognitive function, new treatment options such as lentiviral stem cell therapy warrant further investigation as it has demonstrated that such treatments might reduce glycogen storage the cerebrum and cerebellum as was demonstrated in mice [109].

Formation of antibodies against rhGAA potentially pose a threat to the outcome of classic infantile patients who are life-long dependent on ERT. Since antibodies may counteract the activity of ERT, prevention of anti-rhGAA antibodies in an ERT-naïve setting has been attempted in classic infantile Pompe patients using various immunomodulatory regimes [45, 110-113]. B cells were eliminated using Rituximab (RTX) and T cell to B cell communication was influenced by Methotrexate (MTX) before the first dose of ERT (20 mg/kg every other week) was administered. While anti-rhGAA antibodies remained low in some patients, in others extra rounds of immunomodulation or even continuous B cell suppression with RTX was performed to prevent anti-rhGAA antibody formation. Clinical outcomes of patients receiving immunomodulation were heterogeneous. Some patients still required invasive ventilation and many did not learn to walk. Various patients also received a combination of immunomodulation and higher dose at some point in time. How much immunomodulation or higher dosing contributes to positive clinical effects has not been fully sorted out and needs further investigation. Studies on a combination of a higher dose and immunomodulation in an ERT-naïve setting are also of further interest.

Once anti-rhGAA antibodies have been formed, it is difficult to eliminate them. As many classic infantile Pompe patients are already receiving ERT and have developed antibodies against the ERT, a different strategy is required to eliminate anti-rhGAA antibodies in these patients compared to those who did not form antibodies yet. Several groups have made attempts to reduce high titers by using a Bortezomib (eliminates plasma memory B cells) based regime [79, 110, 112, 114-116]. Also, in these studies the reported clinical outcome is heterogeneous and anti-rhGAA antibodies could not be eliminated in all patients.

SCOPE AND AIMS OF THIS THESIS

So far, many advances have been made for the classic infantile Pompe patients. Many patients survive beyond the first year of life, cardiac hypertrophy reduces, and many patients reach motor milestones that were previously unheard of. Unfortunately, studies have also shown that there are still residual problems and limitations. Close to 50% of patients die early before the age of 3 years and many develop residual muscle weakness. Another concern is the development of antibodies and uncertainties about the long-term cognitive development of patients.

The goal of this thesis is two-fold. The first part of this thesis focuses on the efforts we undertook to improve the clinical outcome in classic infantile Pompe patients. **Chapter 2** presents a study on differences in clinical outcome between children receiving different ERT dosages. Half of the patients received 20 mg/kg every other week from start of treatment, the other half started on 40 mg/kg/week. All patients in this study were CRIM-positive. **Chapters 3 and 4** studies the additional effects of immunomodulation on anti-rhGAA antibody formation in an ERT-naïve setting and after high and sustained anti-rhGAA antibodies have developed in both CRIM-negative and CRIM-positive patients. In **chapter 5** we describe the long-term effects of a higher and more frequent dosing of ERT and the additional effects of immunomodulation on the clinical outcome in both CRIM-negative and CRIM-positive patients.

The second part of this thesis focuses on specific long-term outcome features of our surviving classic infantile Pompe patients. **Chapter 6** focusses of the long-term effect of ERT on cardiac dimensions, function, conduction and rhythm disturbances. **Chapters 7 and 8** provides studies on the long term cognitive outcome from early infancy to adulthood of patients with classic infantile Pompe disease and compares the results with MRI findings. At the end of this thesis the results are discussed in **chapter 9** and recommendations to improve clinical outcome are given.

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Chapter 2

Effects of a higher dose of alglucosidase alfa on ventilator-free survival and motor outcome in classic infantile Pompe disease: an open-label single-center study

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ABSTRACT

Background

Though enzyme-replacement therapy (ERT) with alglucosidase alfa has significantly improved the prospects for patients with classic infantile Pompe disease, some 50% of treated infants do not survive ventilator-free beyond the age of 3 years. We investigated whether higher and more frequent dosing of alglucosidase alfa improves outcome.

Methods

Eight CRIM (Cross Reactive Immunological Material) positive patients were included in the study. All had fully deleterious mutations in both *GAA* alleles. Four received a dose of 20 mg/kg every other week (eow) and four received 40 mg/kg/week. Survival, ventilator-free survival, left-ventricular mass index (LVMI), motor outcome, infusion-associated reactions (IARs) and antibody formation were evaluated.

Results

All 8 patients were alive at study end, seven of them remained ventilator-free. The patient who became ventilator dependent was treated with 20 mg/kg eow. Three of the four patients receiving 20 mg/kg eow learned to walk; two of them maintained this ability at study end. All four patients receiving 40 mg/kg/week acquired and maintained the ability to walk at study end (ages of 3.3-5.6 years), even though their baseline motor functioning was poorer. There were no apparent differences between the two dose groups with respect to the effect of ERT on LVMI, the number of IARs and antibody formation.

Conclusions

Our data may suggest that a dose of 40 mg/kg/week improves outcome of CRIM-positive patients over that brought by the currently recommended dose of 20 mg/kg eow. Larger studies are needed to draw definite conclusions.

BACKGROUND

Pompe disease (glycogen storage disease type II, OMIM #232300) is a rare, autosomal recessive lysosomal storage disorder caused by deficiency of acid a-glucosidase and characterized by lysosomal glycogen storage, mainly in muscle tissue [1]. Depending largely on how much enzyme activity is preserved, it can present at different ages, from soon after birth to late adulthood. Patients with the classic infantile form present in the first months of life with generalized muscle weakness, hypertrophic cardiomyopathy, respiratory problems, and feeding difficulties [2]. If untreated, they usually die before one year of age due to cardio-respiratory insufficiency.

Patients' prospects were significantly improved in 2006, when enzyme-replacement therapy (ERT) with recombinant human acid a-glucosidase (Myozyme®, alglucosidase alfa) was approved. ERT prolongs lifespan, improves cardiac hypertrophy, and enables patients to reach previously unmet motor milestones [3-8]. However, response to treatment varies between patients. When treated with either 20 or 40 mg/kg every other week (eow), approximately half of patients with classic infantile Pompe disease do not survive ventilator-free beyond the age of three years [5]. Similarly, a substantial proportion of patients do not learn to walk, and nearly all retain residual muscle weakness [9-11]. Effective clearance of glycogen from skeletal muscle is reported in only a small number of patients [4-6, 12, 13].

Preclinical studies in mice [14, 15] and clinical studies in infantile patients [3-6, 16] have shown that the reduction in glycogen levels in skeletal muscle is dose-dependent. On the basis of these findings and of the published intracellular half-life of alpha-glucosidase [17-20], we estimated that patients might benefit from a higher and more frequent dose. We therefore treated affected infants with a dose of 40 mg/kg/week, i.e., the dose previously administered to four infants treated with recombinant human acid a-glucosidase from rabbit milk [3, 4]. The safety and efficacy of this higher and more frequent dosing regimen was compared with that of the recommended dose of 20 mg/kg eow.

METHODS

Patients

Classic infantile Pompe disease was defined as symptoms of muscle weakness within six months of birth, hypertrophic cardiomyopathy, and confirmation of total deficiency of acid a-glucosidase (GAA) activity combined with the finding of pathogenic mutations in both GAA alleles. From 2009 on we treated new patients with 40 mg/kg/week. In the current study we compared patients who started treatment with the recommended dose of 20 mg/kg eow (start before 2009) to patients who started with a dose of 40 mg/kg/week (start after 2009) and who had received the treatment for at least 3 years. Data of this ongoing investigator driven study were included until April 1st 2014; or until a dose change. The study was performed independent from industry. The Medical Ethical Committee at Erasmus MC University Medical Center approved the protocols and all parents gave written informed consent. None of the patients received immunomodulation. Only CRIM-positive patients were included. Due to the small number of patients no comparative statistics were applied.

Clinical efficacy

Clinical efficacy was measured by assessing survival, ventilator-free survival, number of hospitalizations for respiratory infections, cardiac dimensions, and motor function. Cardiac dimensions were measured by 2D-guided M-mode echocardiographic tracings (using a Philips iE33 xMAtrix Echocardiography System, Philips Medical Systems, Andover, MA, USA), at baseline and at regular intervals thereafter. Left-ventricular mass index (LVMI) was calculated as a measure for hypertrophic cardiomyopathy (LVMI >+2z-scores [21]). Motor function was examined using the Alberta Infant Motor Scale (AIMS) [22] and the achievement of motor milestones was examined at regular clinical assessments.

Safety

Safety assessments included the monitoring of infusion-associated reactions (IARs). Adverse events that were judged to be possibly, probably or definitely related to ERT were IARs. The severity of each IAR was indexed by clinical judgment as mild, moderate or severe [4].

Before enzyme infusions, blood samples were drawn at regular intervals to measure antibodies to ERT with an enzyme-linked immunosorbent assay (ELISA) [23].

Pharmacokinetic analysis

To determine the activity of acid a-glucosidase in the blood circulation and the rate of alglucosidase alfa clearance in relation to dosing, we measured the activity in plasma during enzyme infusions with 20 mg/kg and 40 mg/kg. Blood samples were drawn before the start of the infusion, at 2 and 3 hours after start, at 15 min before the end, at the end of infusion, and then 15, 30, 60, and 120 min thereafter.

To determine the percentage of the enzyme in the blood that was antibody-bound, patients' plasma samples were incubated in the presence of Protein-A Sepharose beads to bind antibody-bound alglucosidase alfa, and in parallel in the presence of Sepharose beads only (control). After removal of the beads by centrifugation, acid α-glucosidase activity was measured in the supernatant [24]. Pre-infusion serum samples were collected to determine the corresponding patients' antibody titers by ELISA [23].

RESULTS

Patients

We included 8 patients with classic infantile Pompe disease, four of whom were treated with alglucosidase alfa in a dose of 20 mg/kg eow and four with 40 mg/kg/week. The patients' characteristics are summarized in Table 1. Patients in the 20 mg/kg eow dose group started ERT at a median age of 0.9 months (range 0.1-2.2 months) vs. a median age of 3.1 months (range 0.3-4.6 months) in the 40 mg/kg/week group. The median age at study end was 4.1 years (range 1.7-9.4 years) in the 20 mg/kg eow dose group and 3.5 years (range 3.3-5.6 years) in the 40 mg/kg/week dose group. All patients had very severe mutations in the GAA gene

Clinical efficacy

Survival and ventilator-free survival

At baseline, four of the eight patients required supplemental oxygen; 50% in both dose groups. Oxygen supply was discontinued in all patients within months after start of treatment. At study end, one of the four patients in the 20 mg/kg eow dose group had developed respiratory insufficiency and became ventilator dependent at the age of 2.7 years during a pneumonia (Table 1). In the 40 mg/kg/week group none had developed respiratory insufficiency. All patients are alive.

Table 1. Patient characteristics related to infusion-associated reactions (IARs).

	Patient	Gender	Age at start of ERT (months)	Age at study end in months *(years)*	Mutation I
20mg/kg eow	**1**	M	0.1	33 *(2.7)*[#]	c.1460T>C
	2	F	0.5	113 *(9.4)*	c.2481+102_2646+31del
	3	M	1.2	66 *(5.5)*	c.1933G>T
	4	M	2.2	20 *(1.7)*	c.2481+102_2646+31del
	Total				
40mg/kg	**5**	F	0.3	42 *(3.5)*	c.525delT
	6	F	2.4	67 *(5.6)*	c.2481+102_2646+31del
	7	M	3.8	39 *(3.3)*	c.2481+102_2646+31del
	8	F	4.6	41 *(3.4)*	c.378_379del
	Total				

M, male; F, female; eow, every other week; IAR, infusion-associated reaction; ERT, enzyme-replacement therapy, NA, not applicable. [#]Patient developed respiratory insufficiency

Hospital admissions for respiratory infections

After the start of ERT, three of the four patients treated with 20 mg/kg eow were repeatedly hospitalized for respiratory infections or aspiration pneumonias, the number of admissions ranged from 3 to 5. In the 40 mg/kg/week group none of the patients were admitted for respiratory infections or aspiration pneumonias since the start of ERT and all were discharged from hospital within 3 weeks after the start of ERT.

Cardiac outcome

Median baseline LVMI was similar in both groups; in the 20 mg/kg eow group (median z-score +13.5, range z-score 4.9-21.8) and in the 40 mg/kg/week dose group (median z-score +21.4, range z-score 6.4-25.8). LVMI steadily decreased in both dose groups (Figure 1A-B). At study end, LVMI was within normal limits in 3 of 4 patients in the 20 mg/kg eow dose group and in all patients in the 40 mg/kg/week dose group.

Important to note is that one of the patients, treated with 40 mg/kg/week, had severe left-ventricular dilatation and severe mitral valve regurgitation at baseline, which was considered to be life threatening by the treating cardiologist. After 1.6 years of treatment this patient's LVMI had normalized and mitral regurgitation had become moderate. End-diastolic left ventricular internal dimension (LVIDd) and shortening fraction had also become normal [25, 26].

Mutation II	Total no. of IARs possibly related to ERT (no. severe)	ERT duration at first IAR in months (years)	ERT duration at last IAR in months (years)
c.1460T>C	18 (1)	2.9 *(0.2)*	29.5 *(2.5)*
c.2481+102_2646+31del	None	NA	NA
c.525delT	27 (0)	3.2 *(0.3)*	19.9 *(1.7)*
c.525delT	3 (1)	8.1 *(0.7)*	17.3 (*1.4)*
	48 (2)		
c.1933G>A	2 (0)	1.4 *(0.1)*	9.4 *(0.8)*
c.2481+102_2646+31del	70 (6)	0.7 *(0.1)*	37.6 *(3.1)*
c.525delT	10 (0)	0.9 *(0.1)*	10.3 (*0.9)*
c.2104C>T	5 (0)	9.7 *(0.8)*	12.2 *(1.0)*
	87 (6)		

Motor function

At baseline, all eight patients showed symptoms of muscle weakness, including head lag and axial hypotonia; six had AIMS scores below the 5th percentile (2/4 in the 20 mg/kg eow and 4/4 in the 40 mg/kg/week dose groups (Figure 1C-D) .During treatment, seven of the eight patients ultimately approached the maximal AIMS score and learned to walk: 3/4 in the 20 mg/kg eow dose group (median age at walking 16 months, range 15 -17 months), and 4/4 in the 40 mg/kg/week dose group (median age at walking 15 months, range 14-17 months). Over time, some patients lost motor milestones (Figure 1C). One patient who had initially learned to walk lost this skill after becoming ventilator-dependent at the age of 2.7 years. The only patient who did not learn to walk temporarily lost the ability to attain a sitting position after a Respiratory Syncytial Virus infection at the age of 1.3 years. The loss of motor milestones was observed only in the 20 mg/kg eow group and not in the 40 mg/kg/week dose group. At study end, 2 of the 4 patients in the 20 mg/kg eow group were able to walk compared to all 4 patients in the 40 mg/kg/week group. Yet, muscular problems such as facial-muscle weakness, weakness of the neck flexors and ankle dorsiflexors weakness were observed in patients treated with 40 mg/kg/week.

Safety

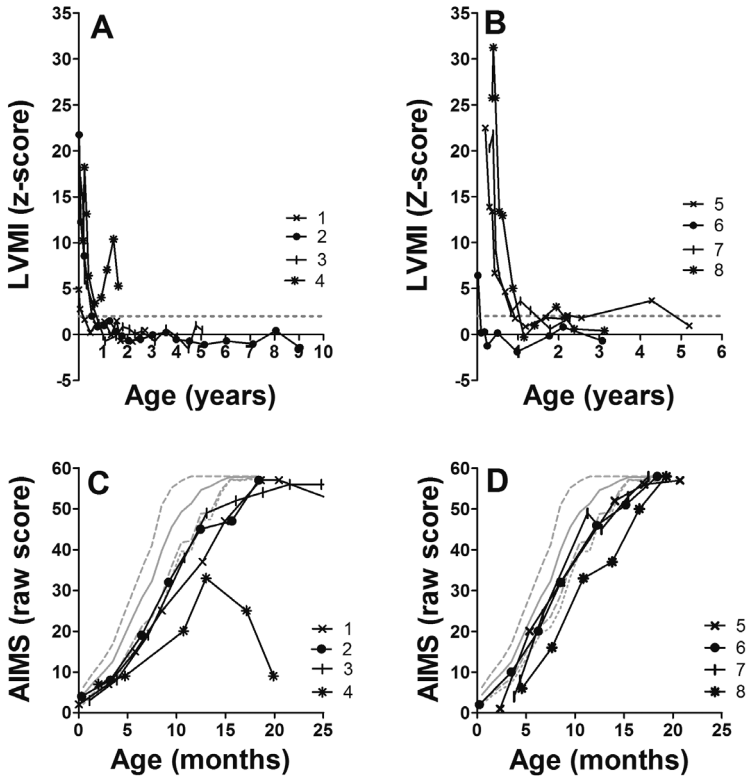
Infusion-associated reactions

IARs were experienced by 3/4 patients treated with 20 mg/kg eow and by 4/4 patients treated with 40 mg/kg/week (Table 1). The number of IARs per patient varied substantially. One patient in the 40 mg/kg/week dose group had 70 IARs (over 50% of all IARs), six of them were severe. Remarkably, the IARs started within minutes of the start of the infusion, when the infusion rate was still slow. Total IgE, serum tryptase and complement levels were within the normal range. Two patients treated with 20 mg/kg eow had one severe IAR each. The most common IARs were exanthema, fever, and decreased oxygen saturation. All IARs could be controlled by slowing the infusion rates and prolonging the duration of the infusion, with or without the administration of antihistamines and/or steroids. No patients discontinued treatment because of IARs, all recovered without sequelae, and premedication could be stopped. At the end of the study, 4/4 patients treated with 40 mg/kg/wk had been IAR-free for at least 1.5 years and all received infusions at home.

Antibody formation

Figure 2A-B shows the antibody titers to alglucosidase alfa of the two groups over the entire study period. In the 20 mg/kg eow dose group the median peak antibody titer was 1:6,250 (range 1:1,250-1:31,250); in the 40 mg/kg/week dose group the median peak was 1:31,250 (range 1:250-1:156,250). Peak antibody titers of patients who started ERT before the age of 2 months ranged from 1:50-1:6,250, those of patients who started ERT later ranged from 1:31,250-1:156,250 (Figure 2C).

Figure 1. Left-ventricular mass index (LVMI) z-scores and Alberta Infant Motor Scale scores over time.

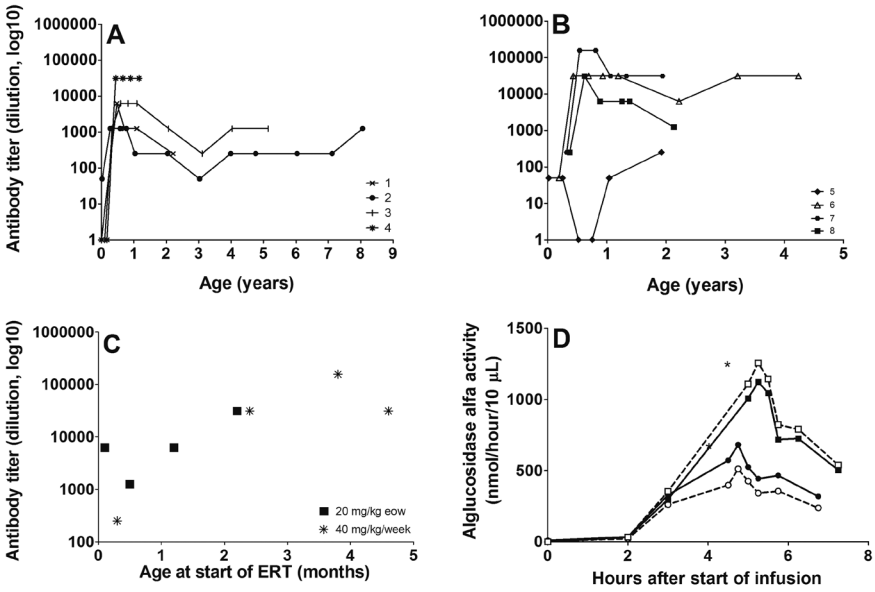


The different symbols represent different patients. LVMI 20 mg/kg eow (A) and 40 mg/kg/week group (B); The dashed grey line represents the upper limit of normal (+2 z-scores). AIMS 20 mg/kg eow (C) and 40 mg/kg/week group (D). The different symbols represent different patients. Grey solid line: p50; dashed grey line: p90 and p10; dotted grey line: p5.

Pharmacokinetic profile

We studied differences in the pharmacokinetics of alglucosidase alfa administrations of 20 mg/kg and 40 mg/kg by giving both doses to the same patient at an interval of one week. A 40mg/kg infusion led to approximately twice the enzyme activity in plasma as compared to a 20mg/kg infusion (Figure 2D). The plasma half-life seemed independent of the dose. Around the time that these experiments were performed, this patient's antibody titer was 1:6,250. Using a Protein-A Sepharose based precipitation method, we could not detect substantial amounts of antibody-bound alglucosidase alfa during enzyme infusion (Figure 2D). Neither could we detect antibody-bound alglucosidase alfa in the plasma of three other patients who received 40 mg/kg/week and had antibody titers ranging from 1:1,250 to 1:31,250 (patients 6, 7 and 8) at the time of investigation.

Figure 2. Antibody titers to alglucosidase alfa and Enzyme activity in plasma using doses of either 20 mg/kg or 40 mg/kg.



Antibody titers to alglucosidase alfa over time in 20 mg/kg eow (A) and 40 mg/kg/week group (B). Peak antibody titers in relation to age at start of ERT. Patients received either 20 mg/kg eow (squares) or 40 mg/kg/week (asterisks) (C). Enzyme activity (D): Blood samples were collected just before the start of infusion (0 h) and at regular time intervals thereafter. A dose of 20 mg/kg (circles) and 40 mg/kg (squares) were given to the same patient (patient 3) at one week's interval after 5.5 years of therapy (titer 1:6250). Closed symbols represent total acid α-glucosidase activity in the plasma; open symbols represent the amount of activity that was not antibody-bound. The activity in the supernatant was measured with MUGlc and is expressed in nmol 4 MU liberated per 10 µl supernatant per hour. NB: Even though the enzyme-activity assay is a standardized and validated assay there is always a slight variation in the figures obtained. All samples were analyzed as part of one experiment. The total set of analyses were performed three times with comparable results.

Dose increase at time of clinical deterioration.

In 3 of the 4 patients in the 20mg/kg eow dose group the dose was increased to 40mg/kg/week (ages 1.7, 2.7 and 5.5 years). This decision was made because the patients experienced life threatening respiratory infections leading to respiratory insufficiency in one of them.

After dose increase, respiratory infections disappeared in 2 patients. In the third patient, who had become ventilator dependent, ventilation remained required in supine position and during respiratory infections during the day. The 2 patients who were not able to walk did not regain walking ability. Patients are all alive five years after dose increase.

DISCUSSION

It is unquestionable that the introduction of enzyme replacement therapy has significantly improved the life expectancy of patients with classic infantile Pompe disease [3-7]. Nevertheless, nearly 50% of the infants treated do not survive ventilator-free [5, 6]. In this study we evaluated the efficacy and safety of a higher and more frequent dosing regimen, which we hoped would improve the patients' clinical outcome.

Preclinical studies in mice have shown a dose dependent uptake of alglucosidase alfa in the range from 10 to 100 mg/kg [15, 16, 18, 27]. In the very first clinical study in which we treated classic infantile patients with recombinant human alpha-glucosidase from rabbit milk, we observed a similar dose dependent effect in that the alpha-glucosidase activity in the skeletal muscle only normalized when the dose was increased from 15-20 mg/kg/week to 40 mg/kg/week [3, 4, 12]. It is known that muscle cells are hard to treat since only a small fraction of infused enzyme actually reaches the muscle cells. Further it is by now generally accepted that treatment needs to be started before irreversible muscle damage has occurred. This combined experience was reason for us to treat patients with a dose of 40 mg/kg/week from start and not to wait until patients deteriorated. Earlier no difference in clinical response was found between infantile patients treated with either 20 mg/kg/eow and 40 mg/kg/eow [5]. This might be attributed to the lower dose and larger dose interval. Another factor that may have played a role is that there were more CRIM-negative patients in the higher dose group [5, 23, 28]. CRIM-negative patients tend to perform poorer. We therefore excluded CRIM-negative patients from the current study.

The most notable contrast we observed between the two dose groups was the difference in overall clinical condition, which was reflected in the difference in hospital admissions for the two groups: while none of the patients treated with 40 mg/kg/week had ever had respiratory infections requiring hospitalization, 3/4 patients treated with 20 mg/kg eow required frequent readmissions. Consequently, one of these patients developed respiratory insufficiency at the age of 2.7 years. Our study results suggest that the 40 mg/kg/week dosing regimen helps to stabilize or improve the respiratory condition of affected infants better.

Similarly, motor function appeared to be better in the 40 mg/kg/week dose group, all of whom learned to walk and maintained the ability to do so. Unlike 3/4 patients treated with 20 mg/kg eow learned to walk and only 2/4 could still walk at the end of the study. The loss of motor milestones in the 20 mg/kg eow dose group was preceded by infections requiring hospital admissions. Importantly walking was not regained in our patients when the dose was increased after deterioration. Recently two studies also reported minor

effects of dose increase when patients perform poorly [8, 29]. It should also be noted that response to ERT varies between patients treated with the same dose. This is illustrated by 1/4 patients treated with the lower dose of 20 mg/kg eow, who performed well until the end of the follow-up at the age of nine years.

With regard to cardiac hypertrophy, both dosing regimens worked equally well, which is explained by the fact that a lower dose is required to correct or prevent the cardiac hypertrophy compared to the skeletal muscle weakness [3, 14, 15]. For the same reason, adults with Pompe disease with residual a-glucosidase activities of up to 25% do not generally develop hypertrophic cardiomyopathy, while they do have skeletal muscle weakness [1].

Although we observed no clear differences in safety parameters, the small numbers do not allow us to draw firm conclusions. While nearly all patients in each dose group experienced IARs the overall number of IARs was higher in the 40 mg/kg/week dose group. This was largely due to a single patient that had had over 50% of the total number of IARs. A similar pattern was observed in the pivotal trials [5, 6]. The patient with most IARs in our study had recurrent episodes of exanthema, coughing and vomiting, occasionally accompanied by saturation drops. Remarkably, the IARs started within minutes of the start of the infusion, when the infusion rate was still slow. Total IgE, serum tryptase and complement levels were within the normal range. While this patient had a relatively high sustained antibody titer, the titer was similar to that of other patients who did not develop as many IARs. At the time of writing, the patient was receiving home-based enzyme therapy without problems, and time since last IAR was over 2 years.

It is well recognized that therapeutic proteins can induce an immunological response that neutralizes the effect of ERT. Three of the four patients treated with 40 mg/kg/week and two of the four treated with 20 mg/kg eow developed a peak antibody titer of 31,250 which was estimated to be the highest titer without significant consequences for ERT at a dose of 40 mg/kg [23]. Using pharmacokinetic studies in the present study, we could not detect substantial amounts of antibody-bound alglucosidase alfa during enzyme infusion in patients whose antibody titers ranged from 1:6,250 to 1:31,250. One patient receiving 40 mg/kg/week had a peak antibody titer of 1:156,250, which later declined to 1:31,250. According to earlier estimates, as much as 54% of the administered enzyme (about 10 mg/kg) is antibody-bound at a dose of 20 mg/kg and a titer of 1:156,250 [23]. If a similar amount (10 mg/kg) were bound upon administration of 40 mg/kg, about 30 mg/kg would theoretically still be available for uptake in the target tissues.

Overall, we found no apparent correlation between the level of antibodies and the dose of ERT, although patients treated with 40 mg/kg/week tended to develop higher antibody titers than those receiving 20 mg/kg eow. This is consistent with a previous study that compared the level of antibody titers between patients treated with 20 or 40 mg/kg eow [28]. In line with previous observations [23, 30] the patient's peak antibody titer seemed to be related to the age at start of therapy.

A further point that requires attention is that muscular problems were still observed in patients treated with 40 mg/kg/week. This may be due to insufficient glycogen clearance. As glycogen also accumulates in neural tissues, including motor neurons of the spinal cord and peripheral nerves [31], we cannot exclude the possibility that neurological damage plays a role as well.

Our study describes a limited number of patients. We have chosen to report on children who received at least 3 years ERT in a dose of 40 mg/kg/week. Inclusion of more patients and longer follow-up will be needed to get the full picture. So far, our group of children starting on 40mg/kg/week seems to have a better outcome than those who started on 20mg/kg eow. Our data suggest that the 40 mg/kg/week has the best effect when applied from start.

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Chapter 3

High sustained antibody titers in patients classic infantile Pompe Disease following immunomodulation at start of enzyme replacement therapy

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ABSTRACT

Background

To evaluate whether immunomodulation at start of enzyme replacement therapy (ERT) induces immune tolerance to recombinant human acid alpha-glucosidase (rhGAA) in classic infantile Pompe patients.

Methods

Three patients (one Cross Reactive Immunological Material (CRIM) negative, two CRIM-positive) were treated with four weekly doses of Rituximab (RTX), weekly Methotrexate and monthly IVIG and ERT at 40 mg/kg/week. Antibody titers were measured using ELISA. Neutralizing effects on rhGAA activity and cellular uptake were determined and combined with pharmacokinetic analysis. Clinical efficacy was evaluated by (ventilator-free) survival, reduction in left ventricular mass index (LVMI) and improvement of motor function.

Results

Immunomodulation induced B cell depletion that was accompanied by absence of antibody formation in all three patients. Upon cessation of RTX treatment, all three patients showed B cell recovery, which was accompanied by formation of very high sustained antibody titers in two patients. Neutralizing effects on infused rhGAA were low to mild/moderate. All patients were alive at study end, learned to walk, and showed (near) normalization of LVMI.

Conclusions

Immunomodulation as recommended in the literature prevented formation of rhGAA antibodies only during B cell depletion, but failed to induce immune tolerance in 2 out of 3 patients.

INTRODUCTION

Pompe disease (Glycogen Storage Disease type II, OMIM: #232300) is an autosomal recessive lysosomal storage disorder caused by deficiency of the enzyme acid-α-glucosidase (GAA), and results in lysosomal glycogen accumulation with prominent pathology in cardiac and skeletal muscle cells [1]. Pompe disease presents as a spectrum of clinical phenotypes. The classic infantile form is the most severe, with residual alpha-glucosidase activity in cultured fibroblasts of less than 1%. About two third of the patients with classic infantile Pompe disease produce inactive GAA protein, and they are therefore called Cross Reactive Immunological Material (CRIM) positive; one-third does not produce any detectable GAA protein and these patients are called CRIM-negative. Hypertrophic cardiomyopathy, generalized muscle weakness and respiratory insufficiency are characteristic features. Without treatment, classic infantile patients die within the first year of life due to cardiorespiratory failure [2-4].

Enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA, alglucosidase alfa, Myozyme) has led to significant improvement of the prospects of classic infantile Pompe patients, but the clinical response to ERT is heterogeneous: nearly half of patients do not survive ventilator-free beyond the age of three years [5-9]. One of the factors that may reduce the efficacy of ERT is the formation of antibodies to rhGAA that neutralize its activity or prevent its cellular uptake [10-13]. Antibodies potentially pose a threat to survival because classic infantile patients are strongly dependent on ERT [14-16]. In general, a CRIM-negative status is associated with high probability to form anti-rhGAA antibodies and a poor clinical outcome [11, 12]. However, many CRIM-positive classic infantile and some childhood/adult onset Pompe patients (which are per definition CRIM-positive), also develop high anti-rhGAA antibody titers. To date the likelihood to form high and sustained antibody titers is difficult to predict [11, 12, 17, 18].

In 2012, prevention of antibody formation was shown by immunomodulation/immune tolerance induction (ITI) at the start of ERT [19, 20]. The protocol consisted of a four-week treatment with Rituximab (RTX), and additional treatment with Methotrexate (MTX) and intravenous immunoglobulin (IVIG). The rationale was to prevent formation of B memory cells at the start of ERT by eliminating B cells with RTX during the initial 4 weeks of ERT. MTX was used as additional immunomodulatory agent, and IVIG supplementation was applied to prevent opportunistic infections.

We implemented this primary immunomodulation protocol [19, 20] in our patients, and monitored the following parameters over a period of at least 2 years: formation of anti-rhGAA antibodies, depletion and repopulation of B cells, effects of antibodies on GAA enzyme activity and cellular uptake, and clinical outcome. Furthermore, we determined the effects of antibodies during infusion with ERT.

MATERIAL AND METHODS

Treatment protocol and blood parameters

Study protocols were approved by the Institutional Review Board and written informed consent was obtained from the parents. Standardized assessments were performed at baseline, on a monthly basis during the first three months, and every three months thereafter as described by van den Hout et al. [6]. Immunomodulation consisted of intravenous RTX 375 mg/m^2/dose, weekly for four weeks followed by intravenous MTX 1 mg/kg/dose based on Messinger et al. and Mendelsohn et al. [19, 20]. The first dose of RTX was administered the day before the first dose of alglucosidase alfa dosed at 40 mg/kg/week. MTX was given directly after RTX one day before the infusion during the first four weeks and from week five onwards weekly MTX was given directly after ERT infusion. Intravenous Immunoglobulin (IVIG) 400 mg/kg was given monthly starting at week four until B cell levels and IgG levels had reached normal values for age. Antibiotic prophylaxis was given to prevent (respiratory) infections. Regular blood analysis consisted of assessing number of neutrophils, number of B and T cells, aspartate transaminase (AST), alanine transaminase (ALT), creatine kinase (CK), and gamma globulin (IgG, IgM, IgA) levels. MTX was temporarily stopped when neutrophil counts were below 0.50x10^9g/l, AST/ALT levels became more elevated than expected on base of the disease course, or when patients had fever (temperature >38.5 °C).

Patients

Patients treated for at least 24 months with ERT and immunomodulation were included. Immunomodulation was performed in patients, regardless of their CRIM status, that were older than 2 months of age at the start of ERT, as these might have the highest risk to develop high anti-rhGAA antibody titers [12]. Here we report on the first three patients from our center that received immunomodulation. Classic infantile Pompe disease was defined as symptoms of muscle weakness within six months after birth, the presence of hypertrophic cardiomyopathy, confirmed deficiency of endogenous α-glucosidase in fibroblasts of <1% of the normal mean, and mutation analysis.

Clinical efficacy

Clinical outcome was evaluated by (ventilator-free) survival. Cardiac dimensions were measured by conventional echocardiography according to the recommendations of the American Society of Echocardiography; left ventricular mass index (LVMI) was calculated by the Devereux formula and indexed by body surface area. An LVMI of > +2SD of age-related peers was considered abnormal [21, 22]. Motor function was assessed by Alberta Infant Motor Scale (AIMS), Bayley Scales of Infant Development II (BSID-II), and by recording motor milestones [23, 24]. Infusion associated reactions (IARs) were noted when they occurred.

Antibody detection

Anti-rhGAA titers were assessed at baseline, four, eight and 12 weeks after start of ERT and thereafter every three months. Standardized antibody analysis was performed by an ELISA as described [12, 17, 18]. Antibody titers were measured in a five-fold serial dilution to determine the antibody titer range. When a positive titer was found, a two-fold serial dilution was used to obtain a more precise titer. The highest titer measured was used. Samples were measured in duplicate per assay, and assays were performed at least twice. The background of the ELISA method was determined to be 1:250 by omitting the coating of the plates with rhGAA as described previously [17].

Pharmacokinetic (PK) profile: effect of antibodies on ERT during infusion

Blood was drawn before start of infusion with alglucosidase alfa, at several preset time points during the infusion, and after completion. Plasma or serum was prepared and stored at −80°C until use. The effect of antibodies during infusion of ERT was determined by capturing antibody-rhGAA complexes using protein A or G Sepharose, followed by analysis of the supernatant on rhGAA enzyme activity by 4-MU assay as described [18] and rhGAA protein content by mass spectrometry (MS) as described [25]. Results obtained using protein A - or protein G Sepharose - were similar.

Neutralizing effect of antibodies in cells

Neutralizing effects of antibodies were measured as described [12, 17]. In short, to fibroblasts from a patient fully deficient for endogenous GAA activity, 20 ml of patient's serum was added together with 200 nmol rhGAA in a total volume of 200 ml Ham's-F10+ medium containing 3mM PIPES and antibiotics. Enzyme activity was measured in medium and in cell lysate and compared to the activity in a fetal calf serum (FCS) sample (set at 100%).

Neutralizing effects of antibodies in vitro

This was performed as described in [12]. In short, 10 nmol rhGAA in 10 ul Phosphate Buffered Saline (PBS) was incubated with various dilutions of patient's sera in the presence of 40ul Protein A/Sepharose for one hour at room temperature. The mixture was centrifuged for two minutes at 12,000 RPM in an Eppendorf centrifuge. Enzyme activity in the supernatant was measured and compared to the results obtained with sera from control individuals.

RESULTS

Patients and clinical evaluation at baseline

Patient characteristics are given in Table 1. Patient 1 was homozygous for c.525delT, which leads to undetectable GAA mRNA or protein expression. This patient was therefore CRIM-negative [26]. Patient 2 was homozygous for c.1551+1G>A. Immunoblot analysis showed that this *GAA* variant led to GAA protein production and the patient was therefore CRIM-positive (Figure 1). This is in agreement with splicing analysis of this variant, which indicated that it causes a perfect skip of exon 10 in which the reading frame remains intact [27]. Patient 3 contained the c.525delT and the c.2481+102_2646+31del (delex18) *GAA* variants, the latter of which expresses a truncated GAA protein, and therefore this patient was also CRIM-positive [28]. Patients started ERT at a relatively late age (5.8; 4.3; and 3.1 months). At start of ERT, all three patients were able to move and lift arms and legs from the surface, showed poor head balance, while none were able to roll over. AIMS scores were clearly below the 5th percentile in all three patients. Two patients required oxygen by nasal prong, the other was monitored at night because of superficial breathing. All required nasogastric tube feeding and had a severe hypertrophic cardiomyopathy (Table 1). The LVMI value was the highest in patient 2, who showed additional dilated cardiomyopathy.

To exclude other possible causes of cardiac disease in this patient, exome sequence analysis of a panel of 49 genes known to be involved in hypertrophic cardiomyopathy was performed (Table 2). No other pathogenic variant was found.

Effect of immunomodulation on B and T cells

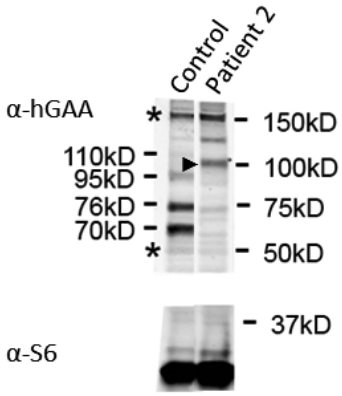
The number of B cells decreased to undetectable levels after start of RTX treatment, and normalized in all patients after RTX was stopped. Time to B cell recovery was 6.7 months (patient 1); 6.7 months (patient 2); and 7.7 months (patient 3) (Figure 2). T cells remained within normal values throughout the study in all three patients (data not shown). During B cell depletion all patients received IVIG and this was continued after B cell recovery until gamma globulin levels remained within the normal range for age (>3.5 g/l). IVIG was stopped in patient 1 at 18.7 months after start of immunomodulation, in patient 2 at 12.6 months. At study end patient 3 still needed IVIG with 2-3 months intervals. These results indicate that all three patients responded to the immunomodulatory treatment with a rapid and transient B cell depletion.

Table 1. Patient Characteristics.

Characteristics	Patient 1	Patient 2	Patient 3
At Baseline			
Gender	Male	Female	Male
Age at diagnosis (months)	5.7	4.0	2.9
Age at start of ERT (months)	5.8	4.3	3.1
GAA variants	c.525delT c.525delT	c.1551+1G>A c.1551+1G>A	c.525delT c.2481+102_2646+31del
CRIM status $	Negative	Positive	Positive
Enzyme activity in fibroblasts / leucocytes ǂ	0.3 / 1.6	0.1 / 1.6	0.4 / 1.3
LVMI at baseline / Z-score ‖	265 / 26.1	753 / 87.8	160 / 12.8
AIMS at baseline (percentile score)	4 (p<5)	3 (p<5)	3 (p<5)
At Study End			
Age in months	34	28.2	27.0
LVMI / Z-score	65 / 0.7	87 / 3.5	72 / 1.6
Maximum motor milestone (age achieved in months)	Walking (21.3)	Walking (18.0)	Walking (17.3)
Number of IARs (number of severe IARs)	15 (3)	0	0
Peak antibody titer (months of ERT treatment)	800,000 (13)	6,250 (14)	200,000 (14)
B-cell recovery (time in months) ^	6.7	6.7	7.7
B-cell level (age at study end) ^	1.25*10E9 (30.2)	0.75*10E9 (25.4)	0.67*10E9 (22.4)
IgG level (age at study end) #	4.6 (30.2)	6.5 (25.4)	3.3 (22.4)

ERT: enzyme replacement therapy; GAA: acid-α-glucosidase; CRIM: cross-reactive immunologic material; LVMI: left ventricular mass index; AIMS: Alberta Infant Motor Scale; IAR: infusion associated reaction; IgG: immunoglobulin G; $ CRIM status in patient 1 and 3 was based on known effects of *GAA* variants. In patient 2, CRIM status was assessed by immunoblot analysis of fibroblasts (see Figure 4). ǂ Enzyme activity in nmol/hr/mg (fibroblasts: 4-MU substrate; leucocytes: 4-MU substrate + acarbose). Normal range fibroblasts: 45-180 nmol/hr/mg. Normal range leucocytes: 6.7-27 nmol/h/mg. ‖ LVMI in g/m^2. ^ Time to B-cell recovery in months after the last dose of RTX. B- cell normal range: 0.2-2.1*10E9. # IgG normal range: 3.5-10.0 g/l

Figure 1. Immunoblot analysis of patient 2.



Cell homogenates were prepared as described in Van Gelder et al 2014. Equal amounts of protein were loaded per lane. The two lanes are: control fibroblasts from a healthy individual and fibroblasts from patient 2. Patient 2 shows a specific band at ~105.8 kD, which is consistent with the size of the 110 kD precursor GAA protein minus 4.2 kD, caused by an in-frame skip of exon 10.

Table 2. Exome sequence analysis of 49 genes involved in hypertrophic cardiomyopathy applied in patient 2.

Genes tested			
MYBPC3	GLA	TTR	CALR3
MYH7	JPH2	VCL	TTN
TNNT2	LAMA4	CTNNA3	ABCC9
TNNI3	LAMP2	DSC2	ACTN2
MYL2	LDB3	DSG2	ANKRD1
TPM1	MIB1	DSP	BAG3
TCAP	MYH6	JUP	CAV3
CSRP3	MYOZ2	PKP2	CRYAB
LMNA	MYPN	SCN5A	DES
TNNC1	NEXN	TMEM43	EMD
ACTC1	PDLIM3	PRKAG2	FLH1
MYL3	PRDM16	RBM20	TAZ
PLN			

Safety of immunomodulation and ERT

The immunomodulation regimen was well tolerated. Patients 1 and 3 showed a mild skin reaction during the first dose of RTX. In Patient 2 the third RTX infusion was skipped because of a concomitant bacterial infection at that time. In patient 1, MTX treatment was temporarily suspended at 11 months after start of immunomodulation for four weeks due to unanticipated increase of AST and ALT levels (Figure 2). MTX was restarted at a lower dose of 0.5mg/kg/week. In total, 9 of the weekly MTX administrations were skipped over the course of 24 months (Figure 2). In patient 3, one dose of MTX was skipped due to low neutrophil count 21 months after start. IARs during alglucosidase alfa infusions occurred only in patient 1. Fifteen mild IARs and three severe IARs were observed (as defined in Table 3). The age at the first IAR was 6.7 months (infusion 5; First asterisk in Figure 2). Antihistamines were administered before the next infusions and the infusion rate was adapted. Antihistamines were stopped at age 8.5 months (infusion 14). Infusion rates were gradually adapted to higher rates, starting at the age of 14 months (8.2 months of ERT). This process was still ongoing at the age of 20.7 months (14.8 months of ERT) when he experienced a new IAR (second asterisk in Figure 2). At that time infusion rates were slowed down again and antihistamines and corticosteroids were started. With this regimen IARs remained variably present until the end of the study period.

Antibody titers

Antibody titers as measured with ELISA are shown in Figure 2. In patient 1 anti-rhGAA antibodies were first detected at 4.5 months of ERT (titer 1: 1,250) and continued to increase to a maximum titer of 1:800,000 at 13 months of ERT. In patient 2 anti-rhGAA antibodies were undetectable during the first nine months of ERT. At 12 months of ERT the antibody titer was 1: 1,250 and increased to an intermediate maximum titer of 1:6250 at 14 and 21 months of ERT. In patient 3 anti-rhGAA antibodies were detected at two months of ERT (1: 6250), and increased to a very high maximum titer of 1:200,000 at 14 months of ERT. The start of antibody formation coincided with B cell recovery. Titers remained high in patients 1 and 3, and fluctuated between intermediate and background levels in patient 2 during follow up (Figure 2). These results indicated that the immunomodulation procedure induced a temporarily suppression of antibody formation during B cell ablation, while it failed to induce immune tolerance.

Table 3. Infusion-associated reactions in patient 1.

Type of IAR	Frequency
General discomfort	12
Fever	9
Shivering	7
Local erythema	8
Quivering lip	4
Hypotension	2
Pallor	9
Tachycardia	9
Desaturation	1
Generalized erythema	2
Regurgitation	2
Swollen ear	1
Total infusions with IARs	**15**

Neutralizing effect of antibodies

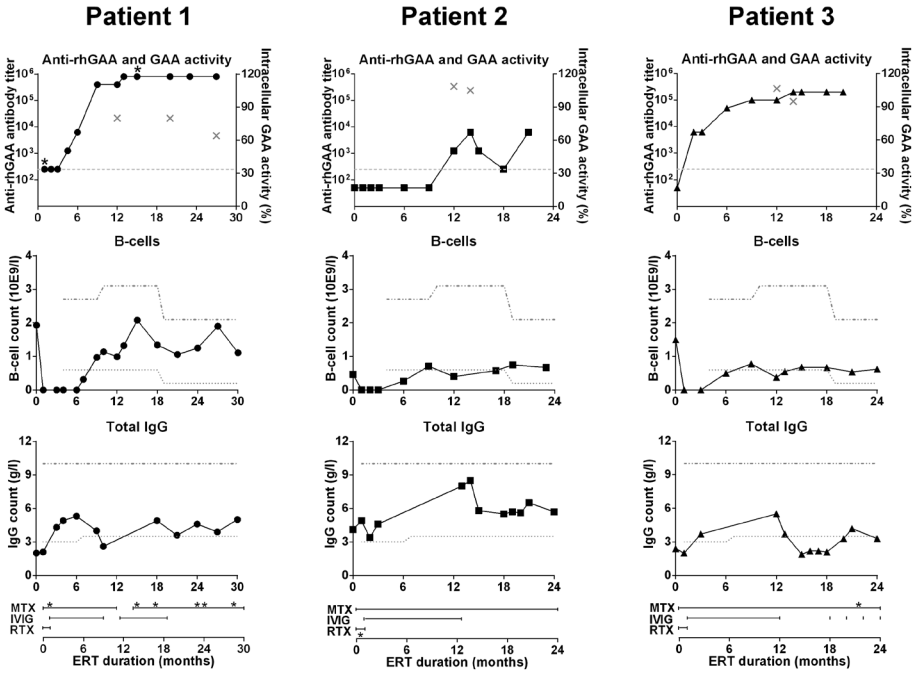
To test for neutralizing effects of antibodies, GAA-deficient fibroblasts were incubated with alglucosidase alpha plus patients' serum, followed by enzyme activity measurements in medium and cell lysates (Figure 2). The latter was taken as the sum of neutralizing effects on cellular uptake and inhibition of enzymatic activity. A reference patient that we reported by us previously [18], served as positive control for detection of neutralizing antibodies and showed an enzyme activity of 29.7% in the cell lysate (data not shown). No neutralizing effects of antibodies in the medium were found (enzyme activities in all case were > 92% of control; data not shown). In patients 1, 2, and 3, enzyme activities in cell lysates were 79.8%, 105% and 94.8% as percentage of control at 20, 14, and 14 months of ERT respectively (Figure 2; grey crosses, right axis), indicating moderate (patient 1) to no (patients 2 and 3) neutralizing effects. However, at 27 months of ERT, neutralizing effects of antibodies in patient 1 were more pronounced and indicated 60% residual enzyme activity in cell lysates.

Pharmacokinetic profile: effect of antibodies on rhGAA during infusion

To determine the effect of anti-rhGAA antibodies on rhGAA enzymatic activity during ERT, serum samples collected at several time points during infusion were used for immunoprecipitation with protein A-Sepharose, followed by measurement of rhGAA activity in the supernatant (Figure 3A). Sepharose only served as control. The analysis was performed at 20, 14, and 14 months of ERT for patients 1, 2, and 3 respectively. The amount of precipitated alglucosidase alfa activity in serum samples collected during infusion was close to zero in all three patients (Figure 3A), suggesting that protein A-bound alglucosidase alfa was either enzymatically inactive or hardly pres-

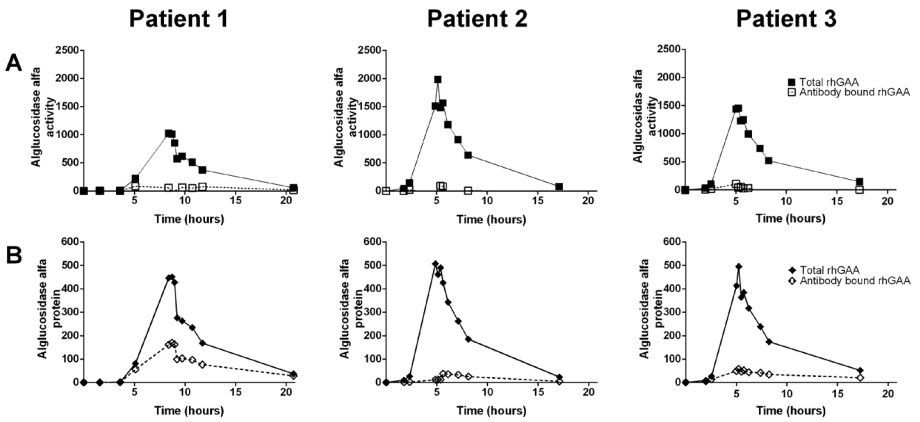
ent. To distinguish between these possibilities, we used mass spectrometry to detect alglucosidase alfa protein independent of enzyme activity, as reported by us recently [25]. Figure 3B shows that the percentages of antibody-bound rhGAA protein were 37.7%, 2.7% and 11.8% in patients 1, 2, and 3 respectively. We conclude that the anti-rhGAA antibodies in these patients preferentially bound to infused rhGAA protein that was enzymatically inactive.

Figure 2. Immunological responses during immunomodulation.



Each column represents a single patient. **Upper row**: Anti-rhGAA antibody titers (black symbols, left Y-axis) and neutralizing effects of anti-rhGAA antibodies (grey crosses on right Y-axis). The intracellular rhGAA activity is determined by incubation of fibroblasts with patient serum plus rhGAA (alglucosidase alfa), and is expressed as percentage of control serum (fetal calf serum). Black asterisk indicates the first and subsequent IAR in patient 1. Dashed grey lines represent background levels of the ELISA. **Second row:** B cells levels; dashed grey lines indicate normal values in serum. **Third row:** Total IgG levels; dashed grey lines indicate normal values in serum. **Bottom row**: Immunomodulation schedule per patient, each line represents treatment duration. In patient 1 and 3 the black asterisk indicates missed MTX injection(s); in patient 2 black asterisk indicates missed RTX infusion.

Figure 3. Pharmacokinetic profile of alglucosidase alfa activity and alglucosidase alfa protein during infusion with ERT.

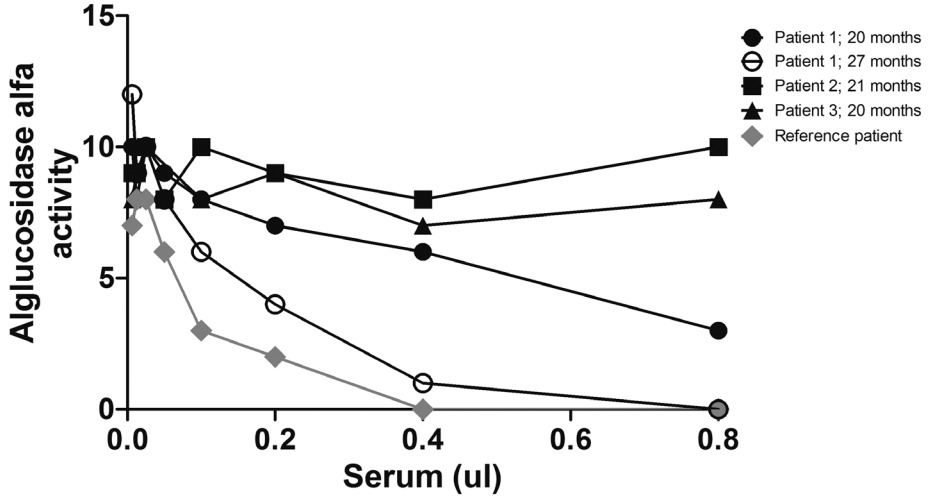


A: Alglucosidase alfa activity measured in serum before and during infusion with rhGAA. Closed symbols represent the total rhGAA enzyme activity. Open symbols represent serum that was first subjected to immunoprecipitation with protein A, which captures rhGAA bound to anti-rhGAA antibodies. The remaining rhGAA enzyme activity in the supernatant was measured and used to calculate the % of antibody bound enzymatically active GAA. Results using protein G were similar.
B: The experiment described in the upper row was now analyzed by mass spectrometry rather than enzyme activity, and reports values for antibody-bound rhGAA protein irrespective of their enzymatic activity. Closed symbols represent the total rhGAA protein in sera; open symbols the antibody-bound rhGAA protein in sera.

Antibody titers using immunoprecipitation

To test the effect of anti-rhGAA antibodies on enzymatic activity in a cell-free assay, we incubated a fixed amount of rhGAA with increasing amounts of patient's serum, followed by immunoprecipitation with protein A and measurement of enzyme activity in the supernatant. Serum from the reference patient with known neutralizing effects of antibodies efficiently precipitated alglucosidase alfa activity (Figure 4; grey line). In contrast, serum from patients 2 and 3 failed to precipitate alglucosidase alfa activity in vitro (Figure 4; squares and triangles). Patient 1 showed moderate capacity to precipitate alglucosidase alfa activity at 20 months of ERT (Figure 4; filled circles). However, when tested seven months later (month 27), serum from patient 1 showed enhanced capacity to precipitate alglucosidase alfa activity (Figure 4; open circles), with an efficiency that was almost as strong as that of the reference patient. We conclude from these data that in patient 1, titers of antibodies with neutralizing effects increased from 20 to 27 months of ERT, while patient 2 and 3 did not show detectable neutralizing antibodies.

Figure 4. Antibody titers determined by immunoprecipitation under cell-free conditions.

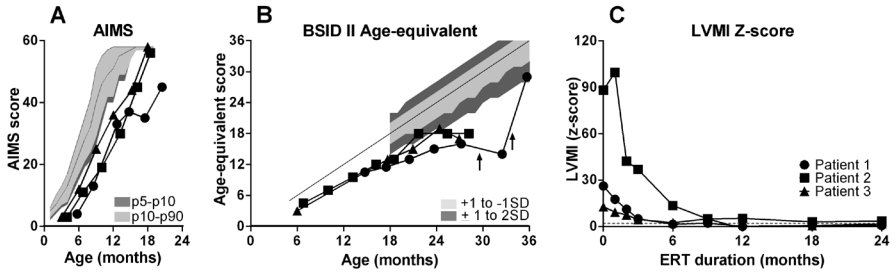


A fixed amount of rhGAA was incubated with various amounts of patient serum *in vitro* in the absence of cells, and anti-rhGAA antibodies were immunoprecipitated with protein A/G. The enzyme activity of rhGAA in the supernatant was used to determine the % of antibody-bound enzymatically active rhGAA (relative to control serum, using the reference patient with known neutralizing antibodies as described by de Vries et al. in 2010). Anti-rhGAA titers at time of analysis were 1: 800,000 (patient 1, both time points); 1: 200,000 (patient 2); and 1: 6,250 (patient 3).

Effects of ERT and immunomodulation on clinical outcome

At study end, all three patients survived ventilator-free. LVMI Z-score decreased significantly in all three patients and normalized completely in patients 1 and 3. It was still slightly elevated in patient 2 at study end (LVMI 87 g/m^2, Z-score 3.5; SD 7.9, Figure 5C) [21], while cardiac dilatation had disappeared and the shortening fraction normalized to 40% (data not shown). Motor function improved. All patients learned to walk independently at ages of 21, 18 and 17 months (patients 1, 2, and 3, respectively), and AIMS and BSID II scores increased accordingly (Figure 5). NG tube feeding could be stopped at ages of 15.8, 10 and 9 months. Oxygen supply and monitoring could be stopped after 3, 13.5 and 6 months of ERT. At the age of 29 months (24 months of ERT), patient 1 temporarily lost the ability to walk after a foot injury (Figure 5B). At the age of 34 months (28 months of ERT) he regained the ability to walk (albeit with lesser stability than before).

Figure 5. Effects of ERT and immunomodulation on clinical outcome.



A Motor function measured by AIMS; **B** Motor function measured by BSID II. Raw scores were translated to age equivalent scores to compare patients. Patient 1 lost the ability to walk at the age of 29 months (first black arrow) and regained this ability at the age of 34 months (second black arrow). **C** LVMI Z-score. Normal values depend on body surface area (BSA) as described by Poutanen et al. For a BSA up to 0.75 normal values are 59.2 g/m^2 with an SD of 7.9 g/m^2.

DISCUSSION

This study was performed to evaluate the effect of immunomodulation on antibody formation in three classic infantile Pompe patients that were naïve to ERT. We found that the immunomodulation protocol induced immune suppression during the first seven months, as evidenced by elimination of B cells and absence of anti-rhGAA antibodies, similar as shown previously [19]. However, while B cell recovery occurred in all three patients after this period, it was accompanied by formation of very high sustained antibody titers in two out of three patients. Patient 1 was the only patient in this study who developed IARs (Table 3), and the majority of IARs occurred when high antibody titers were present, in line with our previous observations [17]. Neutralizing effects of antibodies in vitro were mild to moderate. Clinical response to ERT was evident, as indicated by survival beyond one year of age, normalization of cardiac function, ability to walk, and scores using AIMS and BSID II. We conclude that the protocol for immunomodulation at start of ERT induces a temporarily suppression of antibody formation. Future work should be directed towards the development of protocols that induce sustained immune tolerance.

Overview of literature on antibody formation and clinical outcome

The results of our study and other published studies on primary immunomodulation are summarized in Table 4. Messinger et al. were the first to describe the effects of transient immunomodulation in two ERT naïve patients. One patient remained antibody-free for up to two years following cessation of transient RTX treatment, while the other patient developed a titer of 1:1500 at 7 months, which dropped to 1:400 after 18-24 months [19]. Their group, using a slightly different transient immunomodulation protocol, also reported another patient who had low antibody titer of 1:3,200 at 12 months [29]. On the other hand, a report by Elder et al. found high antibody titers in the first patient five months after receiving transient immunomodulation [30]. This patient died eight months later, and the authors decided to continue RTX treatment in the following four patients to suppress antibody formation. That same year, Banugaria et al. published a decision flow chart in which patients with increasing antibody titers after transient immunomodulation received a second round of immunomodulation, which was applied to two out of seven patients [31]. Broomfield et al. reported on nine patients receiving transient immunomodulation and only one developed an antibody titer [15]. In the present study, two out of three patients with our transient immunomodulation protocol developed very high antibody titers upon B cell recovery. While other reports suggested that transient treatment with RTX plus MTX

may induce long-term immune tolerance [19, 20, 29, 31], our results indicate that this protocol may induce a transient suppression of antibody formation rather than immune tolerance in certain patients. It should be noted that while all of these studies used Rituximab to eliminate B cells, variations existed with respect to additional treatments. For example, the duration and dosage of Methotrexate treatment varied, and in some cases MTX was replaced by Mycophenolate or Sirolimus (Table 4). Also the age at which ERT and immunomodulation was started varied between patients, and ranged between 0.1 to 8 months of age (median 3.1 months). It is unknown to what extent these variations affect the immunomodulatory outcome.

Clinical outcome of previously published patients that received immunomodulation was heterogeneous (Table 4). Of the 24 patients, five patients died (21%), and only six learned to walk (25%). In contrast, in the present study all three patients survived at study end and learned to walk. A likely important factor that contributed to the positive clinical outcome in the present study is the high dosage of rhGAA (40 mg/kg/week), which has previously been shown to have a beneficial effect compared to the standard dosage of 20 mg/kg every two weeks [13]. The patients in the previously published studies received either 20 mg/kg every two weeks or 20 mg/kg/week, Broomfield et al. reported on two patients in whom the dose was increased to 40 mg/kg/week. Another factor to be considered is CRIM status. A negative CRIM status has been associated with a poor prognosis, reduced response to ERT, and tendency to form high antibody titers [10-12]. The majority of published patients in Table 4 were CRIM-negative, in line with the poor clinical outcome of these patients. However, patient 1 of the present study was also CRIM-negative, yet survived and learned to walk, while he started ERT the latest at the age of 5.8 months. Because antibodies in this patient only showed mild to moderate neutralizing effects (discussed below), we hypothesize that the high dosage of rhGAA may have been responsible for his relatively good clinical outcome at study end.

Neutralizing effects of antibodies

Surprisingly, the very high antibody titers detected using ELISA showed mild to moderate (patient 1) to no (patients 2 and 3) neutralizing effects in vitro in cell-based assays (Figure 2), a cell-free assay (Figure 4), and on serum samples collected during infusion with rhGAA (Figure 3A). These assays have been shown to be capable of detecting neutralizing effects of antibodies, as highlighted previously [17, 18].

Whether the immunomodulation protocol has influenced the nature or IgG subclasses of anti-GAA antibodies remains to be investigated in future analysis by comparing sera of patients that underwent immunomodulation or not. It is worrying that in patient 1, neutralizing effects increased from mild (~20%) in the first year to moderate (~40%) around study end (27 months of ERT). A limitation of the present study is the relatively short follow up after cessation of RTX treatment and the low number of patients. Future work should be directed toward longer follow up of formation of neutralizing antibodies and the efficacy of ERT in a larger cohort. To translate neutralizing effects measured in vitro to the situation in patients, one needs to take into account the dosage of ERT. At a higher dosage employed here, it is expected that neutralizing effects will be less severe compared to a regular dosage, as more antibody-free rhGAA will be available.

The inclusion of mass spectrometry as a read out for antibodies revealed a surprise: a considerable % of rhGAA protein was captured by antibodies during infusion in patient 1 (37%), but only a small fraction (2 %) of enzymatically active GAA was captured (comparing Figures 3A and 3B). This suggests that infused rhGAA may undergo denaturation upon prolonged exposure to conditions present in the circulation. In agreement, we found that the half-life of rhGAA enzymatic activity in blood in vitro is only 100 minutes, while full length rhGAA protein remains detectable over prolonged periods [32]. In tissue culture medium and in the cellular fraction we did not find evidence that antibodies from patients promoted degradation of rhGAA, although a larger study is required to fully address this. The ELISA assay cannot distinguish between antibodies directed towards native or denatured proteins, suggesting that ELISA titers per se are insufficient to predict potential deleterious effects of antibodies, and that additional analysis using neutralizing assays are required to more accurately link antibody formation to clinical outcome.

Table 4. Overview of the literature of immunomodulation in classic infantile Pompe patients naïve to ERT

Study	Pt	Allele 1	Allele 2	Age start ERT	Follow up	Alive	Vent. free	Walks
Messinger	3	c.2560C>T	c.2560C>T	16 w	24 m	Yes	Yes	Yes
2012 [19]	4	c.1548G>A	c.525delT	15 w	24 m	Yes	Yes	Yes
Elder	A	c.2560C>T	c.2560C>T	8 m	11 m	No	No	No
2013 [30]	B	c.1396delG	c.1705dup	8 m	36 m	Yes	No[1]	No
	C	c.925G>A	c.925G>A	2.75 m	30 m	Yes	Yes	No
	D	c.1548G>A	not found	6 m	24 m	Yes	Yes	No
	E	c.1933G>A	c.2501_2502del	3 m	22 m	Yes	Yes	No
Banugaria	1	c.2608C>T	c.2608C>T	3.0 m	101 w	Yes	Yes	No
2013 [31]	2	c.546+2T>C	c.546+2T>C	4.1 m	92 w	Yes	NIV	No
	3	c.236_246del	c.236_246del	2.4 m	89 w	Yes	Yes	Yes
	4	c.525delT	c.2560C>T	0.1 m	70 w	Yes	No	No
	5	c.2560C>T	c.2560C>T	0.5 m	59 w	Yes	NIV	No
	6	c.525_526delTG	c.525_526delTG	1 m	51 w	Yes	NIV	No
	7	c.2560C>T	c.2560C>T	1 m	48 w	No	No	No
Stenger 2015 [29]	1	c.2105G>T	c.2512C>T	1.2 m	17 m	Yes	Yes	Yes
Broomfield	6	c.525delT	c.2608C>T	4.7 m	4.2 m	No	No	No
2016 [15]	7	c.2608C>T	c.2608C>T	6.6 m	5.4 m	Yes	Yes	No
	8	c.2237G>A	c.2237G>A	2.4 m	9.6 m	No	No	No
	12	c.2078dup	c.2078dup	6.7 m	8.9 m	Yes	Yes	No
	14	c.2560C>T	c.2560C>T	18 d	17.5 m	No	No	No
	16	c.2237G>A	c.2237G>A	36 w[#]	33 m	Yes	Yes	Yes
	17	c.2560C>T	c.2560C>T	5.2 m	28.4 m	Yes	Yes	Yes
	19	c.525delT	c.2481+102_2646+31del	3.9 m	32.9 m	Yes	NIV	Yes[2]
	22	c.877G>A	c.877G>A	2.2 m	49.4 m	Yes	NIV	No
This study	1	c.525delT	c.525delT	5.8 m	~24 m	Yes	Yes	Yes
2017	2	c.1551+1G>A	c.1551+1G>A	4.2 m	~24 m	Yes	Yes	Yes
	3	c.525delT	c.2481+102_2646+31del	3.1 m	~24 m	Yes	Yes	Yes

IM: immunomodulation; NA: not applicable; ND: not determined; NIV: non-invasive ventilation; RTX: Rituximab intravenous; MTX: Methotrexate.
patient 16 in Broomfield et al was born at 34 weeks gestational age and ERT was initiated at 36 weeks of gestational age.
[1] Patient B became ventilator dependent after follow-up.
[2] Patient 19 lost ability to walk 4 months after gaining it, within follow-up period.
[3] Immunomodulation (IM) treatment used per study:
[4] Ref 15: B cell recovery 4 to 9 months after last RTX; Personal communication, A. Broomfield.
[5] Time after B cell recovery in ref 15 was 4-9 months after last RTX, time given here in age when tested.

IM treatment[3]	IM duration	IM repeat	Time since last RTX	B cell recovery[4]	Peak titer (time after B cell recovery)[5]	Neutralizing effects
1A	5 w	No	30 m	Yes	1:1,600 (2 m)	ND
1A	5 w	No	17 m	Yes	0 (9 m)	ND
2A	5 w	No	10 m	Yes	1:500,000 (5 m)	ND
2B	RTX ongoing	No	<12 w	No	NA	ND
2B	RTX ongoing	No	<12 w	No	NA	ND
2B	RTX ongoing	No	<12 w	No	NA	ND
2C	RTX ongoing	No	<12 w	No	NA	ND
1E	5 w	No	96 w	Yes	0 (81 w)	ND
1E	5 w	No	87 w	Yes	0 (67 w)	ND
1E	5 w	No	84 w	Yes	0 (69 w)	ND
1E	5 w	No	65 w	ND	0 (NA)	ND
1E	5 w	Yes	24 w	No	1:6,400 (before)	ND
1E	5 w	Yes	8 w	No	1:6,400 (before)	ND
1E	5 w	No	43 w	Yes	0 (8 w)	ND
1B	5 w	No	39 w	Yes	1:3,200 (39 w)	ND
1C	5 w	No	~6 m	Yes	0 (>4-9 m)	ND
1C	5 w	No	~3 w	Yes	0 (0.6 y)	ND
1C	5 w	No	~9 m	Yes	0 (>4-9 m)	ND
1C	5 w	No	~16 m	Yes	0 (0.9 y)	ND
1C	5 w	No	~17 m	Yes	0 (>4-9 m)	ND
1C	5 w	No	~31 m	Yes	0 (>4-9 m)	ND
1C	5 w	No	~28 m	Yes	0 (1.8y)	ND
1C	5 w	No	~32m	Yes	1:12,800 (1.3 y)	ND
1C	5 w	No	~49 m	Yes	0 (2.5 y)	ND
1D	MTX ongoing	No	23 m	Yes	1:800,000 (6.3 m)	mild/ moderate
1D	MTX ongoing	No	23 m	Yes	1:6,250 (7.3 m)	No
1D	MTX ongoing	No	23 m	Yes	1:200,000 (6.3 m)	No

1A RTX 375 mg/m^2/dose for 4 doses; MTX 0.4 mg/kg/dose subcutaneous for 9-17 doses; IVIG 500 mg/kg administered once in patient 1.
1B RTX 375 mg/m^2/dose for 4 doses; MTX 15 mg/m^2/dose oral for 9 doses; IVIG 400-500 mg/kg twice.
1C RTX 375 mg/m^2/dose for 1-4 doses; MTX 0.4 mg/kg/dose subcutaneous for 10-18 doses.
1D RTX 375 mg/m^2/dose for 4 doses; MTX 1 mg/kg/week intravenous for the duration of follow up; IVIG 400 mg/kg monthly.
1E RTX 375 mg/m^2/dose for 4 doses; MTX 0.4 mg/kg/dose subcutaneous for 9 doses; IVIG 400-500 mg/kg 5-6 times.
2A RTX 750 mg/m^2/dose for 2 doses; Mycophenolate 300 mg/m^2/day oral; IVIG 500-1000 mg/kg monthly.
2B RTX 375 mg/m^2/dose for 3 doses + repeat RTX every 12 weeks; Sirolimus 0.6-1 mg/m2/day oral; IVIG 500-1000 mg/kg monthly.
2C RTX 750 mg/m^2/dose for 2 doses+ repeat RTX every 12 weeks; Sirolimus 0.6-1 mg/m2/day oral; IVIG 500-1000 mg/kg monthly.

CONCLUSIONS

While immunomodulation of ERT naïve Pompe patients using a combination of Rituximab, Methotrexate, and IVIG has been reported to be associated with prevention of antibody formation[19, 20], we showed that this treatment induces immune suppression during B cell depletion rather than tolerization in 2 out of 3 patients in our study. It will be important to optimize protocols that can provide long-term prevention of antibody formation without compromising general immunity in Pompe patients treated with ERT.

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Chapter 4

Effects of immunomodulation in classic infantile Pompe patients with high antibody titers

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ABSTRACT

Background

To evaluate whether immunomodulation can eliminate high sustained antibody levels, and thereby improve clinical outcome in classic infantile Pompe patients receiving enzyme replacement therapy (ERT) with recombinant human alpha-glucosidase (rhGAA).

Methods

Three patients (two cross-reactive immunologic material (CRIM) negative) with high sustained antibodies received a three-week treatment protocol with Rituximab and Bortezomib, followed by daily Rapamycin and monthly IVIG. Patients received 40mg/kg/week rhGAA. Antibody titers were measured using ELISA. Neutralizing effects on cellular uptake were determined. Clinical efficacy was measured in terms of (ventilator-free) survival, reduction in left ventricular mass index (LVMI) and improvement in motor function.

Results

Before immunomodulation anti-rhGAA antibody titers ranged from 1:156,250 to 1:781,250 and at last assessment from 1:31,250 to 1:156,250. Neutralizing effects of anti-rhGAA antibody titers (observed in two patients) disappeared. Infusion-associated reactions were no longer present. Immunomodulation resulted in substantial increases of aspartate transaminase, alanine transaminase, and creatine kinase levels. The two CRIM-negative patients who could walk at start maintained their ability to walk; the patient who had lost this ability did not regain it.

Conclusions

Immunomodulation protocol used in our study reduced antibody titers to some extent, but did not eliminate them. We observed some positive impact on the clinical stability. We speculate that the high dose of ERT applied from start was the main reason for the good outcome of our CRIM-negative patients.

INTRODUCTION

Pompe disease (Glycogen Storage Disease type II, OMIM #232300), an autosomal recessive lysosomal storage disorder caused by deficiency of acid-α-glucosidase (GAA), results in lysosomal glycogen accumulation in all cell types, but mainly in muscle cells [1]. Pompe disease presents as a spectrum of clinical phenotypes, the classic infantile form being the most severe form [2]. Classic infantile patients have less than 1% of enzyme activity in fibroblasts and present with hypertrophic cardiomyopathy (HCM), progressive generalized muscle weakness, and respiratory difficulties. Without treatment, patients die within the first year of life due to cardiorespiratory failure [3-5].

Enzyme replacement therapy (ERT) with recombinant human alpha-glucosidase (rhGAA, alglucosidase alfa, Myozyme) has improved prognosis for patients by improving survival and improving motor outcome. [6, 7]. Clinical response varies greatly between patients [6, 8-11]. A higher dose of ERT has been shown to positively influence patients' outcome [6, 12, 13]. Antibodies to rhGAA may counteract positive effects of ERT by neutralizing its activity or preventing cellular uptake [12, 14-16]. Cross-reactive immunologic material (CRIM negative) patients, i.e. who produce inactive GAA protein, are more likely to form higher anti-rhGAA antibodies titers than CRIM-positive patients. Generally, CRIM-negative patients have been reported to respond poorly to ERT [14, 16-18]. Despite reports that antibody formation can be prevented successfully by primary immunomodulation (i.e. using a combination of Rituximab (RTX), Methotrexate (MTX) and intravenous immunoglobulin (IVIG) before the first ERT dose) [8, 19-21], some patients still develop high anti-rhGAA antibody titers [21-23].

There have also been attempts at secondary immunomodulation, i.e. eliminating anti-rhGAA antibodies in patients who have developed high titers during ERT. The best reported results involved a protocol using Bortezomib, a proteasome inhibitor that induces apoptosis in plasma cells by dysregulating signaling cascades [24]. So far, the data that has been published is on very few patients, whose outcome also vary [19, 20, 25-28].

We studied the effects of secondary immunomodulation on anti-rhGAA antibody titer formation, the cellular uptake of GAA, the depletion and repopulation of B cells, and clinical outcome using a protocol combining Rituximab, Bortezomib, Rapamycin and IVIG.

METHODS

Patients and treatment protocol

We included three patients with classic infantile Pompe disease with high sustained anti-rh-GAA antibodies. Two patients did not receive immunomodulation previously, while patient 2 did [23]. Classic infantile Pompe disease was defined as symptoms of muscle weakness within 6 months after birth, the presence of HCM, complete deficiency of α-glucosidase in fibroblasts (<1% of normal values), and two very severe mutations in the GAA gene. Patients were already participating in an ongoing study into the effects of ERT, study protocols had been approved by the Institutional Review Board. Written informed consent was obtained from the parents. ERT was dosed at 40 mg/kg/week.

Immunomodulation protocol

Our immunomodulation protocol for patients with high titers was derived from protocols published by Messinger et al., Banugaria et al. and Elder et al. [19, 22, 24]. The following immunomodulation regimen was applied: 3 weekly infusions of RTX 375mg/m^2; 6 twice-weekly doses of Bortezomib 1.3 mg/m^2; monthly IVIG (first dose 1.0 g/kg; subsequent doses of 0.5 g/kg); Rapamycin was commenced at week 4 (10 - 20 kg 1.0 - 1.5 mg/day; 20-30 kg 1.5 - 2.0 mg/day; double dose on first day of Rapamycin treatment). Dose was adjusted on the basis of serum Rapamycin levels (normal range 4-12 µg/l). To reduce the risk of infections, all patients received Azithromycin prophylactically. Regular blood analysis consisted of determining the number of B cells and the levels of aspartate transaminase (AST), alanine transaminase (ALT), creatine kinase (CK), and gamma globulin (IgG, IgM, IgA).

Antibody titer and neutralizing effects

Blood samples were drawn at regular intervals and stored at -80°C until analysis. Anti-rh-GAA antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) as described earlier [16, 17]. Experiments were performed in duplicate and assays were repeated at least twice. Our figures present the highest titers measured. The neutralizing effects of antibodies were determined at least twice per patient by studying their effect on cellular uptake in vitro [16, 17].

Clinical outcome measures

Standardized assessments were performed at start of ERT and every three months thereafter [6], and also at start of immunomodulation. Clinical outcome parameters were (ventilator-free) survival, left ventricular mass index (LVMI, where an LVMI Z-score ≥ +2SD was defined as abnormal); pulmonary function tests; and motor function assessed by Alberta Infant Motor Scale (AIMS); Bayley Scales of Infant Development II (BSID-II); 10-meter run test, and 6-minute walk test (6MWT). Infusion-associated reactions (IARs) were recorded [29-34].

CASE REPORTS

Patient 1.
Patient 1 (CRIM-positive) started ERT at 2.4 months (table 1). At birth she had presented with persistent tachypnea and HCM attributed initially to gestational diabetes in her mother. At time of diagnosis she had severe hypotonia and a prominent head lag. When prone she could not lift her head from the surface and anti-gravity movements of the limbs were not observed (AIMS score was 1). She was still able to drink (weight 6.5 kg +2.0 SD for Dutch children) and did not require nasogastric tube (NGT) feeding. During ERT, LVMI normalized, with the LVMI z-score decreasing from 22.5 to 1.75 during the first 9 months of ERT (figure 3E). She learned to walk unsupported at the age of 15 months. At the age of 2.5 years she developed a transient right-sided facial nerve palsy elicited by a herpes simplex viral infection. From then on, she experienced frequent airway and urinary tract infections, accompanied by transient periods of poorer motor functioning. She maintained the ability to walk until the age of 6 years. From the age of 6.1 years, motor function started to decline. At the age of 6.4 years she was no longer able to stand unsupported.

Patient 2.
Patient 2 (CRIM-negative) started ERT at 5.8 months (table 1). At 5 months he was hospitalized due to feeding difficulties and muscle weakness accompanied by HCM. At time of diagnosis he was able to lift his limbs from the surface, but could not roll over. Due to insufficient oral intake, NGT feeding was started. Primary immunomodulation was started before the first ERT dose [23]. During ERT, LVMI normalized, with the LVMI z-score decreasing from 26.1 to 1.4 during the first 6 months of ERT (figure 3E). NGT feeding could be stopped at age of 21 months. He learned to walk unsupported at 21 months. After a fall he lost the ability to walk for 4 months (age 2.5 years), but then regained it without intervention. Due to the concerns raised by his quality of movement and the reoccurrence of IARs, a second round of immunomodulation was initiated.

Patient 3.
Patient 3 (CRIM-negative) started ERT at 1.9 months (table 1). There were feeding difficulties from birth onwards. Shortly after a chest X-ray at the age of 5 weeks had revealed HCM, she was diagnosed with Pompe disease. At time of diagnosis she could lift her limbs from the surface, could turn her head when in prone position, and could take some support on her legs. Due to insufficient intake, an NGT was placed. After start of ERT, LVMI normalized, with LVMI z-score decreasing from 17.8 (LVMI 200 g/m^2) to 0.1 during the first 9 months of ERT (figure 3). NGT feeding could be stopped at the age of 9 months. She learned to walk unsupported at the age of 11 months, and obtained the maximum AIMS score of 58 at the

age of 12 months (figure 3). After her first birthday, she gradually started to perform more poorly than age-related peers (BSID II scores, figure 3E). She also developed a Gower's sign and her calves became hypertrophic. LVMI increased slightly (LVMI 80.7 g/m^2, z-score 2.7) without functional consequences.

Table 1. Patient Characteristics

	Patient 1	Patient 2^	Patient 3
Baseline and initial response			
Age at start (in months)	2.4	5.8	1.9
Mutations	c.2481+102_2646+31del538 c.2481+102_2646+31del538	c.del525T c.del525T	c.del525T c.del525T
CRIM status	Positive	Negative	Negative
Ventilatory support	No	No	No
LVMI at start in g/m2 (z-score)	237 (22.5)	265 (26.1)	200 (17.8)
Time to LVMI normalization (z-score)	9 months (1.75)	6 months (1.43)	9 months (0.1)
Age pull to stand (in months)	11.6	14.8	9.2
Age walking (in months)	15	21.3	11.7
NGT at start	No	Yes	Yes
Age at which NGT ended (in months)	N.A	21	9
Total number of IARs (total severe IARs)	70 (6)	22 (5)	16 (0)
Age at last IAR (in years)	4.0	3.5	2.1
At start of secondary immunomodulation			
Age in years	6.6	3.5	2.3
Ventilatory support	No	No	No
LVMI in g/m2 (z-score)	70.6 (0.4)	63.2 (0.5)	83.9 (3.1)
Best motor function	Sitting	Walking	Walking
Antibody titer	1:156,250	1:156,250	1:781,250
Enzyme activity in cell lysates	50%	60%	100%
At study end			
Age in years	9.1	5.6	3.8
Ventilatory support	No	No	No
LVMI in g/m2 (z-score)	82.5 (1.3)	65 (0.7)	55 (-0.5)
Best motor function	Sitting	Walking	Walking
NGT/PEG (age in years)	Yes (7.0)	No	No
Last antibody titer (time since last RTX in years)	1:31,250 (0.5)	1:31,250 (2)	1:1561,250 (1.5)
Enzyme activity in cell lysates	100%	100%	100%
B-cell normalization / time since last RTX in months	Yes / 14 No / 5$^{\$}$	Yes / 6	Yes / 3
Last B-cell level*	0	0.85*10^9/L	0.48*10^9/L
IARs since start of immunomodulation	No	No	No

CRIM: cross-reactive immunologic material; LVMI: left ventricular mass index; NGT: nasogastric tube; IAR: infusion-associated reaction; PEG: percutaneous endoscopic gastrostomy tube; RTX: Rituximab.
^ Patient 2 initially received immunomodulation in an ERT naïve setting. *B- cell normal range; for age 2-5 years normal range of 0.2-2.1*10E9, for age 5-10 years normal range of 0.2-1.6*10E9 $^{\$}$After an initial round of immunomodulation patient 2 received a second round 2 years later because of high rhGAA antibodies

RESULTS

Effects of immunomodulation on B cells

After RTX treatment B cells became depleted in all patients (table 1). In patients 1, 2 and 3 time to B cell recovery was 1.2 years, 6 and 3 months, respectively. During B cell depletion all patients received IVIG. Patient 1 who received the first round at the age of 6.6 years received a second round of immunomodulation at 8.5 years. At study end there was no B cell recovery.

Anti-rhGAA antibody titers before and after immunomodulation

Anti-rhGAA antibody titers are shown in figures 1 (from start of ERT) and 2 (after immunomodulation). In patient 1, anti-rhGAA antibodies were first detected at one month of ERT (titer 1:250, figure 1). This increased to a maximum titer of 1:31,250, which was maintained between the ages of 0.4 and 6.2 years. At start of secondary immunomodulation at 6.4 years, her titer was 1:156,250. In patient 2, who had started primary immunomodulation before start of ERT, anti-rhGAA antibodies were first detected at 5.5 months of ERT (titer 1:1,250). These increased to the highest maximum titer of 1:800,000 between the ages of 1.6 and 2.7 years [23]. This patient had received MTX in a dose of 0.5 mg/kg/week until 5 days before start of secondary immunomodulation. At start of secondary immunomodulation at 3.5 years, his titer was 1:156,250. In patient 3, anti-rhGAA antibodies were first detected at one month of ERT (1: 31,250). These increased to a titer of 1:781,250, which was maintained until start of secondary immunomodulation at the age of 2.3 years.

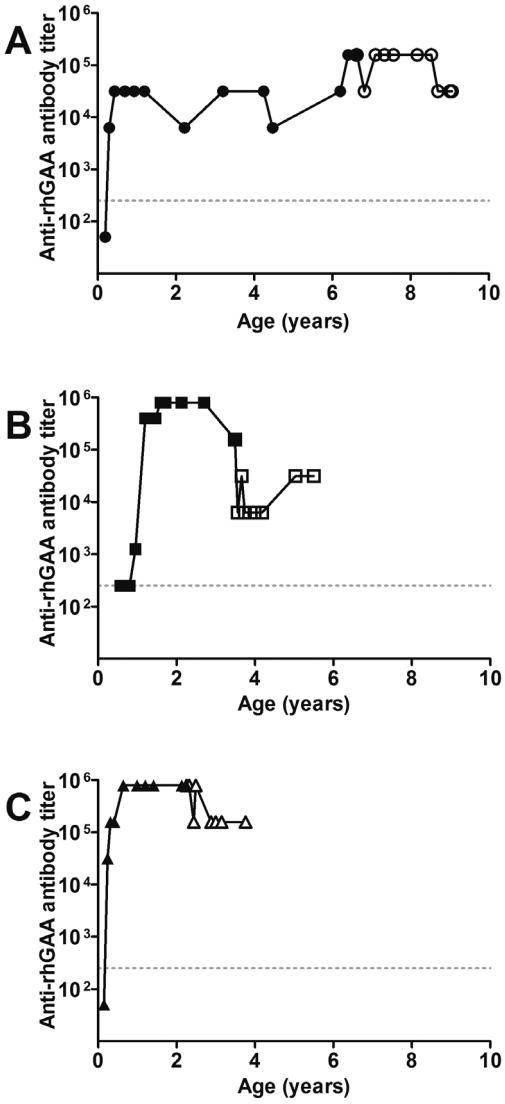
The immunomodulation schedule per patient is shown in figure 2, as well as the effects on anti-rhGAA antibody titer. In patient 1 anti-rhGAA antibody titers decreased from 1:156,250 to 1:31,250 one month after start of immunomodulation, but rose again to 1:156,250 a month later. The high sustained titers were the reason for starting a second round of immunomodulation 2 years later. Titers decreased to 1:31,250. In patient 2, anti-rhGAA antibody titers decreased from 1:156,250 to 1:6,250; his last titer was 1:31,250. In patient 3, anti-rhGAA antibody titers decreased from 1:781,250 to 1:156,250.

Neutralizing effects of antibodies

To test for neutralizing effects of anti-rhGAA antibodies, GAA-deficient fibroblasts were incubated with alglucosidase alpha plus patients' serum. Enzyme activity was measured in medium and fibroblast cell lysates (figure 2). In patient 1, no neutralizing effects of anti-rhGAA antibodies had been observed at the ages of 0.4 and 3.2 years (activity in cell lysates 100% and 83% compared to the activity in controls [16]). At age 6.4 years enzyme activity in cell lysates was 49.7%. In patient 2, enzyme activity in cell lysate decreased from 79.8% at the age of 2.1 years to 60% at the age of 2.7 years [23]. In patient 3 enzyme activity in cell lysate was 93.7% at age of 2.1 years.

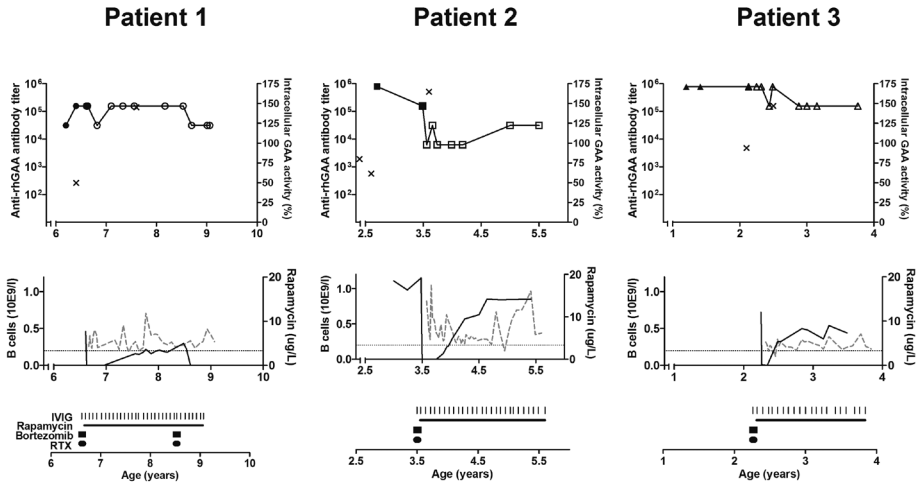
Figure 2 shows the effects of immunomodulation on neutralizing effects. At the ages of 7.6 years (patient 1), 3.6 years (patient 2) and 2.5 years (patient 3) no neutralizing effects of anti-rhGAA antibodies were observed (enzyme activity in cell lysates of 145%, 165%, 147%).

Figure 1. Anti-rhGAA antibody titers



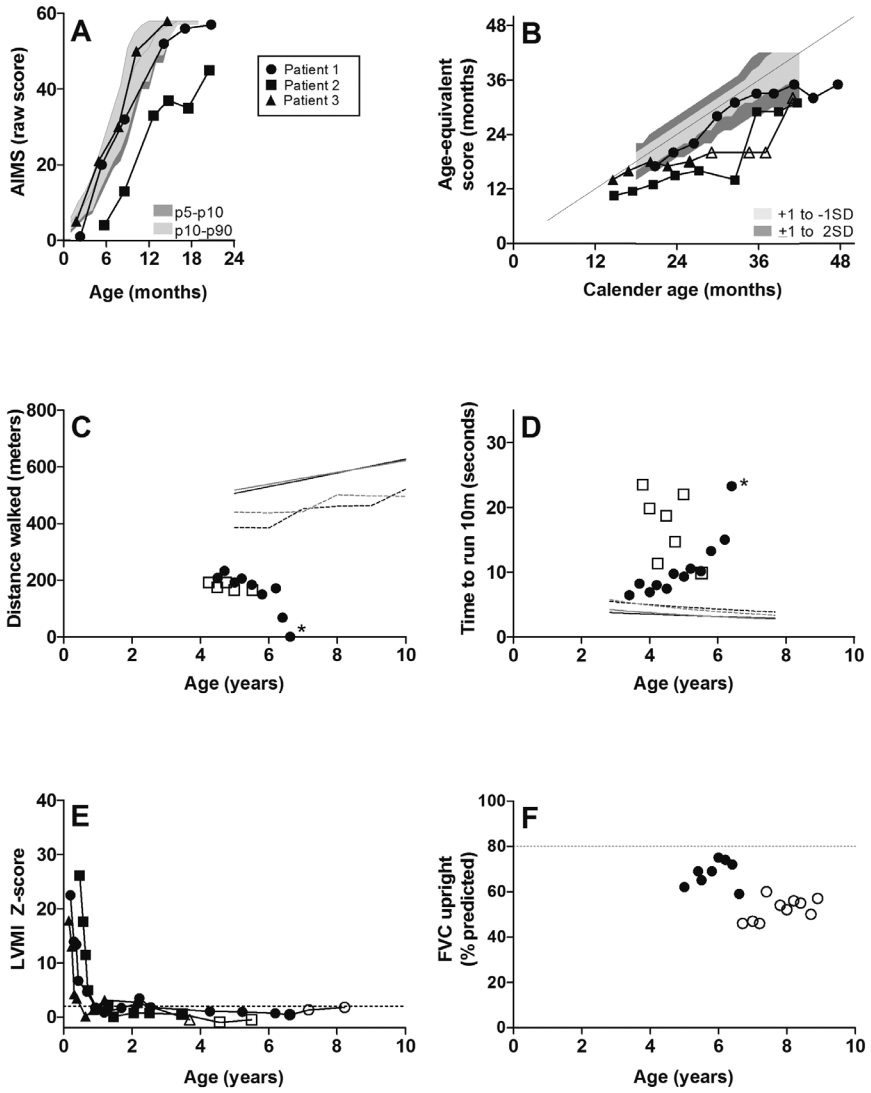
Anti-rhGAA antibody titers per patient during follow-up. Panel A is patient 1. Panel B is patient 2. Panel C is patient 3. Anti-rhGAA antibody titers before immunomodulation are shown as closed symbols. The open symbols represent titers after immunomodulation.

Figure 2. Effects of immunomodulation



Each column represents a single patient. **Upper row:** Anti-rhGAA antibody titer in detail after immunomodulation, as shown previously in figure 1 (line with symbols on the left axis), and neutralizing effects of anti-rhGAA antibodies (crosses, on the right axis). **Middle row:** Serum B cell levels per patient (black line on the left axis) and Rapamycin serum levels per patient (grey dashed line on the right axis). Dotted grey line represents the lower level of normal for B cells for age, which is 0.2*10E9/l. **Bottom row:** Immunomodulation treatment per patient. Verticals stripes represent each individual IVIG infusion. Horizontal line represents the period in which Rapamycin taken. Squares represent each cycle of 6 Bortezomib infusions. Circles represent each cycle of 3 Rituximab infusions.

Figure 3. Clinical outcome



All closed symbols represent measurements taken before secondary immunomodulation; all open symbols represent measurements taken after immunomodulation. **A.** AIMS score per patient during follow-up. Grey areas represent normal values. **B.** BSID II age-equivalent score during follow-up. **C.** Distance walked during 6-minute walk test in patients 1 and 2, patient 3 is too young to perform the 6MWT. Patient 1 lost the ability to walk at the age of 6.6 years (marked with asterisk). **D.** Time to run 10 meters in patients 1 and 2. Patient 1 lost the ability to walk at the age of 6.1 years (marked with asterisk). **E.** LVMI Z-score during follow-up. **F.** FVC % of predicted relate to age in patient 1. Patients 2 and 3 are too young to perform pulmonary function test.

Clinical outcome measures

At study end, all patients were alive, none required ventilatory support (table 1), and LVMI was within normal limits (figure 3E). Patient 1 did not regain the ability to walk (figure 3C); patients 2 and 3 maintained the ability to walk. For patient, 2 the distance walked during the 6MWT remained stable over a period of 1.5 years, and the time needed to run ten meters improved (figure 3D). For patient 3 BSID II scores were within normal limits at the end of the study period (at the age of 3.8 years); before, they had been slightly lower. Pulmonary function tests could be performed only in patient 1 (figure 3F): before immunomodulation, the percentage of predicted of the forced vital capacity (FVC) had ranged between 59% and 75%, after immunomodulation it ranged from 46% and 60%.

Safety and effects on IARs

Before start of secondary immunomodulation, all patients had experienced IARs (table 1). After the start of secondary immunomodulation, no IARs were observed. Immunomodulation was well tolerated. None of the patients experienced serious infectious diseases. Mild erythema was observed at the Bortezomib injection site in all patients. No other adverse events were reported. At last observation, all patients were still receiving daily Rapamycin and monthly IVIG. Over the course of two to three months after the start of secondary immunomodulation, AST, ALT and CK levels all increased in the three patients, the increase in CK levels being the most prominent (from 842 to 3175 U/l (patient 1); from 1537 to 2953U/l (patient 2); and from 3451 to 5363 U/l (patient 3)). At study end, CK levels had not yet reached pre-immunomodulation levels. The increase in CK levels remained unexplained.

DISCUSSION

In this study, we evaluated the effect of a secondary immunomodulation protocol using Rituximab, Bortezomib, Rapamycin and IVIG, to improve or stabilize clinical outcome of three classic infantile patients with high sustained antibody titers. We observed B cell depletion and recovery in all three patients. Before immunomodulation anti-rhGAA antibody titers ranged from 1:156,250 to 1:781,250. At last assessment titers ranged from 1:31.250 to 1:156.250. Thus, secondary immunomodulation did not eliminate anti-rhGAA antibody titers. The neutralizing effects of anti-rhGAA antibodies that were observed in two patients before start of immunomodulation disappeared. Before secondary immunomodulation, all patients had experienced IARs; after none did so. Noteworthy, none experienced serious infectious diseases during immunomodulation. We speculate that IVIG administrations may have contributed to the low infection rate.

It was difficult to fully judge the effect of immunomodulation on clinical outcome parameters. The two CRIM-negative patients who could walk at start maintained this ability. The patient who had lost this ability did not regain it. We observed some positive impact on the clinical stability.

Overview of literature on secondary immunomodulation: effect on antibodies

To compare our results with the other patients with high titers receiving immunomodulation reported in the literature [19, 20, 25-28] we have summarized all reported data (8 patients) in table 2. The first to publish their results were Messinger et al., who reported on two CRIM-negative patients receiving secondary immunomodulation with RTX, MTX and IVIG [19]. These patients' anti-rhGAA antibody titers were substantially lower than those in our patients (maximum titer 1:12,800). The patients remained antibody-free after B cell recovery.

Subsequently Kazi et al. and Stenger et al. [20, 28] reported on the addition of Bortezomib to the Messinger protocol, they treated three patients with high anti-rhGAA antibody titers (1:200,000 – 1:819,200). Three patients had a decline in titers or were antibody-free at their last assessment (0-1:1,200) [20, 28], the fourth patient, who received additional Cyclophosphamide instead of Bortezomib, continued to have high titers (increase from 1:25,600 to 1:204,800; 1:102,400 at last assessment) [25]. Markic et el. and Deodato et al. applied slightly different protocols in two patients (one CRIM-negative and one CRIM-positive) with low anti-rhGAA antibody titers (1:6,400 and 1:3,200). After B cell recovery titers remained low to undetectable [26, 27].

In our study, anti-rhGAA antibody titers in one CRIM-negative patient (patient 2) decreased substantially. Previously, he had received primary immunomodulation that had no effect on antibody titers [23]. When we decided that the same patient should start secondary immunomodulation at the age of 3.5 years, titers were 1:800,000. An additional sample taken at the actual start of secondary immunomodulation was slightly lower (1:156,250) and declined further to 1:6,250. It is unclear whether the decline in titers was due entirely to immunomodulation, or whether a decline was already in progress. At last assessment, the titer had increased to 1:31.250. We observed limited effects on anti-rhGAA antibody titer in our two other patients. We conclude that the overall effect of secondary immunomodulation in our study was more limited than the effect of secondary immunomodulation in the other reports.

Possible consequences of the different immunomodulation protocols used

It must be noted that different secondary immunomodulation protocols were used, and that it is not yet clear how the differences between them may explain the differences in anti-rhGAA antibody formation and elimination. Seven of the eight patients reported in the literature received an initial round of weekly RTX infusions [19, 20, 25, 27, 28]; in six of these patients continued to receive repeated RTX infusions every four to 12 weeks thereafter to a maximum of 52 doses [19, 20, 27, 28]. RTX is a chimeric monoclonal antibody that induces apoptosis of CD20-expressing B cells, but does not eliminate memory B cells. To eliminate memory B cells, Bortezomib was added to the protocol, with Rapamycin to modulate T cell responses and IVIG to overcome the period of immunoglobulin depletion. In addition, Rapamycin may have an impact on glycogen storage by influencing the mTOR pathway and inhibition of glycogen synthase [35]. It is possible that longer and/or more frequent dosing of RTX, Bortezomib could be more effective in preventing immune responses.

We also conclude that there are differences in the definition of "what is a high titer" and when to start immunomodulation. In our earlier studies we did not find inhibitory effects in patients with titers below 1:31,250 [16]. Future research should seek to identify the most successful secondary immunomodulation protocol in patients with high sustained titers.

Overview of the literature on secondary immunomodulation: clinical outcome

As table 2 shows, there are wide variations between the clinical outcome reported for patients receiving secondary immunomodulation. While seven of the eight patients reported in the literature (87.5%) were alive at study end, only one (12.5%) remained ventilator-free and learned to walk (patient 2 of Messinger et al.). This patient was the youngest at start of ERT (age 16 days) and was receiving 40 mg/kg/week of ERT at study end.

Table 2. Overview of the literature on immunomodulation in classic infantile Pompe patients after antibodies have formed

Study	Pt	CRIM	Age at start of ERT	Age at start of IM	IM protocol	IM duration	Current ERT dose[1]	Follow-up since start of IM
Messinger 2012 [19]	1	Neg	7 w	0.5 y	1	40 m	20 eow	4.6 y
	2	Neg	16 d	2 m	1	IVIG ongoing	40 w	3 y
Banugaria 2012 [24]	1	Neg	4.2 m	8.8 m	2	RTX twice	variable	2.5 y
Markic 2013 [26]	1	Pos	5 m	17.5 m	1	46 w	20 eow	3 y
Deodato 2013 [25]	1	Neg	7 m	13 m	3	3 w	20 eow	22 m
Stenger 2015 [20]	1	Pos	23 d	11 m	4	Ongoing	20 eow	13 m
Kazi 2016 [27]	1	Pos	6.0 m	2.4 y	5A	3 y	40 w	5.5 y
	2	Neg	4.2 m	2 y	5B	Ongoing	40 eow	6.9 y
This study 2017	1	Pos	2.4 m	6.6 y	6	Rap/IVIG ongoing	40 w	2.5 y
	2	Neg	5.8 m	3.5 y	6	Rap/IVIG ongoing	40 w	2.1 y
	3	Neg	1.9 m	2.3 y	6	Rap/IVIG ongoing	40 w	1.5 y

[1] Excluding Banugaria 2012 (one patient) and the patients in our study, all patients started ERT dosed at 20 mg/kg every other week. [2] Patient did learn to walk, but lost the ability at the age of 6 years. [3] Titer was previously 1:25,400. [4] Titer was previously 1:800,000. Pt: Patient; CRIM: cross-reactive immunologic material; Pos: Positive; Neg: Negative; ERT: enzyme replacement therapy; w: weeks; m: months; y: years; IM: immunomodulation; eow: every other week; RTX: Rituximab; Vent. free: ventilator-free survival; MTX: Methotrexate; IVIG: intravenous immunoglobulin. Rap: Rapamycin

	Alive	Vent. free	Walks at study end	Titer at start of IM	Number of RTX infusions	B cell recovery	Last known titer (time since B cell recovery)
	Yes	No	No	1:1,600	15	Yes	0 (20 m)
	Yes	Yes	Yes	1:12,800	14	Yes	0 (10 m)
	No	No	No	1:25,600	6	No	1:102,400 (before recovery)
	Yes	No	No	1:6,400	8	Yes	0 (unknown)
	Yes	No	No	1:3,200[3]	1	Yes	1:100 (16 m)
	Yes	No	No	1:200,000	11	No	1:1,200 (no recovery)
	Yes	No	No	1:204,800	19	Yes	0 (2.5 y)
	Yes	No	No	1:819,200	52	Yes	0 (4 w)
	Yes	Yes	No[2]	1:156,250	3	No	1:31,250 (before recovery)
	Yes	Yes	Yes	1:156,250[4]	3	Yes	1:31,250 (2 y)
	Yes	Yes	Yes	1:781,250	3	Yes	1:156,250 (1.5 y)

Immunomodulation (IM) protocol used per study:

1 RTX 375 mg/m^2/dose for 4 weekly iv doses followed by maintenance doses; MTX 0.5 mg/kg weekly oral doses; IVIG 500 mg/kg/month

2 Cyclophosphamide 15mg/kg iv on day 1 followed by 2mg/kg/day iv for 9 days, IVIG 400 mg/kg day 5 through 9; Plasmapheresis day 1, 3 and 5 in week 20, 34 and 56. Between week 34 and 56 oral Cyclophosphamide 2 mg/kg was given. Followed by iv RTX 375 mg/m^2/week in weeks 99 through 102 and in weeks 140 and 141.

3 Plasmapheresis on days 1, 3 and 5. RTX 375 mg/m^2 iv once on day 7, directly followed by IVIG (dose not mentioned), with 4 extra IVIG doses over the following 8 months.

4 RTX 375 mg/m^2/dose iv followed by 10 maintenance doses; Bortezomib 1.3mg/m^2/dose in 2 sessions of 4 iv doses. MTX 0.5 mg/kg for 27 oral doses; IVIG 500 mg/kg for 5 doses.

5A Cyclophosphamide 250mg/m^2 iv twice; RTX 375 mg/m^2/dose in 2 sessions of 4 doses followed by 11 maintenance doses; Bortezomib 1.3mg/m^2/dose in 3 sessions of 4 iv doses; MTX 15mg/m^2 oral doses; IVIG 400-500mg/kg/month

5B RTX 375 mg/m^2/dose for 4 iv doses followed by RTX maintenance doses 70 weeks later; Bortezomib 1.3mg/m^2/dose in 4 sessions of 4 iv doses; MTX 15mg/m^2 oral doses; IVIG 400-500mg/kg/month

6 RTX 375 mg/m^2/dose for 3 iv doses; Bortezomib 1.3mg/m^2/dose for 6 iv doses. Rapamycin daily according to body weight from week 4 onwards; IVIG 500 mg/kg/month

In our patients, the overall clinical outcome was better. Despite the development of high anti-rhGAA antibody titers, both of our CRIM-negative patients learned to walk. After start of secondary immunomodulation – which had been initiated due to a decline in the quality of movements – one patient improved on time tests, and the other, at the age of 3.8 years performed within normal limits of the BSID II, even though she had previously shown some deviation. The CRIM-positive patient, who had lost the ability to walk, stabilized.

The overall good clinical outcome in our CRIM-negative patients may be explained by the higher ERT dose of 40 mg/kg/week. With a higher dosage, more antibody-free rhGAA should be available, and the neutralizing effects of the same titer are likely to be less severe than in patients receiving 20 mg/kg every other week.

In conclusion, immunomodulation protocol used in our study reduced antibody titers to some extent, but did not eliminate them. None of the patients experienced serious infections and occurrence of IARs disappeared. Increases in CK levels remained unexplained. We noticed some positive effects on the clinical stability. The higher dose of ERT used in our study seems to have made an important contribution to the better clinical outcome of our CRIM-negative patients.

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Chapter 5

Effects of higher and more frequent dosing of ERT and immunomodulation on long-term clinical outcome of classic infantile Pompe patients

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ABSTRACT

Background

To compare the long-term outcome of classic infantile Pompe patients treated with 20 mg/kg alglucosidase alfa every other week (eow) to those treated with 40 mg/kg/week, and to study the additional effect of immunomodulation.

Methods

Six patients received 20 mg/kg eow and 12 received 40 mg/kg/week. Five patients were CRIM-negative, two in the 20mg, three in the 40mg group. We compared (ventilator-free) survival, motor outcome, IARs and antibody formation.

Results

Survival was 66% in the 20mg group and 92% in the 40mg group. Ventilator-free survival was 50% and 92%. Both CRIM-negative patients in the 20mg group died, whereas all three are alive in the 40mg group. In the 20mg group 67% learned to walk compared to 92% in the 40mg group. At the age of 3 years 33% and 92% were able to walk. Peak antibody titers ranged from 1:1,250 to 1:31,250 in the 20mg group and from 1:250 to 1:800,000 in the 40mg group. Five patients of the 40mg group of whom two CRIM-negative also received immunomodulation (Rituximab, Methotrexate and IVIG) in an ERT-naïve setting. Immunomodulation did not prevent antibody formation. All patients receiving immunomodulation were alive and able to walk at study end.

Interpretation

This study shows that classic infantile patients treated with 40 mg/kg/week have a better (ventilator-free) survival and motor outcome. All three CRIM-negative patients in the 40mg group were alive and persistent walkers compared to none in 20mg group. Immunomodulation did not prevent antibody formation in our study.

INTRODUCTION

Intravenous enzyme replacement therapy (ERT) with recombinant human enzymes is the treatment of choice for several lysosomal storage disorders (LSDs) [1]. Among them is Pompe disease (Glycogen Storage Disease type II, OMIM: #232300), an autosomal recessive lysosomal storage disorder caused by deficiency of the enzyme acid-α-glucosidase (GAA) [2, 3]. The disease spectrum is broad. The most severe classic infantile form presents shortly after birth with a hypertrophic cardiomyopathy and generalized muscle weakness. Without therapy patients die within the first year of life [4, 5]. Major motor milestones like walking are not achieved, GAA deficiency is profound (<1%) and the *GAA* variants are very severe [3]. One third of the patients are cross-reactive immunologic material (CRIM) negative and do not produce any alpha-glucosidase protein. The alpha-glucosidase protein produced by CRIM-positive patients is not adequately transported to the lysosomes and inactive. The registered dose of recombinant human GAA (rhGAA, alglucosidase alfa) for all patients is 20 mg/kg every other week (eow) [2, 3]. This dose is mainly based on the results of the 1602 trial in which patients received either 20 mg/kg or 40 mg/kg eow [6]. This trial in 18 classic infantile patients lasted 52 weeks and all survived beyond the first year of life. There was no clear difference between outcome of the 20 or 40 mg/kg eow group. A longer follow-up study showed that there is still room for improvement as nearly 50% of patients did not survive ventilator-free beyond the age of 3 years and 56% did not learn to walk [6, 7]. More recent longer follow-up studies report similar results [8-10].

Clinical and preclinical studies have shown that skeletal muscles are difficult to target and that uptake is dose dependent [11-13]. Other factors that have shown to be of influence on outcome are CRIM status and antibody formation [14-17]. CRIM-negative patients have shown to perform poorly and tend to produce high anti-rhGAA antibody titers. The outcome of CRIM-positive patients seems more variable. In these patients high sustained titers have also been reported to have a negative effect [14, 18].

To improve clinical outcome, we have investigated whether a dose of 40mg/kg/week could improve clinical outcome. This dose was earlier applied in our very first clinical trial with recombinant human alpha-glucosidase produced in milk from transgenic rabbits in 1999 [19]. From 2009 onwards, all our newly diagnosed patients received 40 mg/kg/week from the start. In 2016 we published our preliminary results in 8 CRIM-positive patients comparing 20 mg/kg eow with 40 mg/kg/week [17]. Here we present our longer follow-up data in a larger cohort of 18 patients also including CRIM-negative patients. From 2012 onwards, we further investigated whether immunomodulation, using a protocol published by Messinger et al. [20], aimed to prevent production of anti-rhGAA antibodies, in combination with a higher dose had added benefit.

METHODS

Patients and treatment

All patients diagnosed with classic infantile Pompe disease who were treated with rhGAA (Alglucosidase alfa, MyozymeÒ) from the start were included. Classic infantile Pompe disease was defined as symptoms of muscle weakness within six months after birth, the presence of hypertrophic cardiomyopathy. Diagnosis was confirmed by deficiency of endogenous α-glucosidase in fibroblasts of <1% of the normal mean, and mutation analysis [3]. ERT was dosed at 20 mg/kg eow between 2003 and 2009. From 2009 onwards all newly diagnosed patients received 40 mg/kg/week. All patients receiving 20 mg/kg eow were increased to 40 mg/kg/week somewhere between 2009 and 2014, due to clinical deterioration. In 2012 immunomodulation with Rituximab (RTX), Methotrexate (MTX) and intravenous immunoglobulins (IVIG) in an ERT-naïve setting was initiated in newly diagnosed patients older than 2 months of age at time of diagnosis [21]. Antibiotic prophylaxis was given to prevent (respiratory) infections.

Clinical efficacy

Standardized assessments were performed at baseline and every three months thereafter [19]. Study protocols were approved by the Institutional Review Board and written informed consent was obtained from the parents and/or patients. Clinical outcome was assessed by (ventilator-free) survival. Cardiac dimensions were measured by conventional echocardiography; the left ventricular mass index (LVMI) was calculated by the Devereux formula and indexed by body surface area. An LVMI of > +2SD of age-related peers was considered abnormal [22]. Motor function was assessed by Alberta Infant Motor Scale (AIMS), Bayley Scales of Infant Development II (BSID-II), and motor milestones were recorded. From the age of 5 years onwards, motor function was assessed using the 6 minute walk test (6MWT) [23-25]. Results were compared to age-related healthy peers. Infusion associated reactions (IARs) were noted when they occurred. The severity of each IAR was indexed by clinical judgment as mild/moderate or severe.

Antibody detection

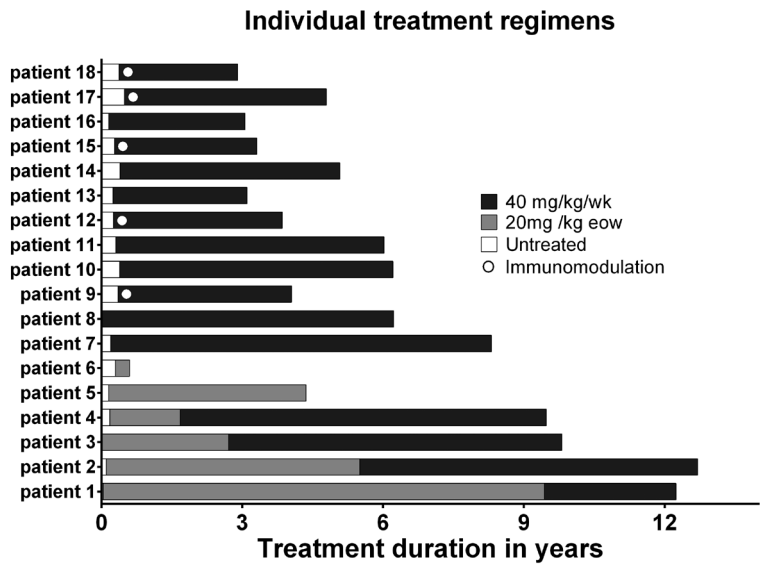
Anti-rhGAA titers were assessed at start of ERT and thereafter at 3, 6, 9, 12, 18, 24 months and from then on yearly. When receiving immunomodulation titers were also determined at 1 and 2 months after start [21]. Standardized antibody analysis was performed by an ELISA as described [16, 26, 27]. Antibody titers were measured in five-fold serial dilutions. Samples were measured in duplicate. Assays were performed at least twice. The background of the ELISA method was determined to be 1:250 by omitting the coating of the plates with rhGAA as described previously [27].

RESULTS

Patients

Eighteen patients were included, nine (50%) were male. Table 1 compares the patient groups treated with 20 mg/kg eow (n=6) and 40 mg/kg/week (n=12). Age at start of treatment was younger in the 20mg group and ranged from 0.1 to 3.6 months (median 1.5 months) compared to 0.3 to 5.9 months (median 3.6 months) in the 40mg group. Two patients in the 20mg group were CRIM-negative and three in the 40mg group. Age at last assessment ranged from 0.6 to 12.6 years (median 9.6 years) and from 3.0 to 8.3 years (median 4.4 years) for both groups. Four patients treated with 20 mg/kg eow had their dose increased to 40 mg/kg/week at ages ranging from 1.5 to 9.4 years either because of serious respiratory infections, becoming ventilator dependent or motor decline. Figure 1 shows the treatment regimen per patient.

Figure 1. Individual treatment protocol per patient



Effects of higher dosing on clinical outcome

At study end survival was 66% in the 20mg group and 92% in the 40mg group (Figure 2). Three patients died (aged 0.6, 3.1, and 4.4 years) due to respiratory failure; both CRIM-negative patients from the 20mg group and one CRIM-positive patient from the 40mg group. Ventilator-free survival was 50% for the 20mg group and 92% for the 40mg group.

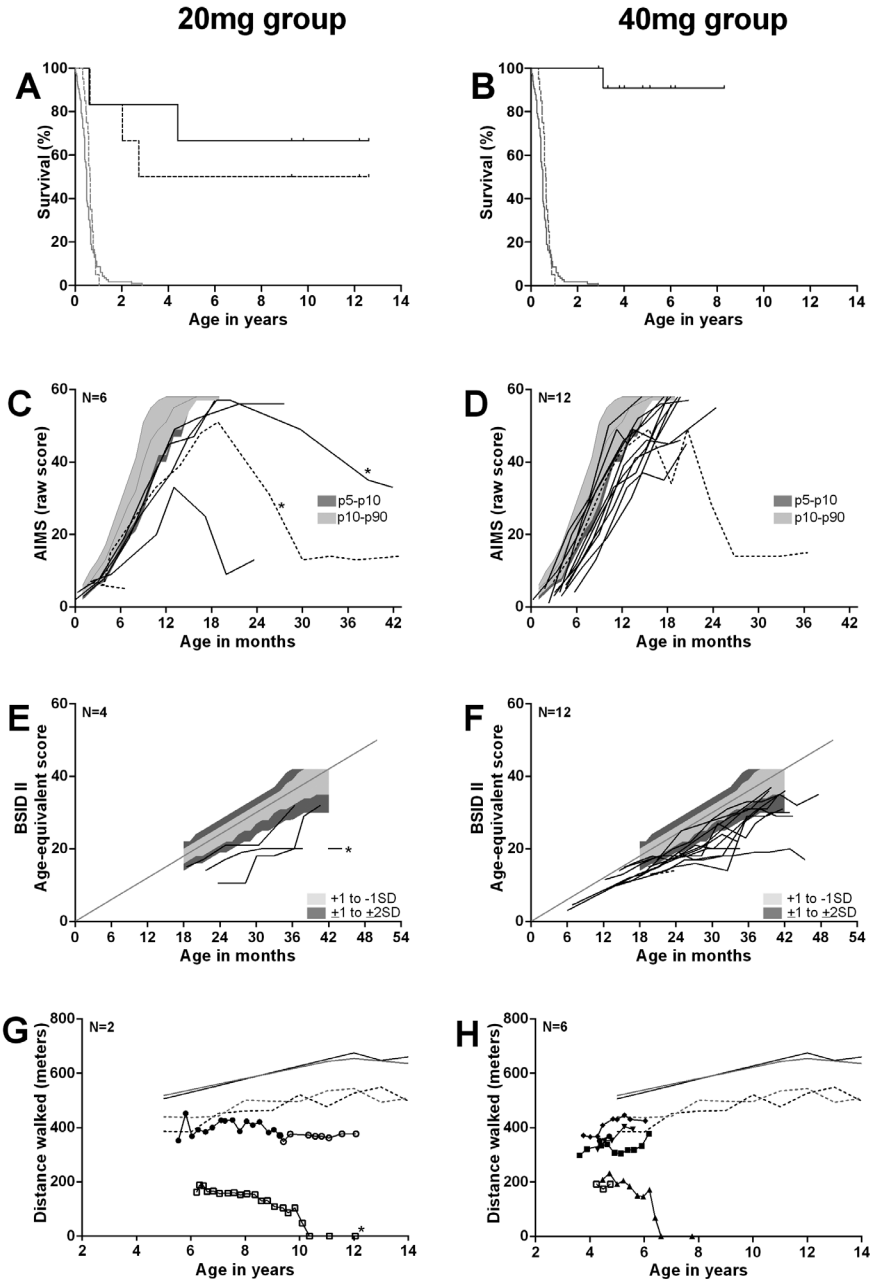
Table 1 Patient characteristics and outcome comparing 20 mg/kg eow with 40 mg/kg/week

	20 mg/kg eow	40 mg/kg/week
Patient Characteristics		
Number of patients	6	12
Males (%)	4 (67%)	5 (42%)
Median age at start in months (range)	1.5 (0.1 - 3.6)	3.6 (0.3 – 5.9)
Number of CRIM-negative (%)	2 (33%)	3 (25%)
ERT dose increase	4 (67%)[1]	N.A.
Age at dose increase in years (median, range)	4.1 (1.5 – 9.4)	N.A.
Outcome		
Median age at last assessment in years (range)	9.6 (0.6 – 12.6)	4.4 (3.0 – 8.3)
Survival (%)	66%	92%
Survival of CRIM-negative patients (%)	0%	100%
Ventilator-free survival (%)	50%	92%
Median LVMI z-score at start (range)	6.15 (2.4 – 8.6)	7.1 (3.0 – 13.7)
Number of patients with LVMI normalization during follow-up (%)	5 (83%)	11 (92%)
Median ERT duration at LVMI normalization in years (range)	0.5 (0.25 – 1.71)	0.5 (0.25 – 1.4)
Best motor milestone (%)		
MMF	1 (17%)	0
Sitting	1 (17%)	1 (8%)
Walking	4 (67%)	11 (92%)
Number of patients walking independently at age 3 years (%)	2 (33%)	11 (92%)
Last motor milestone (number, %)		
MMF	1 (17%)	0
Sitting	4 (67%)	2 (17%)
Walking	1 (17%)	10 (83%)
AIMS at 12 months (median, range)	37 (20 – 45)[2]	39 (20 – 50)
AIMS at 18 months (median, range)	54 (25 – 57)[2]	57 (34 – 58)
BSID-II AE score at 24 months (median, range)	17 (10.4 – 21)[3]	18 (14 – 25)
BSID-II AE score at 36 months (median, range)	20 (20 – 32)[3]	30 (19 – 33)[4]
Median peak antibody titer (range)	1:6,250 (1,250 – 31,250)	1:156,250 (250 – 800,000)
Tube feeding at start (number, %)		
NGT	6 (100%)	9 (75%)
PEG	0	0
Oral	0	3 (25%)
Tube feeding at study end (number, %)		
NGT	1 (17%)	1 (8.5%)
PEG	2 (33%)	1 (8.5%)
Oral	3 (50%)	10 (83%)
Number of patients with IARs (total number of IARs)	5 (64 IARS, 4 severe)	8 (134 IARS, 11 severe)

[1] Dose increase was only applied in the surviving four patients from the 20mg group. [2] AIMS at 12 and 18 months was performed in the five surviving patients. [3] BSID-II was performed in three patients, the two patients requiring invasive ventilation were not tested due to illness. [4] BSID-II at 36 months was performed in the surviving 11 patients, two patients were 34 months of age at time of testing.

CRIM: Cross-reactive immunological material; ERT: Enzyme replacement therapy; LVMI: Left-ventricular mass index; MMF: Minimal motor function AIMS: Alberta Infant Motor Scale; BSID-II AE; Bayley Scales of Infant Development II age-equivalent score; NGT: nasogastric tube; PEG: Percutaneous endoscopic gastrostomy; IARs: Infusion associated reactions

Figure 2. Survival and Motor outcome in patients receiving 20 mg/kg eow or 40 mg/kg/week



A Survival in patients receiving 20 mg/kg eow, grey lines represent the historical cohorts; **B** Survival in patients receiving 40 mg/kg/week, grey lines represent the historical cohorts; **C** AIMS score in patients receiving 20 mg/kg eow; **D** AIMS score in patients receiving 40 mg/kg/week; **E** BSID-II age equivalent score in patients receiving 20 mg/kg eow; **F** BSID-II age equivalent score in patients receiving 40 mg/kg/week; **G** Distance walked during the 6-minute walk test in patients receiving 20 mg/kg eow. The lines without symbols represent the normal values - 0SD (solid line) and -2SD (dashed line) - of healthy peers; **H** Distance walked during the 6-minute walk test in patients receiving 40 mg/kg/week. The lines without symbols represent the normal values - 0SD (solid line) and -2SD (dashed line) - of healthy peers Dashed lines in figures CDEF represent the deceased patients. The asterisks marks the patients who required invasive ventilation.

The ability to walk was achieved by four patients (67%) in the 20mg group and by 11 (92%) in the 40mg group. By the age of three years two (33%) in the 20mg group and 11 (92%) patients in the 40mg group maintained the ability to walk. At end of the study respectively one (17%) and 10 (83%) were persistent walkers.

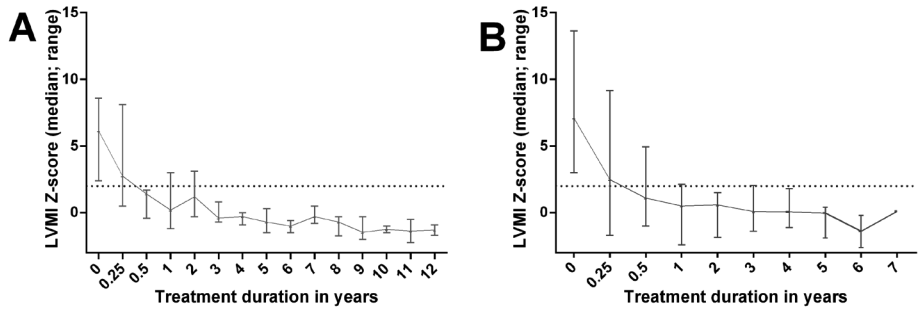
Figure 2 and table 1 show the AIMS and BSID-II scores per treatment group. Although the ranges in AIMS and BSID-II scores were similar, only patients from the 40mg group (n=6 of whom 2 were CRIM-negative) reached the maximum AIMS score of 58. The median BSID-II age-equivalent score at 36 months was higher for the 40mg group, 30 months, compared to 20 months for the 20mg group. Only 50% of patients in the 20mg group compared to 92% of patients in the 40mg group could be adequately tested with the BSID-II at 36 months. The test was not performed in the others either because they had died (n=3) or were ventilator-dependent (n=1). Two patients in the 20mg group and six in the 40mg group performed timed tests, generally commenced at the age of 6 years (Figure 2 G-H). One patient in the 20mg group and 4 patients in 40mg group scored within the normal range for peers at several time points and/or maintained normal scores until study end.

The cardiac response, effect on LVMI, did not show considerable differences between the two groups (Figure 3). The LVMI did not normalize in one patient who died after three months of treatment (20mg group). Two patients in 40mg group had a severe dilated cardiomyopathy at start, which responded well to treatment. In one of those patients, LVMI was still elevated at last assessment +2.05sd (+13.7sd at start). In one patient in the 20mg group, the LVMI normalized fully after dose increase.

Additional effect of immunomodulation in patients receiving 40 mg/kg/week

Five of 12 patients in the 40mg group received additional primary immunomodulation (immunomodulation in an ERT-naïve setting) with Rituximab, Methotrexate and IVIG. This protocol was introduced in 2012 in patients older than 2 months of age. We compared whether there was a difference in clinical outcome between patients treated with 40 mg/kg/week monotherapy (40mg mono group, n=7, CRIM-negative 1) and those treated with 40 mg/kg/week plus immunomodulation (40mg immuno group, n=5, CRIM-negative 2, table 2). The 40mg immuno group was slightly older (range 3.1 to 5.8 months) compared to the 40mg mono group (range 0.3 to 4.8 months) at start.

Figure 3. Cardiological follow-up data



A LVMI z-score score in patients receiving 20 mg/kg eow; **B** LVMI z-score score in patients receiving 40 mg/kg/week. LVMI Z-score were assessed using the Boston Z-scores.

At study end, (ventilator-free) survival was 86% in the 40mg mono group and 100% in the 40mg immuno group (table 2). The patient who died was CRIM-positive. The ability to walk was achieved by six patients (86%) in the 40mg mono group and by five (100%) in the 40mg immuno group. All patients were still able to walk at the age of three years. At end of the study five (71%) in the 40mg mono group and five (100%) in the 40mg immuno group were persistent walkers, including all three CRIM-negative patients. Figure 5 and table 2 show the AIMS and BSID-II scores per treatment group. The ranges in AIMS and BSID-II scores were similar. Also, response on LVMI was similar.

IARs and anti-rhGAA antibody titers

IARs were observed in all groups. Five patients (83%) in the 20mg group and eight (67%) in the 40mg group experienced IARs. Comparison between 40mg mono and 40mg immuno groups revealed that six (86%, CRIM-positive and CRIM-negative) and two (40%, CRIM-negative) patients experienced IARs respectively. In all but two patients (both CRIM-negative), IARs were treated successfully and had not reoccurred for at least 12 months.

When comparing antibody titers between the 20mg and the combined 40mg groups peak titers ranged from 1:1,250 to 1:31,250 in the 20mg group (median 1:6,250) and from 1:250 to 1:800,000 (median 1:156,250) in the 40mg group. Two patients in 20mg group developed high (sustained) titers of 1:31,500, one was CRIM-negative. Figure 4 shows anti-rhGAA antibody titers during follow-up per patient.

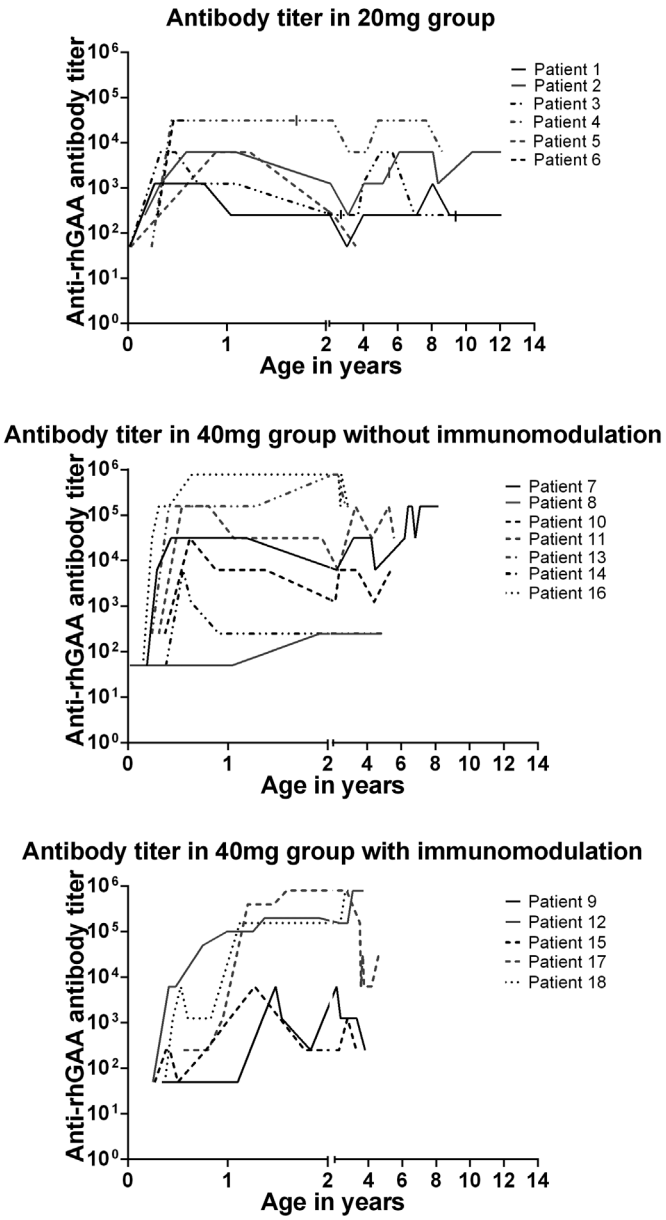
Comparison between the 40mg mono and the 40mg immuno groups showed peak titers ranging from 1:250 to 1:781,250 (median 1:156,250) for the 40mg mono group and titers ranging from 1:6,250 to 1:800,000 (median 1:468,750) for 40mg immuno group. All CRIM-negative patients in 40mg group developed high sustained titers irrespective whether they received immunomodulation; four of the nine CRIM-positive patients developed high sustained titers, one had received primary immunomodulation. After immunomodulation B-cells became depleted in all patients receiving it. B-cell recovery was observed between 5.7 and 7.9 months after the last dose of Rituximab (see for details [21]).

Table 2 Patient characteristics and outcome comparing 40 mg/kg/week with 40 mg/kg/week and immunomodulation

	40 mg/kg/week	**40 mg/kg/week and immuno**
Patient Characteristics		
Number of patients	7	5
Males (%)	3 (43%)	2 (40%)
Median age at start in months (range)	3.1 (0.3 – 4.8)	4.3 (3.1 – 5.8)
Number of CRIM-negative (%)	1 (14%)	2 (40%)
Outcome		
Age at last assessment in years (median, range)	6.0 (3.1 – 8.3)	3.8 (3.0 – 4.8)
Survival (%)	86%	100%
Ventilator-free survival (%)	86%	100%
Median LVMI z-score at start (range)	7.0 (3.0 – 9.6)	7.8 (4.0 – 13.7)
Number of patients with LVMI normalization during follow-up (%)	7 (100%)	4 (80%)
Median ERT duration at LVMI normalization in years (range)	0.9 (0.25 – 0.75)	0.5 (0.25 – 1.4)
Best motor milestone (number, %)		
MMF	0	0
Sitting	1 (14%)	0
Walking	6 (86%)	5 (100%)
Number of patients walking independently at age 3 years (%)	6 (86%)	6 (100%)
Last motor milestone (number, %)		
MMF	0	0
Sitting	2 (29%)	0
Walking	5 (71%)	5 (100%)
AIMS at 12 months (median, range)	41.5 (27 – 50)	36 (20 – 49)
AIMS at 18 months (median, range)	58 (34 – 58)	56 (35 – 58**)**
BSID-II AE score at 24 months (median, range)	18 (14 – 25)	18 (15 – 19)
BSID-II AE score at 36 months (median, range)	31 (30 – 33)	23 (19 – 31)
Peak antibody titer (median, range)	1:156,250 (250 – 781,250)	1:468,750 (6,250 – 800,000)
Antibody titer at last assessment (median, range)	1:31,250 (250 – 781,250)	1:93,750 (250 – 781,250)
Tube feeding at start (number, %)		
NGT	4 (57%)	5 (100%)
PEG	0	0
Oral	3 (43%)	0
Tube feeding at study end (number, %)		
NGT	1 (14%)	0
PEG	1 (14%)	0
Oral	5 (71%)	5 (100%)
IARs in number of patients (total number of IARs)	6 (110 IARS, 6 severe)	2 (24 IARs, 5 severe)

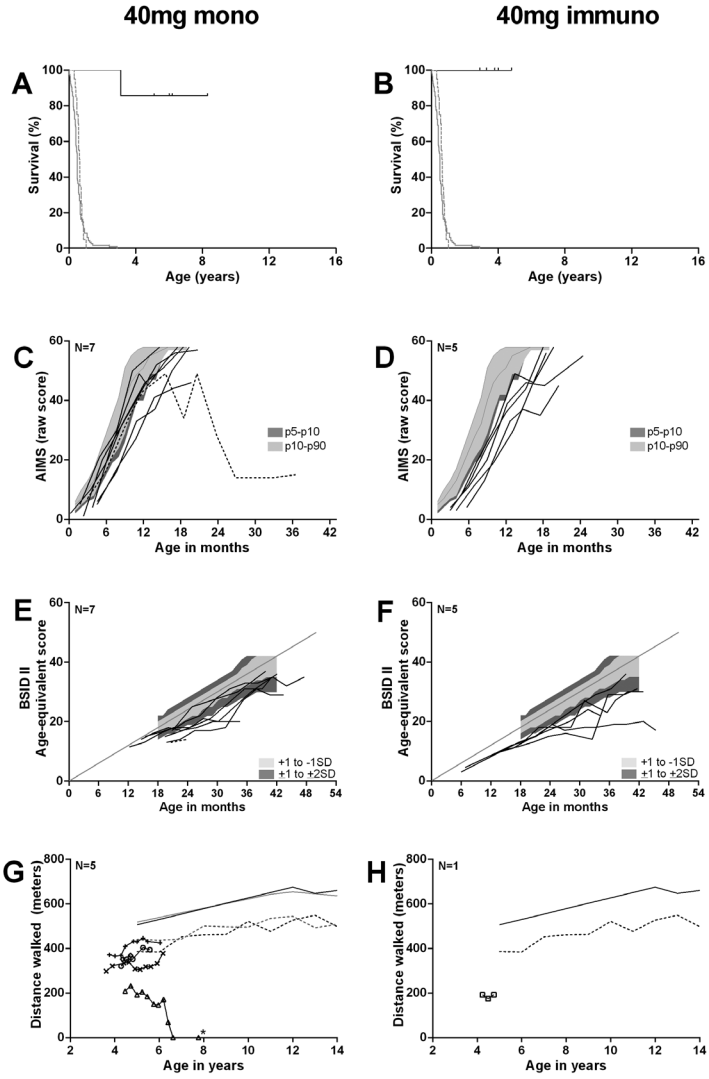
CRIM: Cross-reactive immunological material; ERT: Enzyme replacement therapy; LVMI: Left-ventricular mass index; MMF: Minimal motor function AIMS: Alberta Infant Motor Scale; BSID-II AE; Bayley Scales of Infant Development II age-equivalent score; NGT: nasogastric tube; PEG: Percutaneous endoscopic gastrostomy; IARs: Infusion associated reactions

Figure 4. Anti-rhGAA antibody titers in patients receiving 20 mg/kg eow compared to 40 mg/kg/week monotherapy and 40 mg/kg/week and immunomodulation.



Each line represents an individual patient. Patients 5, 6, 16, 17 and 18 are the CRIM-negative patients. **A** Anti-rhGAA antibody titers in patients receiving 20 mg/kg eow; **B** Anti-rhGAA antibody titers in patients receiving 40 mg/kg/week monotherapy. **C** Anti-rhGAA antibody titers in patients receiving 40 mg/kg/week and immunomodulation in an ERT-naïve setting.

Figure 5. Survival and Motor outcome in patients receiving 40 mg/kg/week monotherapy (40mg mon) or 40 mg/kg/week with immunomodulation (40mg immuno)



A Survival in patients receiving 40mg monotherapy, grey lines represent the historical cohorts; **B** Survival in patients receiving 40mg and immunomodulation, grey lines represent the historical cohorts; **C** AIMS score in patients receiving 40mg mono therapy; **D** AIMS score in patients receiving 40mg and immunomodulation; **E** BSID-II age equivalent score in patients receiving 40mg monotherapy; **F** BSID-II age equivalent score in patients receiving 40mg and immunomodulation; **G** Distance walked during the 6-minute walk test in patients receiving 40mg monotherapy. The lines without symbols represent the normal values - 0SD (solid line) and -2SD (dashed line) - of healthy peers; **H** Distance walked during the 6-minute walk test in patients receiving 40mg and immunomodulation. The lines without symbols represent the normal values - 0SD (solid line) and -2SD (dashed line) - of healthy peers. Dashed lines in figures C and E represents the deceased patient. The asterisks in figure G marks the loss of ambulation in that patient.

DISCUSSION

The primary aim of this study was to investigate the role of higher ERT dosing on clinical outcome. ERT has generally improved short-term survival of patients, but with longer follow-up it has become evident that there are still considerable unmet clinical needs as 50% of the reported patients do not survive ventilator-free at the age of 3 years and even lower numbers obtain or maintain the ability to walk [6, 7]. The current study shows that classic infantile patients receiving an ERT dose of 40 mg/kg/week from start have a better survival and ventilator-free survival (92% for both outcomes) than patients who started on 20 mg/kg eow (66% and 50%). They also have a better motor outcome: 92% achieved the ability to walk and 83% are still able to walk at study end (median age 4.4 years) compared to 67% and 17% respectively in the 20mg group (median age 9.6 years). At the age of 3 years 92% and 33% were able to walk. Most notable was that all three CRIM-negative patients in the 40mg group were alive at study end and able to walk compared to none in 20mg group. Five patients of the 40mg group (of whom two of the three CRIM-negative) also received immunomodulation in an ERT-naïve setting. Immunomodulation did not prevent antibody formation in our study. All patients receiving immunomodulation were alive and persistent walkers at study end.

Studies from various countries have reported the long-term outcome of patients with classic infantile Pompe disease: the UK, Germany and Italy, and the pivotal 1602 trial [6-10]. Most of the patients (n=102) in these combined studies started ERT on 20 mg/kg eow. The maximum follow-up of patients in these studies ranged from 3 years to 13.6 years. The median follow-up duration of the 1602 extension study was the shortest (2.3 years). Reported survival ranged from 56% to 66% and ventilator-free survival was 29% to 50% in the various studies. The ability to walk was achieved in 25% to 44%, and 19% to 39% of patients were persistent walkers at study end. The maximum follow-up in our study was 12.6 years for 20mg group and 8.3 years for the 40mg group. The clinical results of our 20mg group are similar to the results published in literature (66% for survival, 50% for ventilator-free survival, 67% walkers and 17% persistent walkers). In our study, the surviving four patients of the 20mg group were all increased to 40 mg/kg/week at ages ranging from 1.5 to 9.4 years because of clinical deterioration. The dose increase could be the reason why these patients are still alive, experienced limited infections and that so far no additional patients became ventilator dependent. Unfortunately, it could not prevent that only one is a persistent walker.

A potential explanation for the better outcome in the 40mg group is that a higher dose is required to obtain sufficient uptake by muscle cells and transport of rhGAA to the lysosomes, and a more frequent dose to maintain a sufficient GAA activity level in the

lysosomes for glycogen clearance [11, 28]. The estimated intracellular half-life of GAA after uptake based on preclinical studies is 3-7 days [29]. Fourteen days between infusions may be too long. It should be noted that ERT in other LSDs such as MPS I, II, IV and VI are also administered weekly [1]. This could explain why in the 1602 trial no obvious clinical benefit of 40 mg/kg eow over 20 mg/kg eow was observed [6, 7]. It was further noted by the authors of the 1602 trial that there was an overrepresentation of CRIM-negative patients receiving 40 mg/kg eow which may have also contributed to the limited effect.

Regarding antibodies, we found anti-rhGAA antibody titers in both groups: peak titers ranging from 1:1,250 to 1:31,150 in the 20mg group and 1:250 to 1:800,000 in the 40mg group. High sustained antibody titers of ≥1:31,500 developed in four of the nine CRIM-positive and all three CRIM-negative patients in the 40mg group and in one CRIM-positive patient in the 20mg group. This might indicate that antibody titers were generally higher in the higher dose group, but it should also be noted that these patients were generally older at start. Previously we found that patients with titers <1:31,250 did not show inhibitory effects of anti-rhGAA antibody titers in enzyme uptake experiments and immunoprecipitation [16] and calculated that in a patient with a titer of 1:156,250, with a blood volume of 80ml/kg and an ERT dose of 20 mg/kg, 54% of the infused enzyme (about 10 mg/kg) could possibly be bound to antibodies [16, 26]. Theoretically, with a higher dose more antibody-free rhGAA will be available and the anticipated neutralizing effects of antibodies less severe. This may explain why we observed a good clinical outcome in most of our patients despite high titers and why all our CRIM-negative patients in 40mg group are currently alive and persistent walkers, where other studies rarely report survival in CRIM-negative patients.

The second aim was to investigate whether immunomodulation could further improve clinical outcome. In patients receiving immunomodulation in an ERT-naïve setting in combination with a high dose we achieved 100% ventilator-free survival and all are persistent walkers. It should be noted that this group of patients was the youngest of the groups that we compared with the shortest follow-up. Immunomodulation has become a major focus in the treatment of classic infantile Pompe patients to improve clinical outcome [9, 20, 21, 30-32]. We applied immunomodulation protocols in an ERT-naïve setting similar to what was reported in literature. The effect of our immunomodulation regimen on the antibody titers was limited. There are several reports that were successful in eliminating and/or preventing antibody formation. Of the 24 patients receiving immunomodulation in an ERT-naïve setting survival was 80%, ventilator-free survival 71%, and 25% learned to walk (see [21] for review). Most of these patients were CRIM-negative and all patients started ERT on 20 mg/kg eow, yet some were receiving a higher dose at study end. This shows that standard dose in combination with immunomodulation not always leads to a good outcome. We hypothesize that a higher more frequent ERT dose is the main reason for

the good clinical outcome of our patients. Immunomodulation may have contributed to the clinical stability of our patients, but it did not prevent antibody formation. It should be noted however that the higher dose cannot overcome all limitations. Many patients in the 40mg group still have residual muscle weakness and some also lose motor milestones. Thus, the road to a cure for classic infantile patients does not end here. This also includes the development of better immunomodulatory regimens.

Finally, we found high sustained antibody titers and IARs in both dose groups, in both CRIM-positive and CRIM-negative patients. The relationship between the antibody titers, and IARs is very complex and warrants further investigation.

In conclusion, the current study shows that our classic infantile patients receiving 40 mg/kg/week from start have a better survival, ventilator-free survival and motor outcome than patients receiving 20 mg/kg eow. Most notable, all three CRIM-negative patients in the 40mg group were alive and able to walk at study end irrespective of whether they received immunomodulation.

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Part 2

Chapter 6

Cardiac outcome in classic infantile Pompe disease after 13 years of treatment with recombinant human acid alpha-glucosidase

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ABSTRACT

Background

Cardiac failure is the main cause of death in untreated classic infantile Pompe disease, an inheritable metabolic myopathy characterized by progressive hypertrophic cardiomyopathy. Since the introduction of enzyme replacement therapy (ERT), survival has increased significantly due to reduced cardiac hypertrophy and improved cardiac function. However, little is known about ERT's long-term effects on the heart.

Methods

Fourteen patients were included in this prospective study. Cardiac dimensions, function, conduction and rhythm disturbances were evaluated at baseline and at regular intervals thereafter.

Results

Treatment duration ranged from 1.1 to 13.9 years (median 4.8 years). At baseline, all patients had increased left ventricular mass index (LVMI) (median LVMI 226 g/m^2, range 98 to 599 g/m^2, Z-score median 7, range 2.4-12.4). During the first four weeks, LVMI continued to increase in six patients. Normalization of LVMI was observed in 13 patients (median 30 weeks; range 3 to 660 weeks). After clinical deterioration, LVMI increased again slightly in one patient. At baseline, PR interval was shortened in all patients; it normalized in only three. A delta-wave pattern on ECG was seen in six patients and resulted in documented periods of supraventricular tachycardias (SVTs) in three patients, two of whom required medication and/or ablation. One patient had severe bradycardia (35 beats/minute).

Conclusion

This study shows that ERT significantly reduced LVMI, and sustained this effect over a period of 13.9 years. The risk for rhythm disturbances remains. Regular cardiac evaluations should be continued, also after initially good response to ERT.

INTRODUCTION

Before the introduction of enzyme replacement therapy (ERT), cardiac failure was the main cause of death in patients with the classic infantile form of Pompe disease (OMIM 232300), a lysosomal storage disorder caused by deficiency of the enzyme acid alpha-glucosidase (EC 3.2.1.20). A hallmark of the disease is the progressive accumulation of glycogen, predominantly in skeletal and cardiac muscle, but also in various other tissues throughout the body. Pompe disease presents as a spectrum of clinical phenotypes, with the severest classic-infantile form leading to complete deficiency of alpha-glucosidase. Patients present with progressive generalized myopathy and cardiac hypertrophy. Motor milestones are not achieved, and patients rarely survive beyond one year of age. Cardiac failure is the main cause of death [1-4].

Since the introduction of ERT with recombinant human alpha-glucosidase (rhGAA, alglucosidase alfa), survival has improved significantly, due mainly to reduced cardiac hypertrophy and improved cardiac function [5-9]. However, little is known about the long-term effects of ERT on the heart. The present report describes the effects of ERT on cardiac size, cardiac function, and conduction pattern in fourteen classic-infantile Pompe patients over a treatment period up of 13.9 years.

MATERIAL AND METHODS

Patients

This study is part of an ongoing prospective clinical study investigating the safety and efficacy of ERT in classic-infantile Pompe patients. Classic-infantile Pompe disease was confirmed by profound deficiency of α-glucosidase in fibroblasts (<1%), mutation analysis, and the presence of hypertrophic cardiomyopathy. For this study of long-term effects, we only included patients who had been receiving ERT for at least 12 months. ERT doses ranged from 20 mg/kg every other week to 40 mg/kg weekly.

During the study period 5 additional patients were diagnosed. Four did not start ERT because of poor respiratory and/or motor condition or because parents decided not to start ERT. The ages of these patients at time of diagnosis ranged from 2.6-4.6 months. LVMI ranged from 171 to 523 g/m^2 at time of diagnosis and were in the same range as the study cohort, none of these patients were ventilator dependent. All untreated patients died before the age of one year.

Study design

The study was performed at Erasmus MC University Medical Center - Sophia Children's Hospital Rotterdam (Netherlands), and was approved by the Institutional Review Board. Written informed consent was obtained from parents or legal guardians. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Institutional Review Board. Standardized assessments were performed before the start of ERT and every three months thereafter.

Echocardiography

Conventional echocardiography with trans-thoracic M-Mode, two-dimensional echocardiography and conventional echo-Doppler measurements was performed by an experienced sonographer (JP) using a Philips iE33 Echocardiography System (Philips Medical Systems, Andover, MA, USA). Recordings were made at baseline and at regular intervals thereafter according to the recommendations of the American Society of Echocardiography [10]. The following parameters were documented: left ventricular internal cavity dimension in diastole (LVIDd), inter-ventricular septum thickness in diastole (IVSd), left ventricular posterior wall thickness in diastole (LVPWd), and left and right ventricular pre-ejection periods (LVPEP and RVPEP). These values were compared with normal values according to the Boston Z-scores [11]. Left ventricular mass index (LVMI) was calculated using the Devereux formula and indexed by body surface area [12]. Left ventricular hypertrophy was considered to be present if the LVMI Z-scores were >+2SD [11] .

Systolic function was examined by calculating the shortening fraction (SF). Values between 28-44% were considered normal [13]. To assess diastolic function, we used conventional Doppler imaging to examine the E/A ratio. The resulting measurements of the peak early (E) and late (A) transmitral filling velocities were then compared with published reference values. [14]. In January 2007, to optimize the assessment of diastolic function, pulsed wave Tissue Doppler Imaging (PWTDI) was added to the study protocol as an evaluation method. PWTDI velocities from the standard apical four-chamber views were acquired as described [10] and compared with corresponding measurements in age and sex-matched healthy controls.

Relative wall thickness (RWT) was calculated as RWT = ((IVSd + LVPWd)/LVIDd). A cut-off value of 0.42 was used to divide left ventricular (LV) hypertrophy into concentric (RWT > 0.42) and eccentric hypertrophy (RWT < 0.42) [15]. Patients without LV hypertrophy were classified as normal (RWT < 0.42) or concentric remodeling (RWT > 0.42).

Electrocardiography

Standardized 12-lead electrocardiograms (ECGs) were made using a Mortara ELI 350 ECG machine (Mortara Instrument Inc., Milwaukee, USA.) At regular intervals, we obtained ECGs for all patients in order to determine the PR interval, the QT interval corrected for heart rate, the LV voltages, and any rhythm or conduction disturbances. The QT interval was measured in lead II, V5, or V6. The corrected QT interval was determined by dividing the measured QT interval by the square root of the RR interval (Bazett's formula). LV voltages were calculated using the sum of the R wave in lead V6 and the S wave in lead V1. Pediatric reference values were obtained from Rijnbeek et al.; these consisted of a Dutch cohort from Rotterdam and were corrected for age and heart rate [16]. Two investigators examined all ECGs (CvC, IF).

Statistical analysis

For mitral valve E/A ratio, Z-scores were calculated as the difference between the measured value and the mean reference value divided by the standard deviation from the reference value. Z-scores > 2 or < -2 were considered abnormal. Measurements of PWTDI velocities and mitral valve E and A velocities at each patient's last assessment were compared with corresponding measurements in age and sex-matched healthy controls who have participated in an echocardiographic normal value study of our institute [17]. Variables were summarized using descriptive statistics comprising median and range. Differences in cardiac dimensions over time were evaluated using the Wilcoxon rank signed test. P-values < 0.05 were considered statistically significant. Statistical analyses were performed using SPSS version 21.0.

RESULTS

Patients

Fourteen patients were treated for more than 12 months and included in this study; their baseline characteristics are summarized in table 1. Age at start of treatment ranged from 3 days to 8.3 months (median age 2.7 months). At study end, the duration of ERT ranged from 1.1 to 13.9 years (median 4.8 years). All patients had fully deleterious variations in the GAA gene, and profound deficiency of alpha-glucosidase activity. The commonest variations were c.2481+102_2646+31del, which leads to an in-frame deletion of exon 18, and c.del525T, which leads to an unstable messenger with no GAA-protein production. Together they accounted for 15 of the 24 variations found in the GAA gene. Two patients were cross-reactive immunologic material (CRIM) negative, which implies that the variants on both alleles did not result in any GAA protein production.

At start of therapy, all patients had concentric LV hypertrophy (Figure 1, Table 2). Two patients had LV outflow-tract obstruction, and two others had accelerated mid-ventricular velocities of the left ventricle. Four patients (patients 2, 3, 4 and 12) experienced symptoms of congestive heart failure. At start, ten used cardiac medication (Table 1), at study end five still needed medication. Systemic blood pressures and renal function were within normal limits. All patients were hypotonic and most showed clearly reduced scores on the Alberta Infant Motor Scale (Table 1). Two patients were in an end-stage condition of the disease (patients 2 and 4). Four patients required oxygen via nasal prong/cannula (patients 4, 7, 8 and 13); one other patient was ventilator dependent (patient 2). During follow-up, four other patients became ventilator dependent. Later, two of these patients (both CRIM negative) died, at 4.4 and 4.3 years. Neither patient died due to cardiac complications. Patient 3 died after a period of unexplained hyperthermia, possibly due to brainstem dysfunction. Patient 5 died due to respiratory failure during a viral infection.

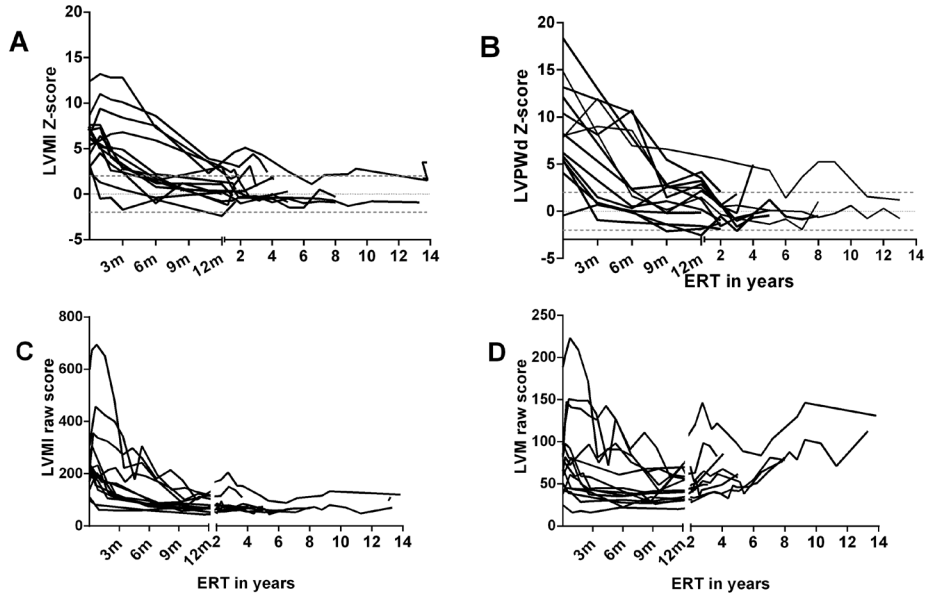
Echocardiography

LV morphology

At baseline, LVMI was profoundly elevated in all patients (median LVMI 226 g/m^2, range 98 to 599 g/m^2; median Z-score 7, range 2.4-12.4; Table 2). The LVPWd was elevated at baseline in 13 patients. Figure 1 shows the effect of ERT on LVMI and LVPWd over time, both show similar effects during follow-up. In eight patients, LVMI decreased immediately after start of ERT. In the remaining six patients it continued to increase for the first four weeks (median increase 79.0 g/ m2 (range 12.4-149 g/m^2)), and then declined.

At the last assessment, the median LVMI was 70.8 g/m^2 (range 48.2 to 119.6 g/m^2, median Z-score, 0.3 range -0.9 to + 2.4); in 13 patients it had normalized. Figure 2 shows the normalization of the LVMI in patients 9. Median time to normalization was 30 weeks (range 3 to 660 weeks). LVMI also normalized in patient 12, who had had dilated hypertrophic cardiomyopathy at start. One patient who never reached fully normal values died at the age of 4.3 years.

Figure 1. Left ventricular cardiac dimensions over time



A) LVMI Z-scores **B)** LVPWd Z-scores **C)** LVMI raw scores **D)** LVM raw scores
Each of the 14 patients is represented by a different line. The dashed grey lines represent the upper and lower limit of normal (Z-score of +2 and -2) and normal value (Z-score of 0).

Cardiac systolic and diastolic function

At baseline, four patients had decreased left ventricular SF (Table 2). SF normalized during treatment. At baseline diastolic function of the LV as measured by E/A ratio was normal in all patients. At follow-up, two patients had abnormalities in diastolic function: patient 3 showed a decline in diastolic function before she died; patient 2, who had end-stage disease, had abnormal E/A ratios in most measurements.

Table 1 Clinical features

Pt	Gender	Age at diagnosis (months)	Age at start ERT (months)	Weight (kg)/ Height (cm) at start ERT	Age at study end in months (years)	Survival*	Weight (kg)/ Height (cm) at study end
1	M	0.7	3.8	7.8/76	171 (14.2)	Alive	51/175
2	F	3.6	7.2	8.3/73	174 (14.5)	Alive	55/165
3	F	0.6	3.0	5.8/60	51 (4.3)	Deceased	18.9/110
4	F	6.2	8.3	5.7/62	174 (14.5)	Alive	33.6/146
5	M	0.2	1.9	4.0/52	53 (4.4)	Deceased	16.7/107
6	M	0.7	1.2	3.6/52	103 (8.6)	Alive	40/132
7	F	0.2	0.5	4.0/50	98 (8.2)	Alive	32/134
8	M	0.1	0.1	3.2/46	69 (5.8)	Alive	19.6/113
9	M	2.0	2.2	4.8/59	64 (5.3)	Alive	21.7/115
10	F	2.3	2.4	6.3/66	52 (4.3)	Alive	23.4/114
11	F	0.1	0.3	4.2/52	27 (2.2)	Alive	13/88
12	F	4.4	4.6	5.8/65	26 (2.2)	Alive	13/91
13	M	3.8	3.8	61/67	24 (2.0)	Alive	13.4/88
14	M	2.9	3.0	5.4/62	17 (1.4)	Alive	10.2/82

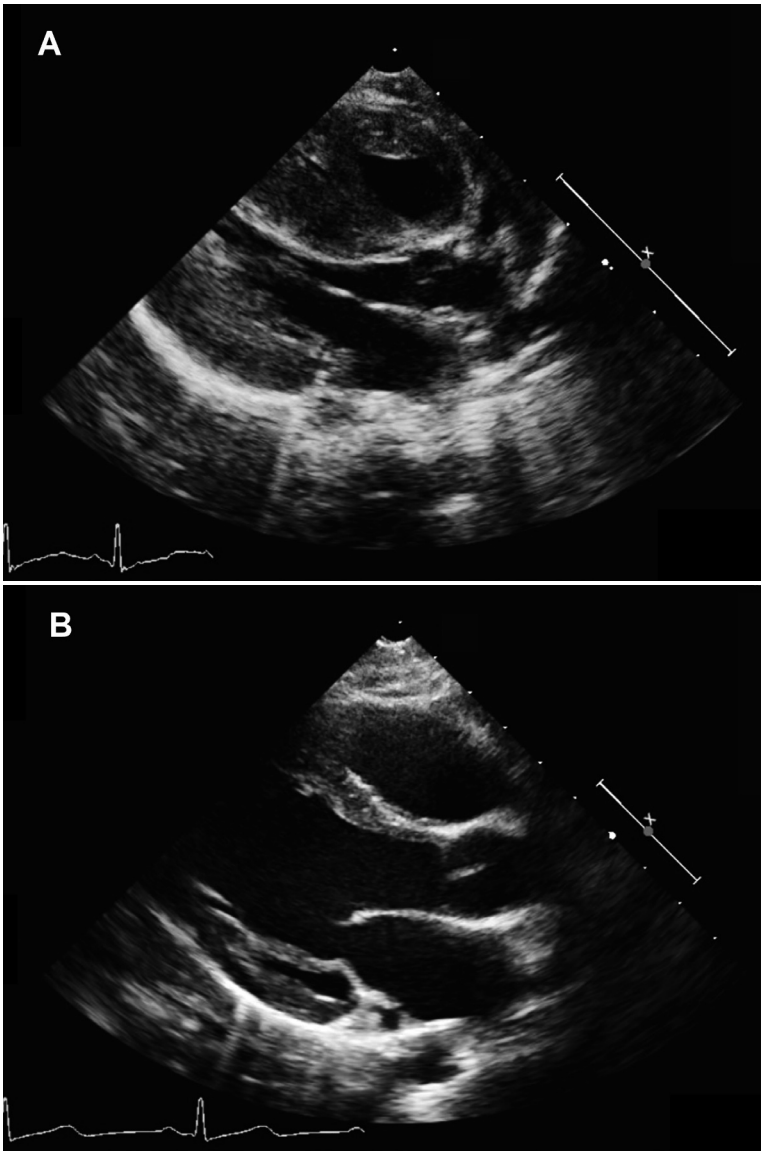
§ = Invasive ventilation started before start of ERT. * = Two patients died; their deaths were not related to cardiac events. ‡ = Lost the ability to walk after becoming ventilator dependent. ^ = baseline cardiac medication. Patients 2,3,4 and 12 experienced symptoms of congestive heart failure, in the other patients medication was started as standard treatment for hypertrophic cardiomyopathy

Mutations	CRIM status	Baseline cardiac medication^	Start Invasive ventilation (years)	AIMS at baseline (p-value)	Maximal motor milestone
c.2481+102_2646+31del c.1799G>A	+	None	-	10 (22)	Persistent walker
c.1115A>T c.525delT	+	Diuretics	0.6§	5 (0)	Tetraplegic
c.525delT c.525delT	-	Diuretics	2.2	4 (1)	Sitting
c.1913G>T c.1548G>A	+	Diuretics, ACE-inhibitor and Digoxin	0.9	5 (0)	Tetraplegic
c.2741delinsCAG c.2741delinsCAG	-	Diuretics	2.0	6 (25)	Walking‡
c.del525T c.1933G>T	+	Beta-blocker	-	3 (1)	Persistent Walker
c.2481+102_2646+31del c.2481+102_2646+31del	+	ACE-inhibitor	-	4 (36)	Persistent Walker
c.1460T>C c.1460T>C	+	None	2.7	2 (3)	Walking‡
c.525delT c.2481+102_2646+31del	+	None	-	7 (12)	Bum scoots
c.2481+102_2646+31del c.2481+102_2646+31del	+	Beta-blocker	-	1 (0)	Persistent Walker
c.525delT c.1933G>A	+	None	-	2 (3)	Persistent Walker
c.2104C>T c.379_380del	+	Diuretics and ACE-inhibitor	-	6 (0)	Persistent Walker
c.2481+102_2646+31del c.525delT	+	Diuretics and beta-blocker	-	4 (0)	Persistent Walker
c.2104C>T c.2481+102_2646+31del	+	Diuretics	-	7 (5)	Pulls to stand

Diastolic function measured by TDI

At last assessment, we obtained TDI values for 10 patients. The results are shown in Table 3. The early mitral inflow velocities (E') were significantly lower and the tricuspid A' velocities were significantly higher than in healthy age-matched controls. In the patient group, MV E/E' ratios were slightly increased.

Figure 2. Echocardiographic parasternal long axis view of patient 9



Electrocardiography

Echocardiographic parasternal long axis view of patient 9 at A) baseline and B) after 5 years of treatment. Note the major difference in both septal and posterior wall thickness.

During follow-up, we evaluated a total of 118 ambulant ECGs from 14 patients. At baseline, PR interval had been normal in nine patients and shortened in five (median PR interval 80 ms, range 60 to 100 ms; Table 2). At study end, the PR interval was within normal limits in six patients and was shortened in eight.

At baseline, LV voltages had been substantially increased (median 4.8 mV, range 2.2 to 8.6 mV); after start of ERT they declined (median 3.9 mV, range 1.7 to 6.2 mV). All patients had also had repolarization disturbances (strain). This normalized in all patients, except in patients 2, 3 and 5, in whom LVMI was slightly increased at last assessment or who showed increased value(s) of LVMI in the measurement(s) before the final one. Four patients (patient 1, 9, 10 and 13) had an incomplete right-bundle-branch block.

At baseline, the corrected QT-interval was normal in ten patients (median: 390 ms range 340 to 430 ms), and shortened in four (patients 1, 4, 13 and 14). At study end, corrected QT-interval was normal in ten patients, shortened in three (patients 4, 5 and 11), and increased in one (patient 2).

Delta waves, suggestive for Wolff-Parkinson-White (WPW) pattern, were found in six patients (patients 2, 4, 6, 7, 9 and 10). In the years after start of therapy, arrhythmias had been documented in three patients (patients 2, 4 and 5). Holter ECGs were performed in four patients (patients 2, 4, 7 and 12). Multiple episodes of supraventricular tachycardia (SVT) were documented in patients 2 and 4. Patient 5 had one episode of SVT, in the days before he died. The tachyarrhythmia were successfully converted by adenosine; in patient 2, rhythm control was established by Sotalol, and in patient 4 it was established by Atenolol and Flecainide. Due to frequent relapses, patient 4 underwent ablation of three aberrant pathways. In patient 2, the most recent Holter ECG revealed severe sinus bradycardia (range of 35 bpm to 97 bpm) and over 6000 pauses were recorded over 24 hours, with a maximum duration of 3.5 seconds. Due to the patient's poor clinical condition, it was decided not to implant a pacemaker.

Table 2 Cardiological parameters

	Baseline							
Pt	**LVM**	**LVM vs BSA**	**SF**	**IVSd**	**LVPWd**	**LVIDd**	**RWT**	**PR interval**
1	**65.2**	**171 (5.4)**	45	**0.9 (5.5)**	**1.0 (8)**	2.7	**0.7**	0.1
2	**83.3**	**203 (6.4)**	42	**1.1 (8)**	**1.0 (7.7)**	2.7	**0.8**	0.08
3	**96.2**	**308 (8.7)**	**25**	**1.4 (13.8)**	**1.1 (10.5)**	2.4	**1.0**	0.07
4	**188.4**	**599 (12.4)**	**10**	**1.8 (19.6)**	**1.6 (18.4)**	**3.7**	**0.9**	0.1
5	**77.0**	**296 (8.7)**	64	**1.4 (15.1)**	**1.2 (13.2)**	1.2	**2.2**	**0.1**
6	**42.0**	**191 (6.1)**	35	**0.9 (7.3)**	**0.9 (8.2)**	2.1	**0.9**	0.09
7	**50.8**	**231 (6.9)**	44	**0.95 (7.9)**	**1.30 (14.9)**	1.7	**1.3**	**0.08**
8	**19.8**	**98 (2.4)**	39	**0.75 (5.1)**	0.38 (-0.5)	**1.2**	**0.9**	0.08
9	**37.8**	**140 (4.4)**	55	**1.1 (9.7)**	**0.79 (5.8)**	1.9	**1.0**	0.08
10	**78.2**	**237 (7.2)**	37	**1.4 (13.3)**	**0.82 (5.7)**	2.6	**0.9**	**0.08**
11	**27.5**	**110 (3.2)**	38	**0.85 (6.2)**	**0.75 (5.5)**	1.6	**1.0**	0.06
12	**81.5**	**263 (7.6)**	**25**	**0.89 (6)**	**0.71 (4.1)**	**3.7**	**0.43**	**0.07**
13	**74.6**	**220 (7.0)**	32	**1.1 (8.9)**	**0.85 (6.2)**	**2.2**	**0.9**	**0.08**
14	**73.8**	**238 (7.5)**	**20**	**0.97 (7.4)**	**1.2 (12.1)**	**2.5**	**0.9**	0.08
Median	*74.6*	*225.5 (7)*	*37.5*	*1.0 (8.0)*	*0.95 (7.9)*	*2.3*	*0.9*	*0.08*
Range	*19.8-188.4*	*98-599*	*10-64*	*0.8-1.8*	*0.4-1.6*	*1.2-3.7*	*0.4-3.1*	*0.06-0.1*

IVSd: inter-ventricular septum thickness in diastole in centimeters; LVIDd: left ventricular internal cavity dimension in diastole in centimeters; LVM vs BSA: Left ventricular mass indexed for BSA; LVPWd: left ventricular posterior wall thickness in diastole in centimeters; SF: Shortening fraction in percentage; RWT: Relative wall thickness.

Table 3. Pulsed Doppler and Pulsed-wave Tissue Doppler velocities at final assessment**.**

	Patients		
	n	*Median*	*Range*
Age (years)	10	5.0	1.0-14.0
Pulsed Doppler velocities			
Mitral valve E velocity (cm/sec)	10	98.8	74.8-127.8
Mitral valve A velocity (cm/sec)	10	58.2	31.8-81.1
Mitral valve E/A ratio	10	1.7	1.0-4.0
Pulsed-wave tissue Doppler velocities			
Mitral valve lateral E' velocity (cm/sec)	10	14.2	7.9-17.9
Mitral valve lateral A' velocity (cm/sec)	10	5.5	3.2-7.0
Mitral valve E/E 'ratio	10	6.9	5.3-11.5
Tricuspid valve lateral E' velocity (cm/sec)	8	12.7	10.7-19.9
Tricuspid valve lateral A' velocity (cm/sec)	9	9.7	3.6-13.4

* institutional reference values

Wilcoxon rank singed test.

				Study end				
	LVM	**LVM vs BSA**	**SF**	**IVSd**	**LVPWd**	**LVIDd**	**RWT**	**PR interval**
	112.7	70.4 (-0.9)	41	0.8 (-0.7)	0.8 (-0.3)	4.6	0.3	0.08
	191.3	119.6 (1.5)#	49	1.1 (1.5)	1.0 (1.4)	1.0	0.4	**0.1**$_{\top}$
	82.8	**109 (2.4)***	46	**1.0 (4)**	**1.0 (5)**	3.0	**0.7**	**0.08**
	129	111.3 (1.7)	41	**0.9 (2.9)**	0.8 (1.9)	NA	**0.7**	**0.08**$_{\top}$
	49.14	78 (0.8)*	57	0.6 (0)	0.6 (0.8)	3.0	**0.5**	0.12
	81.4	67.8 (-0.9)	37	0.7 (-0.6)	0.6 (-1.4)	0.8	0.4	0.25$^{\perp}$
	78.2	71.1 (-0.3)	32	0.1 (0.6)	0.8 (0.6)	3.7	0.4	**0.05**$^{\perp}$
	44.4	57.6 (-0.9)	46	0.5 (-1.2)	0.6 (-0.5)	3.4	0.3	**0.08**
	61.7	74.3 (0.3)	37	0.7 (0)	0.6 (0.1)	3.7	0.4	0.12$^{\perp}$
	85.8	63.3 (1.8)	38	0.9 (1.9)	0.6 (-0.4)	4.1	0.4	**0.08**$^{\perp}$
	32.0	48.2 (-0.8)	36	0.5 (-0.4)	0.4 (-1.3)	3.1	0.3	0.09
	41.5	74.1 (0.5)	35	0.5 (-0.6)	0.5 (-1.2)	3.6	0.3	0.08
	36.1	64.5 (-0.2)	39	0.6 (-0.5)	0.47 (-1)	3.1	0.4	**0.08**
	30.9	65.7 (-0.03)	32	0.7 (1.6)	0.5 (-0.2)	2.6	**0.5**	**0.08**
	49.1	*70.8 (0.3)*	*38.5*	*0.7 (0.0)*	*0.6 (-0.3)*	*3.1*	*0.4*	*0.08*
	30.9-191.3	*48.2-120*	*32-57*	*0.5-1.1*	*0.4-1.0*	*0.8-4.6*	*0.3-0.7*	*0.05-0.3*

Abnormal values are marked in bold; NA = not available; Z-score between brackets; * = Deceased; # = this patient had abnormal values in most measurements, but last two measurements were normal; $_{\top}$= confirmed WPW; $^{\perp}$= Deltawaves on ECG

Controls*			
n	*Median*	*Range*	*P#*
14	5.7	1.7-7.5	.9
14	100.4	78.8-120.9	1.0
14	54.9	36.5-67.1	.3
14	1.8	1.4-3.1	.2
13	18.7	16.5-24.0	***.00***
13	5.3	3.8-12.5	.7
13	5.2	3.9-6.6	***0.01***
13	12.9	10.6-15.2	.9
13	6.8	4.9-9.8	.02

Statistically significant values are marked in bold/italics

DISCUSSION

Due to a positive effect of ERT on cardiac hypertrophy, many patients with classic-infantile Pompe disease treated with ERT have now survived far beyond their first year of life. [5-8, 18-20]. Studies particularly focusing on the long-term consequences on cardiac structure, rhythm and function have not been performed, which was the reason for this study.

Effect of ERT on left ventricular mass and systolic function

At start of ERT, all patients in our study had prominent hypertrophic cardiomyopathy, with LVMIs of up to 599 gram/m^2 (Z-scores of up to + 12.4). As hypertrophy was already present in patients who had been diagnosed shortly after birth, it may – as noted by others [21, 22] – have developed during pregnancy. Earlier we found in untreated patients that the extent of hypertrophy was related to age [2]. Extraordinary one of our patients had severe dilated cardiomyopathy as well as hypertrophy.

Our study showed that ERT led to a significant effect on LVMI, LVPWd and systolic function in all patients (Figure 1). Reduction of LVMI and LVPWd were seen in previous studies with a shorter follow-up [8, 23, 24], new is that this effect is preserved for almost 14 years of ERT. LVMI normalized in 13 patients. Those in whom it did not fully normalize (n=2), or in whom it increased slightly (n=1), either died, or were in the end stage of disease at the start of ERT with a very high baseline LVMI. In none of these patients was cardiac failure the cause of death. At study end, systolic LV function was normal in all patients. A study by Chen et al. limited to 12 months of ERT also showed that LV function improved [25].

Effect of ERT on diastolic function

Using conventional echocardiography, we found no diastolic dysfunction at baseline. However, pseudo-normalization of E/A ratio cannot be ruled out. As PWTDI appears to be more sensitive than conventional echocardiography in detecting abnormalities in diastolic function, we added this measurement to our follow-up protocol. At the end of the follow-up period, patients had significantly lower E' velocities and a trend to higher E/E' ratios at the level of the mitral valve compared to controls. This was also observed by Chen et al. after one year of ERT [25]. This suggests that patients with Pompe disease who receive ERT have abnormal relaxation of the left ventricle, and also increased filling pressures. However, these data are difficult to interpret, since, in healthy young children, E' values and E/E' ratios also change significantly. In future studies, new imaging techniques such as speckle tracking might help to detect diastolic dysfunction at an early stage [26].

Effect of ERT on ECG parameters and cardiac rhythm

In line with the effect of ERT on LVMI, we found a reduction in LV voltages. Repolarization disturbances also disappeared. Importantly, despite treatment with ERT, the short PR and WPW pattern remained present. At start of ERT, 36% of patients had had a short PR interval; at the end of the study, 57% did. At end of study, six patients had a WPW pattern.

A short PR interval is often found in patients with classic infantile Pompe disease [27, 28], it is also found in other storage diseases affecting the heart such as Fabry disease and Danon disease, and in patients with PRKAG2 mutations. [29, 30].

The mechanism underlying this short PR interval is not completely understood; an interesting hypothesis was raised in 1982 by Bharati et al, who correlated electrophysiological abnormalities with pathological postmortem findings in the conduction system of untreated patients with classic-infantile Pompe disease. They found marked glycogen infiltration and vacuolization and an increase in cell size of the Purkinje cells and suggesting that the short PR interval reflected an enhancement of conduction caused by the deposition of glycogen [31]. The electrophysiological finding that the atrium-His interval was shortened and the His-ventricular interval was normal, while enlarged cells filled with glycogen were found in all parts of the conduction system including the bundle of His, challenges this hypothesis. Another theory was proposed by Arad et al. [32], who studied a mouse model with a human PRKAG2 mutation, a disease that leads to glycogen accumulation in the cytoplasm of cardiomyocytes. They observed that the annulus fibrosis was disrupted by glycogen-filled cardiomyocytes, allowing atrioventricular activation by bypassing the AV node. This disruption might also explain the short PR interval and the WPW pattern, and the fact that patients were prone to developing SVTs or severe bradycardia.

Various publications report that arrhythmias, including fatal arrhythmia such as SVT and ventricular fibrillation (VF), may occur in patients with classic-infantile Pompe disease [9, 28, 33, 34]. Three of our patients developed spontaneous periods of SVTs, which required medical intervention. These findings may relate to the observed WPW pattern. While our patients with recurrent SVTs were already severely affected at start of therapy, we cannot rule out that patients who respond well may also develop SVTs or other rhythm disturbances. We therefore advise regular monitoring of all patients receiving long-term ERT.

LIMITATIONS OF THE STUDY

This study has several limitations. Due to the rarity and severity of the disease, research in Pompe disease is restricted to cohort studies with relatively small sample sizes. In addition, increasing insight into the treatment of Pompe disease has led to adaptations in dosage regimens specifically because of skeletal muscle weakness. With respect to cardiac hypertrophy we observed a good response in all patients in our group regardless of the dose. This does not rule out that a higher dose may have had additional benefit in some patients. For several reasons some patients did not receive ERT, this may have influenced our data in part, it should be noted however that patients included in the study and treated with ERT represented the entire spectrum of disease severity of classic infantile Pompe disease. Because PWTDI was not yet available at the start of the study, our follow-up of diastolic function is limited and should be interpreted with caution.

CONCLUSION

This study shows that ERT has a significant effect on cardiac dimensions, which was maintained for almost 14 years. During the first weeks of treatment, before it starts to decline and eventually reaches normal values, LVMI may continue to increase. The time to normalization differs between patients: in some patients LVMI remained slightly elevated or showed a secondary increase. Two patients died, but not because of cardiac failure. In most patients, the effect decrease in LVMI was reflected in reductions of LV voltages and the disappearance of repolarization disturbances. Patients remained at risk of rhythm disturbances. At study end, 57% of patients had a short PR interval, 43% had a WPW pattern and 21% had SVTs, indicating that regulatory cardiac monitoring of patients remains warranted.

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Chapter 7

Cognitive decline in classic infantile Pompe disease – an under acknowledged challenge

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Classic infantile Pompe disease is a progressive lysosomal glycogen storage disorder, which, if untreated, leads to severe skeletal muscle weakness, inability to achieve any motor milestones, and death in the first year. In 1999 we reported the first successful use of enzyme replacement therapy (ERT).[1] Since then, patients' outcome with respect to survival, cardiac function, and motor performance has improved significantly. The oldest patient is currently 16 years old. Although glycogen storage occurs in the brain as well, and ERT cannot pass the blood-brain barrier, so far clinical studies show normal to mildly delayed cognitive development.[2, 3]

CASE REPORT

A nine-year-old classic infantile Pompe patient presented with increasing behavioral problems. He had presented at birth with persistent oxygen need and a hypertrophic cardiomyopathy (cardiac ultrasound left ventricular mass index 176.7 gr/m2; Z score 16.7). At five weeks of age, we confirmed the diagnosis. Blood tests showed an alpha-glucosidase deficiency in leukocytes and two mutations in the GAA gene (c.525delT and c.1933G>T, cross-reactive immunologic material-positive on immunoblotting, highest measured antibody was low, titer 1:6250). Immediately after his diagnosis, he started ERT at a dose of 20 mg/kg/every two weeks. The cardiac hypertrophy disappeared (age 9.9 months) and he learned to walk (age 16.8 months). He had moderate hearing loss of 50 to 70 dB compensated sufficiently by hearing aids. Between three and five years of age, he developed signs of residual muscle weakness such as ptosis, an extraocular motility disorder, and weakness of the dorsal flexors of the feet, hands, and finger extensors. Therefore, we increased his ERT dose to 40 mg/kg/weekly at age 5.5 years. From age 6 he experienced increasing difficulties with walking. At age 9, he could walk short distances without support.

Neuropsychological development was normal to mildly delayed until age 6, as described previously.[2] At the age of 9 years, we repeated the neuropsychological tests due to behavioral problems, like uncorrectable teasing and wanton behavior at school. These tests showed scores in the range of moderate intellectual disability (Wechsler Intelligence Scale for Children–III total IQ 48), with a specific decline in his processing speed and on the performance intelligence. Brain MRI (Figure 1) demonstrated severe symmetrical white-matter abnormalities extending deep into the subcortical white matter also involving the capsula interna and externa. Additional biochemical analyses ruled out other neurodegenerative or leukodystrophic disorders. In retrospect, we conclude that a mismatch between the cognitive expectations of the patient's surroundings and his own cognitive abilities explained this child's behavioral problems.

DISCUSSION

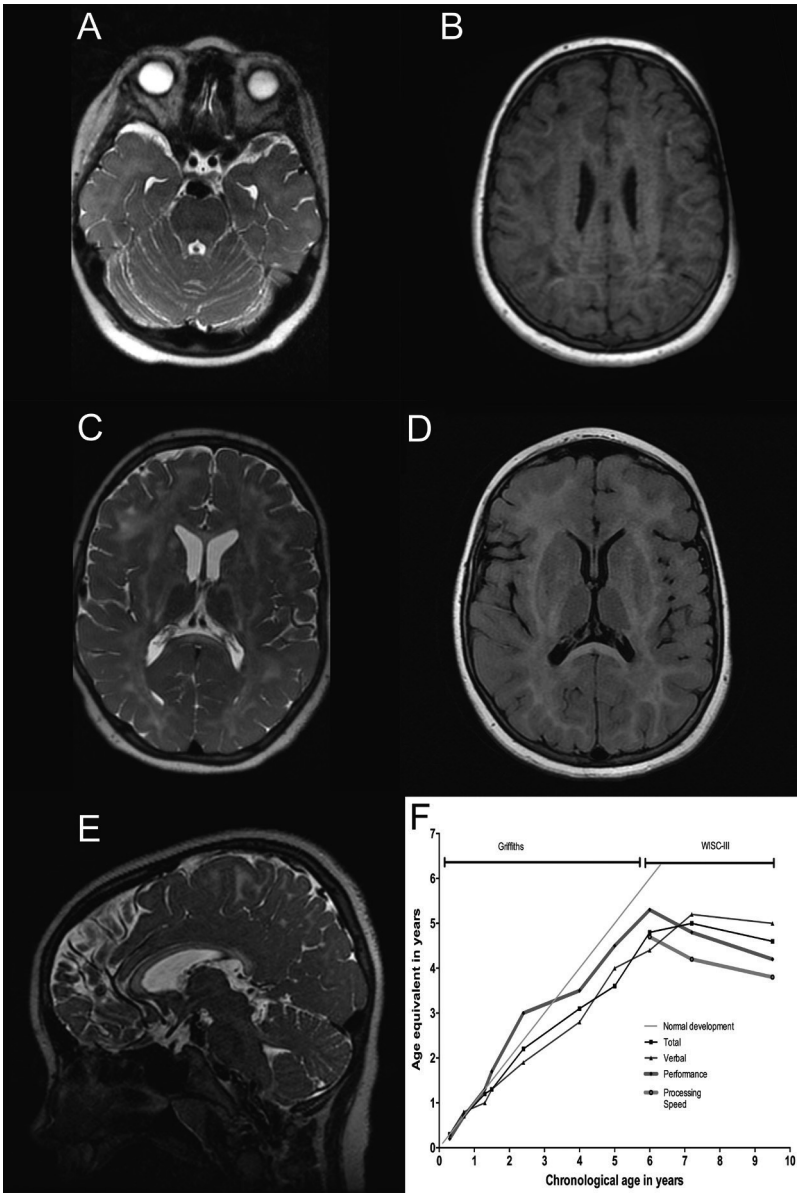
Earlier reports on the occurrence of periventricular white-matter abnormalities in classic infantile Pompe disease [2, 4, 5] suggested that these abnormalities were restricted and fairly stable over time,[2, 4] and that cognitive development in school-aged children ranged from normal to mildly delayed.[2, 3] Extensive white matter changes were reported once in literature with a cognitive follow-up limited to 44 months. This patient had a stable developmental delay (Bayley at 32 months showed a developmental age of 22 months, Snijders-Oomen nonverbal intelligence test at 44 months showed a developmental age of 35 months). [5]

Halting of cognitive development combined with extensive white matter abnormalities as found in our patient is a potential new feature of classic infantile Pompe disease. The deterioration of the processing speed and performance intelligence suggests that the process in the white matter might have been progressive in this moderately affected patient. We want to alert physicians that the consequence of the glycogen storage in the brain on cognition may be larger than expected before. We therefore advise to monitor cognitive development closely in classic infantile Pompe disease. This under-acknowledged challenge of the brain may have important consequences for the development of next generation therapeutic strategies that are currently under development.

ACKNOWLEDGEMENT

We would like to thank David Alexander (Erasmus Medical Center Rotterdam, for editing the manuscript for non-intellectual content) and Laurens Groenendijk (Erasmus Medical Center Rotterdam, for preparing the figure), and Carin van Gelder (Erasmus Medical Center Rotterdam, for collecting data).

Figure 1. White-matter abnormalities and cognitive decline in a patient with classic infantile Pompe disease.



A. Axial T2 (3 mm). **B**. Axial T1 (3mm). **C**. Axial T2 (3mm). **D.** Axial T2 fluid-attenuated inversion recovery (4 mm). **E.** Sagittal T2 (3 mm); All show signal abnormalities of the white matter, both periventricular and subcortical, with sparing of the U-fibers, and with involvement of the capsula interna, externa, claustrum, the corpus callosum, and the corticospinal tracts in the brainstem. The cerebellar peduncle superior and the decussation at the mesencephalon level are also involved. **F.** Decline in cognitive development in a patient with classic infantile Pompe disease. Griffiths = Griffiths Mental Development Scales, WISC-III = Wechsler Intelligence Scales for Children, Third Edition, Dutch version. Total = Total Intelligence, Verbal = Verbal Intelligence, Performance = Performance Intelligence, Processing Speed = Processing Speed Index.

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Chapter 8

Classic infantile Pompe patients approaching adulthood: a cohort study on consequences for the brain

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ABSTRACT

Background

Although the development of enzyme replacement therapy (ERT) for classic infantile Pompe disease has drastically improved patients' prospects, the long-term consequences of glycogen storage in the CNS are still unknown.

Methods

Using neuropsychological tests and brain MRIs, we prospectively assessed a cohort of 11 classic infantile Pompe patients aged up to 17 years.

Results

From approximately age 2 onwards, brain MRIs showed involvement of the periventricular white matter and centrum semiovale. After age 8, additional white-matter abnormalities occurred in the corpus callosum, internal and external capsule, and subcortical areas. From age 11, white-matter abnormalities were also found in the brainstem. Although there seemed to be a characteristic pattern of involvement over time, there were considerable variations between patients, reflected by variations neuropsychological development. Cognitive development ranged from stable and normal to declines that lead to intellectual disabilities.

Conclusions

As treatment enables patients with classic infantile Pompe disease to reach adulthood, white-matter abnormalities are becoming increasingly evident, affecting the neuropsychological development. Therefor we advise to expand follow-up programs to capture CNS involvement in larger, international patient cohorts, to incorporate our findings in the counseling of parents before start of treatment and to include the brain as an additional target in the development of next-generation therapeutic strategies for classic infantile Pompe disease.

INTRODUCTION

Classic infantile Pompe disease is a progressive inheritable myopathy caused by a deficiency of the lysosomal enzyme alpha-glucosidase. This leads to an accumulation of glycogen that mainly affects skeletal muscles and the heart. As patients have severe mutations in the GAA gene, enzyme activity is less than 1% in cultured fibroblasts. Characteristically, they present before the age of six months with a hypertrophic cardiomyopathy, progressive generalized muscle weakness, and respiratory problems. Untreated infants die before the age of one year [1]. In 1999, the first patients with classic infantile Pompe disease were treated with recombinant human alpha-glucosidase. In 2006 ERT (enzyme replacement therapy) was registered [2]. Over the years, ERT has been demonstrated to significantly improve survival, cardiac, and motor outcome [2-5]. The first surviving infants treated in our center are now on the threshold of adulthood.

A limitation of ERT is that it cannot pass the blood-brain barrier. However, small amounts of glycogen also store in the brain [6-12]. Therefore, we looked at the potential consequences of glycogen storage in the CNS using neuropsychological tests and brain MRIs from start of therapy. Previously we found that intelligence ranged from normal to mildly delayed. Early development was easily underestimated if motor functioning was poor [5, 13-15]. Brain MRIs (magnetic resonance imaging) showed predominantly periventricular white-matter abnormalities [15]. This was confirmed by a limited number of other studies in relatively young patients [16-18].

As no studies have related long-term neuropsychological follow-up results to the brain abnormalities in patients up to adulthood, we had two objectives: (i) to use brain MRI and neuropsychological tests to study the long-term consequences of glycogen storage on the CNS, and (ii) to relate imaging results to our findings on neuropsychological functioning in the oldest surviving patients.

METHODS

Patients

ERT with recombinant human alpha-glucosidase started in 1999 with a small group of patients. For the current follow-up study, we included the 11 oldest classic infantile patients of our current cohort (start of ERT between 1999 and 2009). Four initially received recombinant human alpha-glucosidase form rabbit milk. Since 2003 all patients were treated with ERT derived from CHO cells. The dose ranged from 20 mg/kg every other week to 40 mg/kg/week. Inclusion criteria were: GAA activity of <1%; severe mutations in the GAA gene; start of symptoms before six months; and hypertrophic cardiomyopathy at diagnosis. Study protocols had been approved by the Institutional Review Board. Written informed consent was obtained from the children's parents.

MRI

We performed magnetic resonance imaging (MRI) of the brain at least once per patient using a 1.5T system, or a 3 T system (EchoSpeed; GE Healthcare, Milwaukee, WI), and a dedicated 8-channel head coil. MRIs were scanned according to a standardized protocol including T1-weighted, T2-weighted, and FLAIR images. MRIs were scored by assessing white matter changes in several anatomical regions, including (i) the supratentorial region: frontal and occipital periventricular white matter, the centrum semiovale, corpus callosum, external capsule, posterior and anterior limb of the internal capsule (PLIC and ALIC); (ii) subcortical white matter, and U-fibers, and (iii) the infratentorial region: decussation, and cortical spinal tract at the brain-stem level. We also assessed ventricle size (lateral ventricles and fourth ventricles), and abnormal signal intensity and/or volume loss of the nucleus dentatus, basal nuclei and thalami. All MRIs were rated by two independent evaluators: a pediatric neuroradiologist (M.H.L), and a pediatric neurologist (J.M.P. vd H.). If opinions diverged, consensus was reached after deliberation.

Neuropsychological functioning

As early developmental tests in classic infantile Pompe patients can be highly influenced by motor functioning, [13, 15] we focused on the neuropsychological test in patients over the age of five years. Patients underwent regular neuropsychological assessments. These were intended to assess the following:

1. Age five years: early development: Griffiths Mental Developmental Scales
2. From six years onwards: intelligence; the most recent Dutch version of the Wechsler Intelligence Scales for Children – Third edition (WISC-III). The WISC-III-NL is divided into two scales or into three factors. The scales are verbal intelligence and performance intelligence. The factors are verbal comprehension, performance organization, and processing speed. For tetraplegic children, we used the Raven Progressive Matrices.

In a subgroup of patients we tested memory (Rey Auditory-Verbal Learning Test (RAVLT), Memory for Designs (NEPSY - Second Edition), language (Boston Naming Test, Comprehension of Instructions (NEPSY-II)), attention (Dot Cancellation Test, Inhibition (NEPSY-II)), executive functioning (Verbal Fluency (NEPSY-II), Digit Span (WISC-III-NL)), visual spatial functioning (Geometric Puzzles (NEPSY-II), Design Copying (NEPSY-II), Rey Complex Figure Test) processing speed (Processing Speed Index (WISC-III-NL)), behavior (Child Behavior Checklist (CBCL)). All tests were administered in their most recent normed and validated Dutch versions at time of assessment. The children were assessed by two pediatric neuropsychologists (F.A.; B.E). Two patients were tested outside our hospital, once each. One patient due to an MRSA infection. This patient was tested with the most recent Dutch version of the Wechsler Non-verbal Scales (WNV-NL). The other patient due to initiation of therapy in Germany was assessed with the most recent Dutch version of the Snijders Oomen Nonverbal Intelligence Test-Revised (SON-R 2½-7). As these two time-points were important to determine development over time, we decided to include these into the data set.

Statistics

Patients' test results were compared with the normative data for the Dutch population. The mean score for the intelligence tests is 100, with a standard deviation (SD) of 15 points. An Intelligence Quotient (IQ) above 85 indicates normal development, a score between 84-70 indicates mild developmental delay, and a score below 70 indicates intellectual disability. A disharmonic intelligence profile was defined according to the appropriate table of the instruction manual (using the 95% confidence interval). A significant decline in IQ was defined as a loss of more than 30 IQ points (>2 SD) or a decline in raw scores, and a declining tendency as a loss of more than 15 IQ points (>1 SD) [19]. For purposes of comparison, all neuropsychological tests were converted into Z-scores**.** A neuropsychological test score of more than 1.5 SD below a child's total IQ represents a weaker domain [19].

RESULTS

Patients

Table 1 summarizes the patient characteristics. The current age of the patients ranged from 7.6 to 17.7 years. Motor outcome varied. Six of the eleven patients achieved the ability to walk, two of whom were still able to do so at the last evaluation. Three patients had minimal motor functioning and were respirator dependent. Three patients died at the respective ages of 4.3, 4.4 and, 15.6 years.

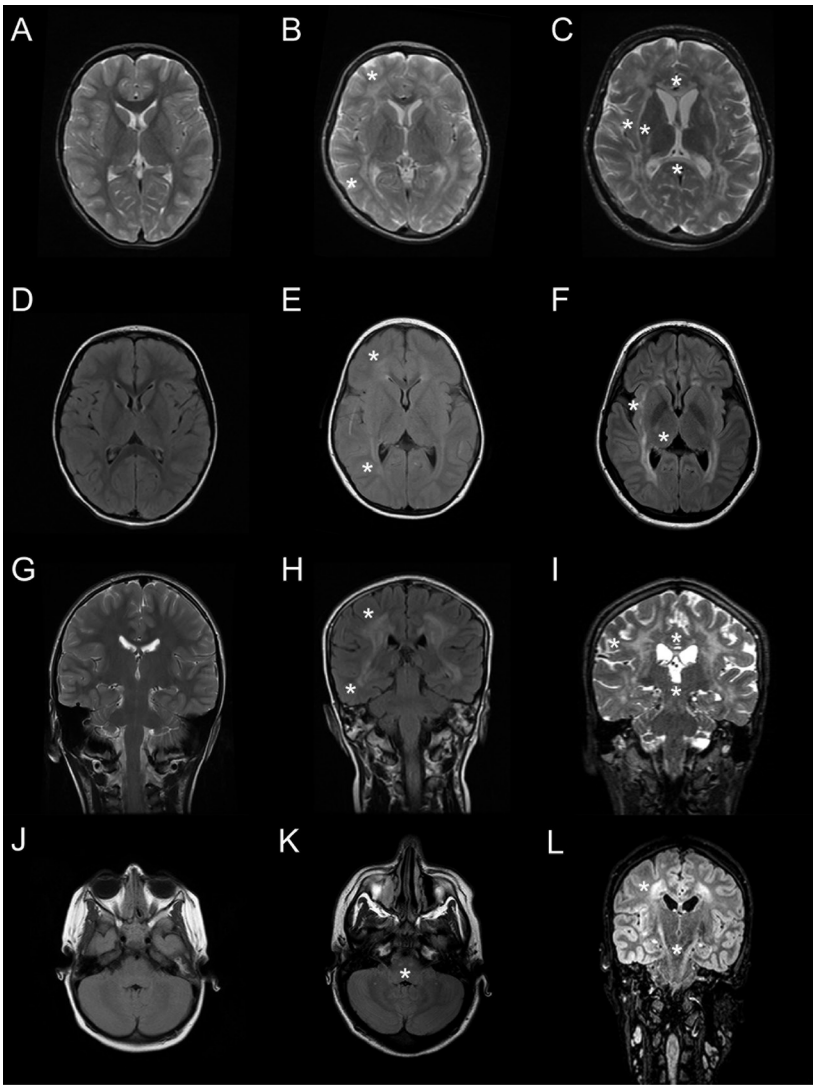
MRI

In total, 21 brain MRIs were performed in 11 patients. The patients' ages at the time of MRI ranged from 0.6 – 17.1 years. Five patients (age range 0.6 – 8.5 years) had one MRI. Six patients (age range 2.7 – 17.1 years) had two to four MRIs. The interval between the first and last MRI was 1.8 - 8.7 years. Table 2 presents the scores of all brain MRIs. At all ages, all patients had white-matter abnormalities in the centrum semiovale. Hyperintensities in the frontal and occipital periventricular areas started to appear at the age of 2.7 years. A tigroid hyperintensity-pattern developed in the occipital region at various ages.

After the age of 8.8 years (11 MRIs, five patients) MRIs started to show additional white-matter abnormalities in the subcortical areas, with sparing of the U-fibers (all patients and all MRIs), and with involvement of the external capsule (5/5 patients, 10/11 MRIs), of the PLIC (4/5 patients, 8/11 MRIs), and of the corpus callosum (4/5 patients, 9/11 MRIs). In the corpus callosum, hyperintensities were first observed in the splenium, and later in the genu and truncus. Abnormalities in the basal ganglia were found in three out of these five patients.

From the age of 11.3 years onwards (four patients, six MRIs), hyperintensities were noticed in the decussation (4/4 patients, 6/6 MRIs) and the corticospinal tracts (4/4 patients; 5/6 MRIs). In addition, all patients had widening of the lateral ventricles (4/4 patients; 6/6 MRIs). The fourth ventricle was widened in only two patients (2/4 patients; 3/6 MRIs). To illustrate the progressive nature of brain involvement in classical infantile Pompe disease. Figure 1 shows the sequential MRIs of the two patients with the longest follow-up.

Figure 1. Long-term MRI follow-up in patients with classic infantile Pompe disease.



A. Axial T2-weighted image of a healthy control subject aged 6 years. **B.** Axial T2-weighted image of patient 1, aged nine years. The arrows indicate periventricular white-matter abnormalities. **C.** Axial T2-weighted image of patient 1, aged 17 years. The arrows show additional involvement of the capsula externa, PLIC and corpus callosum. Note that the ventricles are also mildly widened. **D.** Axial Flair-weighted image of a healthy control subject, aged 6 years. **E.** Axial Flair-weighted image of patient 4, aged 5 years. The arrows indicate periventricular white-matter abnormalities. **F.** Axial Flair-weighted image of patient 4, aged 13 years. Arrows show the spread of white-matter abnormalities towards the capsula externa. The PLIC is slightly involved as well. **G.** Coronal T2-weighted image of a healthy control subject aged 9 years. **H.** Coronal Flair-weighted image of patient 1 (aged 9 years), showing involvement of the periventricular white matter. **I.** Coronal reformatted T2-weighted image of patient 1, aged 17 years. Arrows indicate spread towards the subcortical areas and corticospinal tracts. **J, K, L** Two transversal images and one coronal Flair-weighted image of patient 4 at the ages of 5 years (J) and 13 years (K,L). Image K and L show involvement of the corticospinal tracts, which is not seen on image J.

Table 1. Patient characteristics

Patient	Age at start ERT	Current age	Age Invasive ventilation	Mutations
1	0.3	17.4	-	c.2481+102_2646+31del538 c.1799G>A
2	0.6*	17.7	0.6	c.1115A>T c.525delT
3	0.2.	4.3 †	2.1	c.525delT c.525delT
4	0.7*	17.7	0.9	c.1913G>T c.1548G>A
5	0.2	4.4 †	2.0	c.2741delinsCAG c.2741delinsCAG
6	0.1	11.8	-	c.del525T c.1933G>T
7	0.04	11.3	-	c.2481+102_2646+31del538 c.2481+102_2646+31del538
8	0.001	9.0	2.7	c.1460T>C c.1460T>C
9	0.2	8.6	-	c.525delT c.2481+102_2646+31del538
10	0.2	7.6	-	c.2481+102_2646+31del538 c.2481+102_2646+31del538
11	0.2	15.6 †	3.2	U

All ages are stated in years (see also Ebbink et al., 2012); ERT = enzyme replacement therapy; CRIM = cross-reactive immunological material, LVMI = left ventricle mass index, P = positive; N= negative; U = unknown; MMF = minimal motor function; n: number of MRIs.

	CRIM status	Last LVMI Z-score <2	Hearing aids #	Impaired vision #	Best motor milestone	Last motor function
	P	Yes	Yes	Yes	Walking	Walking
	P	Yes	Yes	Yes	MMF	MMF
	N	No	Yes	No	Sitting	Sitting
	P	No	Yes	Yes	MMF	MMF
	N	Yes	Yes	No	Walking	Sitting
	P	Yes	Yes	Yes	Walking	Sitting
	P	Yes	Yes	Yes	Walking	Walking
	P	Yes	No	Yes	Walking	Sitting
	P	Yes	Yes	Yes	Standing	Sitting
	P	Yes	Yes	Yes	Walking	Sitting
	U	Yes	Yes	Yes	Sitting	MMF

† = deceased;
* = Patients 2 and 4 started therapy in the end stage of the disease; both had minimal motor function and became ventilator dependent before the age of one year.
# Hearing and vision were sufficiently compensated at time of neuropsychological testing.

Table 2. Results of the standardized scoring of MRI abnormalities in patients with classic infantile Pompe disease.

Patient	Age (years)	PWM centrum semiovale	PWM frontal/occipital	Corpus callosum	PLIC	ALIC	External capsule	Subcortical	U fibers	Deccusation	Corticospinal	Widened lateral ventricles	Widened 4th ventricle	Basal ganglia
2	0·6	1	0	0	0	0	0	0	0	0	0	0	0	0
5	1·5	1	0	0	0	0	0	0	0	0	0	0	0	0
10	2·7	1	1	0	0	0	0	0	0	0	0	1*	0	0
3	4·3	1	1	"1"	1	0	0	0	0	0	0	1	0	0
4	5·1	1	1	0	0	0	0	0	0	0	0	0	0	0
10	5·5	1	1	0	0	0	0	0	0	0	0	1*	0	0
4	5·8	1	1	0	0	0	0	0	0	0	0	0	0	0
10	7·3	1	1	0	0	0	0	0	0	0	0	1*	0	0
9	7·5	1	1	0	0	0	0	0	0	0	0	0	0	0
8	8·5	1	1	0	0	0	"1"	0	0	0	0	0	0	0
4	8·8	1	1	0	0	0	1	"1"	0	0	0	0	0	0
7	9·1	1	1	1	1	0	1	"1"	0	0	1	0	0	0
6	9·5	1	1	1	1	0	1	1	0	1	0	0	0	1
1	9·8	1	1	1	0	0	0	1	0	0	0	"1"	0	0
7	11·1	1	1	1	1	0	1	1	0	0	1	0	0	0
6	11·3	1	1	1	1	0	1	1	0	1	1	"1"	0	1
11	13·7	1	1	1	1	0	1	1	0	1	1	1	1	1
4	13·8	1	1	0	0	0	1	"1"	0	1	1	1	0	0
11	15·3	1	1	1	1	0	1	1	0	1	1	1	1	1
1	15·5	1	1	1	1	0	1	1	0	1	0	1	0	1
1	17·1	1	1	1	1	0	1	1	0	1	1	1	1	1

0 = normal, "1" = slightly abnormal, 1 = abnormal. *left ventricle.

PWM = periventricular white matter, PLIC = posterior limb of internal capsule, ALIC = anterior limb of internal capsule,

The shades of grey reflect the severity of involvement: from light (restricted) to dark (widespread).

Intelligence

Table 3 presents the total IQ scores and the scores on subscales of all patients from the age of five years onwards. A total of 34 intelligence tests were performed in nine patients. The age at the latest assessment ranged from 6.0 to 16.2 years. The total intelligence scores over the period ranged from <45 to 121.

Due to the various ages and varying degrees of motor disability, it was not possible to test all patients with the same instrument. Two tetraplegic patients were tested with the RAVEN (patient 2 at ages five, ten and 13 years; patient 4 at ages 12 and 14 years). During follow-up, development was stable in both; one had a normal intelligence and the other a mild developmental delay. Two other patients (patients 8 and 11) were tested with the Snijders Oomen Nonverbal Intelligence Test - Revised and the Wechsler Nonverbal Scale of Ability (age six and seven years). Their total intelligence scores lay in the range of intellectual disability.

Five patients (patients 1, 6, 7, 9, and 10) underwent multiple testing with the WISC-III-NL between the age of 5 and 16 years. Figure 2 shows the total IQ (TIQ); total verbal (TVIQ); total performance IQ (TPIQ); and processing-speed index (PSI) over time. The TIQ, TVIQ and TPIQ of the two youngest patients remained stable over time (patient 9 and 10); one patient had a normal development and the other a mild delay. The three patients with the longest follow up declined in their IQ-scores. Patient 1 had a declining tendency in TIQ and TVIQ from normal to mildly delayed. Patient 6 had a significant decline in TIQ and a declining tendency on TVIQ and TPIQ from mildly delayed to intellectual disability. Patient 7 had a declining tendency on TIQ and TPIQ from mildly delayed to intellectual disability. All five patients declined significantly in their processing-speed index. Patients 1, 7 and 10 had disharmonic profiles at the age of 6 years (TVIQ > TPIQ). Patient 1 became harmonic after a decline in TVIQ, and patients 7 and 10 remained disharmonic.

Follow-up MRI and IQ (patients 1, 6, 7. 9, 10)

To study the relationship between MRI and IQ, the IQs of the patients assessed with the Griffiths and WISC were related to brain MRIs performed at similar ages (time between MRI and IQ assessment < 1 year, range 0.5-9 months). In patients with involvement of the centrum semiovale and periventricular white matter only, intelligence was normal to mildly delayed (patients' ages 2.7 - 7.5 years). In those with additional white-matter abnormalities, the TIQ scores indicated problems ranging from mild developmental delay to intellectual disabilities (ages of patients 9 - 17 years). In these patients, a slight increase in white-matter involvement co-occurred with a declining tendency in their TIQ and TPIQ (patient 7), TVIQ (patient 6), and PSI (patients 1, 6 and 7).

Additional neuropsychological domains

Additional neuropsychological evaluations were performed in seven patients above the age of five years. Attention and visual-spatial integration were tested in the five patients who were able to perform the WISC III-NL (patients 1, 6, 7, 9 and 10). All five patients had problems in their sustained attention, but not in their selective attention. Visual-spatial integration problems were found in three patients (3/5).

Working memory, memory, language, and executive functioning were tested in six patients. These tasks, most of which were verbal, could also be performed in one fully tetraplegic patient. Working memory was abnormal in five patients (5/6). Two patients had memory problems (2/6). No specific problems were found with regard to language (0/6) and executive functioning (0/6). The Child Behavior Checklist was administered to the parents of three patients. Social problems were found in two patients. At the ages of 12 (patient 1) and 5 (patient 6) years there were no signs of behavioral problems on the CBCL. At the ages of 16 and 7 years, 4 and 2 years later, mild affective and mild oppositional behavior was reported in patient 1 and mild symptoms that could be suggestive of attention deficit hyperactivity disorder were reported for patient 6. Mild social problems were found in both patients.

Table 3. Subtest scores of intelligence tests in patients with classic infantile Pompe disease.

Patient \ Age		5	5.8	6	7	9	10	11	12	13	14	16
1	TIQ	97[a]		95	78	76[e]	74	78	82		79[e]	74
	TPIQ	88[a]		88	82	82[e]	81	86	88		92[e]	75
	POI				82	91[e]	85	93	93			82
	TVIQ	108[a]		101	79	75[e]	73	78	81		72[e]	79
	VCI				75	72[e]	78	83	80			80
	PSI			104	99	75	77[e]	72	77		67[h]	60[h]
2	TIQ	80[d]					76[d]			75[d]		
3	TIQ											
4	TIQ								92[d]		93[d]	
	TVIQ								87[e]		93[e]	
5	TIQ											
6	TIQ	75[g]	77[a]	75	68	48[f]	<45[f]	<45[f]				
	TPIQ	90[g]	78[a]	81	64	<55[f]	<55[f]	<55[f]				
	POI			85	71	49	<48	<48				
	TVIQ	80[g]	68[b]	74	76	<55[f]	<55[f]	<55[f]				
	VCI			78	80	56	<54	<54				
	PSI			65	55[h]	<55[f,h]	<55[f]	<55[f]				
7	TIQ	108[g]		84[e]	79	80	64					
	TPIQ	95[g]		78[e]	72	67	<55[f]					
	POI			83[e]	79	71	53					
	TVIQ	118[g]		93[e]	90	96	82					
	VCI			93[e]	92	97	80					
	PSI			77	75[h]	70[h]	62[h]					
8	TIQ				65[c]							
9	TIQ	81[g]			74	74						
	TPIQ	100[g]			75	70						
	POI				73	73						
	TVIQ	116[g]			79	82						
	VCI				82	88						
	PSI				102	77[h]						
10	TIQ	108[g]		116	121							
	TPIQ	103[g]		89	107							
	POI			85	112							
	TVIQ	152[g]		137	128							
	VCI			144	128							
	PSI			108	94[h]							
11	TIQ			54[a]								

Age = age in years.

TIQ = total intelligence test score, TPIQ = total performance intelligence, POI = perceptual organization index, TVIQ = total verbal intelligence, VCI = verbal comprehension index, PSI = processing speed index.

a = Sneijders Oomen Nonverbal Intelligence Test- Revised, b=WPPSI-III-NL (short version), VIQ, c = Wechsler Nonverbal Scale of Ability, d = Raven Progressive Matrices (short version), e = short version of WISC-III-NL, f= floor effect, g= Griffiths Mental Developmental Scales, h = decline in raw scores on PSI

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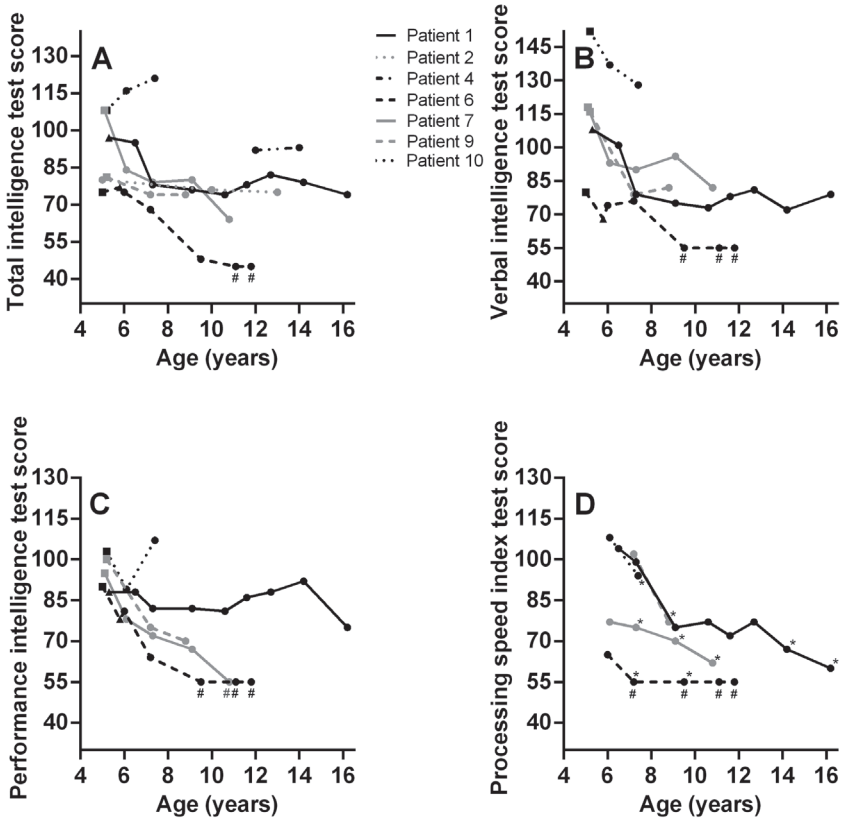
Figure 2. Intelligence-test scores of patients with classic infantile Pompe disease between the ages of 5 and 16 years



* = decline in raw scores in processing-speed index, # = floor effect, ▲ = SON-R 2 ½ -7, ■ = Griffiths Mental Developmental Scales, ● = WISC-III-NL.

DISCUSSION

With now over 17 years of experience with ERT in patients with classic infantile Pompe disease insights in long term outcome are gradually expanding. As a result of enzyme replacement therapy, survival and motor performance have improved significantly, and cardiac hypertrophy has resolved in most patients. One of the limitations of ERT is that it cannot cross the blood brain barrier. As time passes, our maturing patient population shows that the disease is not only a muscle disease, but also affects the brain.

Brain

We noted a characteristic three-stage pattern of white-matter involvement that evolves from periventricular to subcortical and from superior to inferior. For explicatory reasons, we subdivided the process, which is likely to be gradual, in three stages. In stage one (starting around age two), all the patients we evaluated had periventricular white-matter involvement at the level of the centrum semiovale. In stage two (from age eight onwards) the white-matter abnormalities expanded to the subcortical areas and internal and external capsule. In stage three (from age 11 onwards), infratentorial white-matter areas also became involved.

Although there seemed to be a characteristic pattern of involvement over time the extent of the abnormalities, and the rate of progression from stages one to three varied between patients. For example, brain MRIs in five patients aged between 8.5 and 9.8 showed that some had more abnormalities than others at the same age. Brain involvement appeared to be independent of motor functioning. For example, one patient who learned to walk within the normal age limits showed more abnormalities on the MRI than a patient who became tetraplegic before the age of one. Although white matter changes were seen in the capsula interna, we did not note spasticity as reported by Broomfield et al [20].

On the basis of all of these findings we conclude that the white-matter abnormalities on MRI in classic infantile Pompe patients indicate a varied but slowly progressive pattern of white-matter involvement. This finding is new. To date, predominantly periventricular white-matter abnormalities were described that are consistent with our stage one [15-17, 21]. The lack of description of progression towards more extensive white-matter involvement in earlier reports is explained by the absence of reports on MRI in children after the age of nine years, which is when we observed progression towards stages two and three.

Nonetheless, we note variations in the rate at which the white-matter affliction progresses. These explain the three cases reported (one by our own group) that indicate more extensive white-matter disease at a relatively young age (similar to stage two) [22-24]. Additionally, on brain MRIs we found white-matter abnormalities in the frontal regions. Theoretically this could lead to behavioral problems, this is an important aspect for future studies.

Neuropsychological profile

The variations in brain involvement are reflected by the wide range of intelligence-test scores, i.e., from normal intelligence to intellectual disabilities. This range is now wider than in earlier studies. The development over time varies from stable to neuropsychological decline. These findings contrast with those of our previous study and of four other studies on intelligence in classic infantile Pompe disease, where cognitive development was stable, ranging from normal to mildly delayed [13, 15, 16, 25]. Only one case report on a four-year old patient and a recent case report on a nine-year-old expressed concerns about 'a not yet fully described CNS phenotype' [23].

Prompted by the potential effect of the disease on intellectual performance shown by MRI, we performed additional neuropsychological tests, whose results showed a consistent neuropsychological profile. Due to visuospatial problems, patients appeared to be at risk of a disharmonic intelligence profile. All patients also had a lower processing speed and problems in their sustained attention, and several had problems with their working memory and social interaction.

The question remains whether neuropsychological outcome can theoretically be related to the white-matter involvement we found in our Pompe patients. Firstly, the white-matter abnormalities in our patients included the frontal and parietal areas. A recent conceptual model related intelligence to the interaction between the prefrontal and parietal cortex, which relies greatly on its white-matter connectivity [26]. This fronto-parietal integration network is believed to be involved not only in intelligence but also in other neuropsychological functions, such as attention, working memory, and processing speed [19]. As the white-matter abnormalities in our patients develop within this network, it is conceivable that intelligence and these specific neuropsychological functions are affected in our patients.

A limitation of our study is that the group of patients is relatively small, although it can be considered to be large for a rare disease like classic infantile Pompe disease. A strength of the study is that we could include long-term follow-up data from the four patients who were one of the first to start ERT in 1999 (of whom 3 are still alive) and from 7 others that started ERT before 2009.

IN CONCLUSION

As our patient population with classic infantile Pompe disease matures into adulthood, knowledge of this initially fatal muscle disease is broadening. It seems the brain is now becoming the next puzzle in the treatment. We advise, to expand follow-up programs to capture CNS/brain involvement in larger, international patient cohorts, to include the current knowledge in the counselling of parents before start of treatment and to include the brain as an additional target in the development of next-generation therapeutic strategies for classic infantile Pompe disease.

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Chapter 9

General Discussion
and
Future Perspectives

GENERAL DISCUSSION

It has been eighty-six years since the first publication on what is now called Pompe disease. Whether dr. Pompe realized when he published his thesis in 1936 that his discovery would lead to the vast number of researchers working in the field of Pompe disease and the amount of data they have collected over the years in their combined research papers, remains a mystery.

Over the years much has been learnt on the etiology and pathophysiology of Pompe disease. The effects of enzyme replacement therapy (ERT) on the short-term outcome, the survival and motor function revealed major clinical benefits. Now, the first long-term effects of ERT on clinical outcome are being published. The results have answered many questions, but also led to new questions and new treatment options aimed at to further improve the clinical outcome.

The primary aim of studies described in this thesis was to evaluate the long-term outcome of classic infantile Pompe patients (treated with ERT and immunomodulation) with the ultimate goal to improve the treatment of classic infantile Pompe patients. The results have been described in two parts; In **part one** the effects of a higher and more frequent ERT dose on (ventilator-free) survival and motor function were presented by comparing patients who started treatment on 20 mg/kg every other week (eow) with patients who started on 40 mg/kg/week. Part one also described the additional effects of immunomodulation on clinical outcome and anti-rhGAA antibody formation in an ERT-naïve setting (using a treatment protocol which combines Rituximab (RTX), Methotrexate (MTX) and intravenous immunoglobulin (IVIG)) as well as in a situation that high sustained anti-rhGAA antibodies had developed after treatment with ERT (using a treatment protocol which combines RTX, Bortezomib, Sirolimus and IVIG). In **part two** the long-term effects of ERT on cardiac parameters and cognitive development were evaluated.

First the main findings of this thesis are summarized. Later, the results are compared to the results found by our colleagues from around the globe. Finally, the future perspectives to further improve treatment and clinical outcome in the classic infantile Pompe patients are discussed.

MAIN FINDINGS

The main findings of this thesis are summarized in Table 1. The main findings of part one of this thesis is that patients who start on a higher and more frequent dosing of 40 mg/kg/week have a better ventilator-free survival and motor outcome than patients who start on 20 mg/kg eow. This effect is best seen in the outcome of the CRIM-negative patients: All three CRIM-negative patients in the 40mg group were alive and persistent walkers at study end compared to none in 20mg group. The added effect of immunomodulation on clinical outcome was less evident. Immunomodulation in an ERT-naïve setting prevented antibody formation only during B cell depletion, but when B cells reappeared high antibody titers developed in two of the three patients and it did not induce immune tolerance. In patients with high and sustained anti-rhGAA antibody titers, immunomodulation did not eliminate antibodies.

In part two it was demonstrated that the positive effects of ERT on cardiac size and function sustain over 13 years of treatment, but it was also shown that patients are and remain at risk for rhythm disturbances. And finally, it was shown that in the long run Pompe disease may affect the cognitive development. The cognitive development in classic infantile patients of whom the first now have reached adult age/are approaching adult age ranged from normal intelligence to intellectual disabilities. On brain MRIs the white-matter abnormalities that were previously observed, progressed over time. The extent of white matter abnormalities and the rate of progression varied between patients.

Table 1. Main findings of the studies in classic infantile patients described in this thesis

	Main Results	Future Perspectives
	PART 1	
Chapter 2 Double dosing 8 patients	- CRIM positive Patients treated with 40 mg/kg/week show better (ventilator-free) survival and motor outcome Than those treated with 20 mg/kg/eow - Higher dose possibly leads to higher anti-rhGAA antibody titer.	- Studies on the effects of dosing in larger cohorts are recommended.
Chapter 3 Primary immunomodulation	- Immunomodulation did not prevent anti-rhGAA antibody formation in an ERT naive setting. - Survival and motor outcome was good despite high titers.	- Improved immunomodulation protocols are needed for ERT naïve patients - Studies should include assays that study the neutralizing effects of anti-rhGAA antibodies. Studies in an international setting are recommended
Chapter 4 Secondary immunomodulation	- Immunomodulation did not eliminate anti-rhGAA antibodies that had been formed during ERT (only one patient showed a decrease of the titer). - Despite high titers both CRIM-negative patients were persistent walkers	- Improved immunomodulation protocols are needed for patients with high antibody titers - Studies should include assays that study the neutralizing effects of anti-rhGAA antibodies. Studies in an international setting are recommended
Chapter 5 Long-term outcome of double dosing	- Patients receiving 40 mg/kg/week had a better Survival, Ventilator-free survival and motor outcome than those treated with 20 mg/kg/eow, including CRIM negative patients - Patients on 40 mg/kg/week developed higher anti-rhGAA antibody titers. - Effect of immunomodulation is inferior to the effect of higher dosing on clinical outcome	- Studies on the long-term outcome of dosing in both CRIM positive and CRIM negative patients an international setting with patients on different dosages. - These studies should also address how antibody formation can be prevented
	PART 2	
Chapter 6 Long-term cardiological outcome	- Positive effects of ERT on cardiac hypertrophy sustain over at least 13 years. - ERT does not prevent rhythm disturbances; patients remain at risk for SVTs among other rhythm disturbances.	- Cardiological follow-up tests should be performed at least yearly. - Include diastolic function tests in follow-up (TDI and speckle tracking) to diagnose the potential emerging diastolic dysfunction at an early stage.

Table 1. Continued

	Main Results	Future Perspectives
	PART 2	
Chapter 7 Cognitive decline case report	- While cognitive development was earlier concluded to be normal or mildly delayed cognitive decline was observed in a 10-year-old patient. - Widespread white-matter abnormalities were found in subcortical areas, internal and external capsule and infratentorial areas on brain MRI.	- Studies on the cognitive development and white matter abnormalities are needed in a larger cohort.
Chapter 8 Long-term cognitive outcome	- Cognitive development ranged from normal to mild cognitive delays and some may show cognitive decline. - White-matter abnormalities were progressive over time, but the rate of progression varied between patients.	- International studies on the relationship between white-matter abnormalities and the cognitive function in classic infantile Pompe patients are needed - New treatment options are required that do not only target the muscles but also the CNS.

rhGAA: recombinant human alpha-glucosidase; ERT: enzyme replacement therapy; SVTs: supraventricular tachycardias; MRI: magnetic resonance imaging; eow: every other week; TDI: tissue doppler imaging.

EFFECTS OF (HIGHER) DOSING ON CLINICAL OUTCOME

Preclinical tests to the first clinical trials

From the first implementation of enzyme replacement therapy the dose to apply has been a matter of debate. The dose (15 to 20 mg/kg/week) applied in our very first clinical trial [1] (which was in fact the first trial performed worldwide) was based on preclinical studies. In the pre-clinical phase there were two production systems of alpha-glucosidase that were being tested; Chinese Hamster Ovarian Cells (CHO-cells) [2, 3] and milk from transgenic animals [4, 5]. The CHO-cell derived enzyme showed uptake in cultured fibroblasts and muscle cells from Pompe patients and lysosomal glycogen was cleared after 24 hours (dose 2000 nml/hr/ml). The enzyme activity and glycogen concentration remained within normal limits for 7 days [2, 3, 6]. In the Pompe knock-out mouse model, developed at the Erasmus MC [7, 8], a single injection of 100 µg of rhGAA from CHO-cells showed uptake of the enzyme in muscle tissue [4]. In the same study, Bijvoet et al. also published that enzyme derived from the milk of transgenic animals was taken up by heart and skeletal muscle tissue. Bijvoet et al. later reported that a single dose of 17 mg/kg led to normal alpha-glucosidase activity in muscle cells (but not in the brain) [5]. Long-term treatment (6 months) alpha-glucosidase showed that a dose of 17 mg/kg was insufficient to correct alpha-glucosidase activity in skeletal and heart muscle; a decrease in glycogen was not seen [5]. A dose of 68 mg/kg did decrease glycogen content in skeletal and muscle cells. These results from the preclinical tests showing that glycogen clearance was possible, was of great importance for the classic infantile Pompe patients as patients without treatment rarely survive beyond the first year of life [9, 10]. In the first clinical trials different dosing regimens and enzymes were applied (Table 2). Van den Hout et al. and Klinge et al. used enzyme derived from the milk of transgenic animals, Amalfitano et al. from CHO-cells [1, 11-13].

Van den Hout et al. included four patients who were all alive at 12 months of age and all had improved motor functioning [1]. Despite this major clinical benefit, the authors were cautious as two patients required invasive ventilation at the start of or just shortly after the start of treatment and could not be weaned off ventilation while on ERT. In all patients, alpha-glucosidase activity remained below normal values in muscle cells while on a 15-20 mg/kg/week dose. Because some patients did deteriorate the dose was then increased to 40mg/kg/week between 14 and 21 weeks. The results from the pre-clinical studies by Bijvoet et al. [5] showing that a higher dose led to a higher activity in muscle supported this decision. This increase led to normal alpha-glucosidase activity in muscle biopsies in all four patients, but histological assessment revealed that lysosomal glycogen storage was still present in three patients and normal muscle morphology was not obtained [1, 12, 14]. In the fourth patient (the youngest patients at start of treatment), normal alpha-glucosidase activity in muscle tissue led to normalization of muscle morphology. This patient learned

to walk. Several years later other authors found similar results in muscle biopsies that supported the idea that a higher and more frequent dose is required to correct alpha-glucosidase activity and improve muscle morphology [15, 16].

Klinge et al. describes the effect of a 40 mg/kg/week dose in two CRIM-positive patients [13]. Both patients remained ventilator-free after 12 months of treatment, but neither learnt to walk. In muscle biopsies some muscle fibers showed focal restoration filled with normal-appearing sarcomeres, other fibers showed moderate or even extensive vacuolization. Klinge et al. also observed that the younger patients at start of treatment had a better clinical outcome, but they could not relate a poor clinical outcome to CRIM status of anti-rhGAA antibody formation.

The effect of CHO-cell derived enzyme in three patients is described by Amalfitano et al. [11]. Patients received 5 mg/kg twice a week (total dose of 10 mg/kg/week). Two patients were CRIM-negative, they required invasive ventilation and did not learn to walk. The third patient (a CRIM-positive patient) did learn to walk. In retrospect this patient only had minimal cardiac involvement (the LVMI was only +2SD at start of treatment), which does not rule out that this patient had a milder phenotype as described by Slonim et al [17] which could explain the better clinical outcome. Overall, Amalfitano et al. related the poor clinical outcome in two of his patients to the age at start, CRIM status and antibody formation. One CRIM-negative patient from this study was later described by Hunley et al. and Banugaria et al [18, 19]. From these publications it became clear that this patient (patient 1 in Amalfitano et al) had a change in dose after 20 weeks of treatment from 5 mg/kg twice weekly to 10mg/kg twice weekly that was again increased to 10 mg/kg five times per week at week 56 of follow-up. This patient also received immunomodulation with Cyclophosphamide, plasmapheresis, intravenous immunoglobulins (IVIG and Rituximab (RTX) to eliminate anti-rhGAA antibodies. During treatment, patient 1 developed a nephrotic syndrome with subepithelial immune complex depositions. The initial conclusion from the authors that an rhGAA dose from CHO cells of 5 mg/kg twice weekly is sufficient to correct cardiac hypertrophy and improve skeletal muscle function might not be the case.

Follow-up studies using CHO-cell derived enzyme were performed by Kishnani et al. (Duke University) [20-22]. In 2006, they published results from a 52-week follow-up period of eight classic infantile Pompe patients (two CRIM-negative) receiving ERT dosed at 10 mg/kg/week [20]. Survival was 75% and 38% learnt to walk. All eight patients developed anti-rhGAA antibodies, with three patients developing a high titer (1:100,000). In the extension phase of this trial survival was only 33%; the two surviving patients were older than 3 years of age at study end and persistent walkers. The age at death for the deceased patients in

the extension study ranged from 23.5 to 33.8 months. Anti-rhGAA antibody formation and older age at start of treatment were given as the foremost reason of the poor long-term outcome of part of the patients given by the authors.

This study was followed by the AGLU-1602 trial that compared clinical outcome in patients receiving either 20 mg/kg eow with patients receiving 40 mg/kg eow [21]. Eighteen patients (four CRIM-negative) were included, nine in each dose group. Follow-up duration was 52-weeks. All 18 patients were alive after 52 weeks, six patients required invasive ventilation and seven learned to walk. The authors found no differences in clinical outcomes between the two dose groups. Sixteen patients were then enrolled into the 2009 extension study and were treated with their original ERT dose [22]. The median treatment duration was 2.3 years (range 1.1-3.0 years). Of these 16 patients, 13 (81%) were alive at study end (three CRIM-negative patients died). Nine (56%) survived ventilator-free and seven (44%) learned to walk. Fourteen patients (88%) developed anti-rhGAA antibody titers, in six (38%) of them high titers (titer ≥1:31,250) were observed. Five of six patients that developed high titers were in the 40 mg/kg eow group. The authors concluded their article that there was no real clinical benefit of the higher dose, but moreover that it might even increase the risk of high antibody titers. Unfortunately, there was also an overrepresentation of CRIM-negative patients in the 40 mg/kg eow group making it difficult to interpret the data. Of the four CRIM-negative patients in their study, three were enrolled in the 40mg group. Two of CRIM -negative patients in the 40mg group died during the study, the third CRIM-negative patient in the 40mg group died after the study concluded. The CRIM-negative patient in the 20mg group also developed high titers and died. As a CRIM-negative status is associated with a poor clinical outcome and the risk of developing high anti- recombinant human alpha-glucosidase (rhGAA) antibody levels[22-24, 26-30], the conclusion stated by the authors that there was no real clinical benefit of 40 mg/kg eow over 20 mg/kg eow must be interpreted with caution.

The results from the first three clinical trials and dose finding studies (AGLU-1602 trial) combined led to the marketing approval of human recombinant alpha-glucosidase (rhGAA) from CHO-cells for Pompe disease, with the recommended dose for all Pompe patients of 20 mg/kg eow. As a consequence, all newly diagnosed patients had to be started on 20 mg/kg/eow. Unfortunately, not all patients responded well. Based on the earlier results of "rabbit milk" trial, supported by the results from muscle biopsies and preclinical studies, all classic infantile Pompe patients at the Erasmus MC now receive 40 mg/kg/week.

Table 2. Outcome values of initial clinical trials, AGLU 1602 trial and long-term follow-up studies

	Year	Follow-up duration	N	CRIM -	Starting dose	Increase dose
Van den Hout et al.[1]	2000	36 weeks	4	1	15-20 mg/kg/week	40mg/kg/week
Amalfitano et al.[11]	2001	14-17 months	3	2	5mg/kg twice a week	10mg/kg 2 to 5 times weekly[A]
Van den Hout et al.[12]	2004	4 years	4	1	15-20 mg/kg/week	40mg/kg/week
Klinge et al.[13]	2005	12 months	2	0	40mg/kg/week	No
Kishnani et al.[20]	2006	52 weeks	8	2	10 mg/kg/week	20 mg/kg eow or weekly [B]
Kishnani et al.[21]	2007	52 weeks	18	4	9 on 20mg/kg eow 9 on 40mg/kg eow	No
Kishnani et al.[22]	2009	1.1-3.0 years	16	4[C]	8 on 20mg/kg eow 8 on 40mg/kg eow	No
Broomfield et al.[23]	2015	0.5-13.7 years	33	12	20 mg/kg eow	40 mg/kg eow or weekly (in some)
Hahn et al.[24]	2015	0.7-10 years	23	2	20 mg/kg eow	30 mg/kg eow to 40 mg/kg/week (in some)
Parini et al.[25]	2018	0.5-11.5 years	28	7	20 mg/kg eow	20 mg/kg/week to 40mg/kg/week (in some)
Van Gelder et al.[26]	2015	0.3-13.7 years	11	3	15-40 mg/kg/week	40mg/kg/week (in most)
This thesis 20mg	2018	0.6-12.6 years	6	2	20 mg/kg eow	40mg/kg/week (all)
This thesis 40mg	2018	3.0-8.3 years	12	3	40mg/kg/week	No

Eow: every other week; N.A.: not applicable; AB: antibody.
[A] See Hunley et al.[18] , many changes in patient 1. [B] Dose increase during the extension study (follow-up duration ranged from 4 months to 3 years duration).

	Survival	Vent. Free survival	Normal LVMI	Walking	Persistent walker	Peak AB ≥1:31,250
	100%	50%	Decreased in all	N.A.	N.A.	AB found
	100%	33%	33%	33%	N.A.	33%
	75%	25%	25%	25%	N.A.	75%
	100%	100%	Decreased in all	0	N.A.	50%
	75%	63%	25%	38%	25%	38%
	100%	66%	Decreased in all	39%	N.A.	16%
	81%	56%	44%	N.A.	44%[D]	38%
	61%	39%	48%	36%	33%	10%
	57%	43%	96%	39%	22%	8%
	61%	29%	54%	25%	19%	11%
	73%	55%	64%	55%	36%	55%
	66%	50%	83%	67%	17%	33%
	92%	95%	92%	92%	83%	58%

[C] 3 of the 4 CRIM negative patients were included in the 40mg group and developed high anti-rhGAA antibody titers. [D] 7 learned to walk of the 16 patients in the extension trial. The two patients would did not enroll in the extension trial both did not learn to walk.

Results of the first long-term outcome studies

From 2015 onwards, long-term follow-up studies were published with classic infantile Pompe patients receiving several years of treatment (with patients starting treatment on 20 mg/kg eow) [23-25].

Broomfield et al. observed an overall survival of 61%, a ventilator-free survival of 39% and 12 patients (36%) learned to walk, of whom 11 patients (33%) were persistent walkers [23]. The median ERT duration in his cohort was 3 years and 10 months, with a maximum follow-up of 13 years and 7 months. This study also included nine CRIM-negative patients receiving immunomodulation in an ERT-naïve setting, which are described in the next paragraph. These nine immunomodulation patients started ERT on a higher dose of 20mg/kg/week for 12 weeks before lowering the dose to the recommended dose of 20 mg/kg eow. Starting ERT on a higher dose before lowering the dose to the standard dose might have its effect on the clinical outcome of patients, but as these nine patients also received immunomodulation and most were CRIM-negative patients these effects are difficult to determine. In three patients the ERT dose was increased to 40 mg/kg/week because of a clinical decline. Not one of these three showed an improvement in motor function after dose increase, which might indicate that the dose increase was performed too late.

Hahn et al. observed an overall survival of 56%, a ventilator-free survival of 43% and nine (39%) patients learned to walk of whom five patients (22%) were persistent walkers [24]. In this retrospective study, the median age of the patients at the time of data collection was 5.2 years (range 3.6 to 10.2 years). In about half of the patients the ERT dose was increased after clinical deterioration (loss of motor function or frequent infections or ventilator dependency), after dose increase the authors found no improvement of motor function. As in the study by Broomfield et al, here it might also suggest that the dose increase was performed too late.

Parini et al. observed an overall survival of 61%, a ventilator-free survival of 29% and seven patients learned to walk (25%) of whom five patients (19%) were persistent walkers [25]. The median ERT duration in this cohort was 6 years (range 0.5 to 11.5 years). In nine patients ERT dose was altered due to poor clinical outcome or because of IARs; only one of these nine patients was a persistent walker. This study also included one patient receiving immunomodulation in an ERT-naïve setting and two patients receiving immunomodulation after high anti-rhGAA antibodies were formed in the overall outcome; the results are described in the next paragraph.

The results found by Broomfield et al., Hahn et al. and Parini et al. are different from the results found in this thesis. **Chapters 2 and 5** describe how the survival, ventilator-free survival and motor outcome of patients who started ERT on 40 mg/kg/week is much better than that of patients who started on 20 mg/kg eow. Patients who started ERT on 20 mg/kg eow had an overall survival of 67%, a ventilator-free survival of 50% and four (67%) patients learned to walk. Whereas patients who started on 40 mg/kg/week had an overall survival of 92%, a ventilator-free survival of 92% and 11 (92%) patients learned to walk. At the age of three years, 2 patients (33%) were still able to walk in the 20mg group compared to all 11 patients (92%) who learned to walk in the 40mg group. At study end, these percentages were 17% and 83%. The median age at study end in the 20mg group was higher at 9.6 years compared to 4.4 years in the 40mg group. While the differences in age at study end might explain some of the differences found between the two groups, it does not explain the better outcome of the CRIM-negative patients. All three CRIM-negative patients in the 40mg group were alive at study end and were persistent walkers at the ages of 4.8, 3.1 and 3.0 years, whereas both CRIM-negative patients in the 20mg group had died. These last results are different from the results of the CRIM-negative patients described in the literature as they had a 100% mortality when treated with the standard dose.

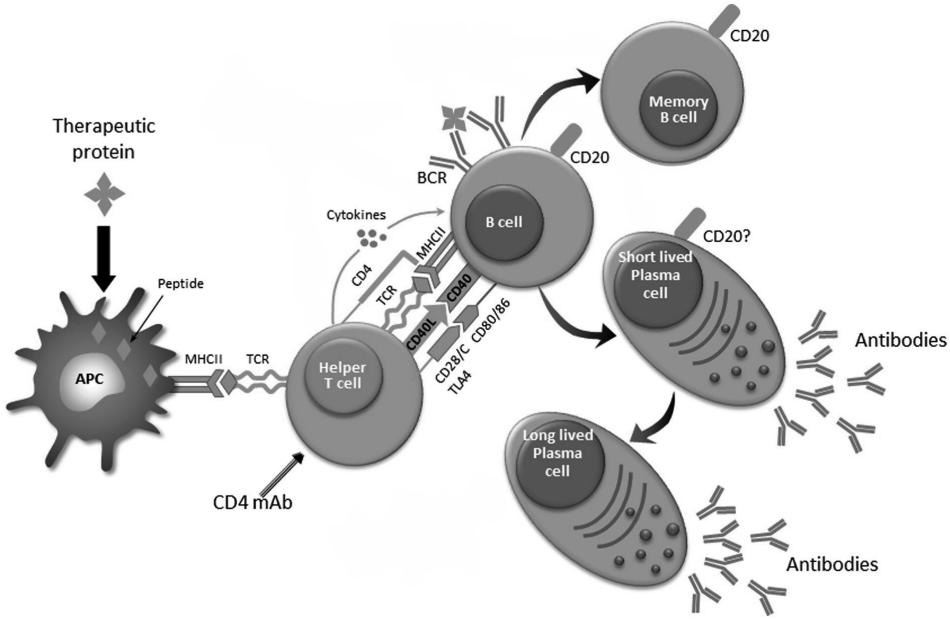
The four surviving patients of the 20 mg/kg eow had their dose increased to 40 mg/kg/week either because of serious respiratory infections, becoming ventilator dependent or after motor decline. While the dose increase did not improve their motor abilities (they remained weaker than patients who started on 40 mg/kg/week), it is most likely the reason why these patients are still alive, have less frequent respiratory infections on the higher dose and overall seem to have a more stable disease course.

EFFECTS OF ANTI-RHGAA ANTIBODIES ON CLINICAL OUTCOME

The discussion that antibody formation could pose a real threat to the efficacy of treatment was already ongoing before the clinical trials started. Because the classic infantile Pompe patients either have no residual enzyme activity, produce no alpha-glucosidase protein at all or produce an inactive altered alpha-glucosidase protein, the human body can see the infused recombinant enzyme as foreign and thus builds an immune response. To understand the development of anti-rhGAA antibodies, understanding of the immune system is key. The human immune system consists of the innate and adaptive immune systems. The innate immune system is also known as the in-born immune system and is the first and immediate line of defense against infections [31]. The cells of the innate immune system (among others the macrophages, dendritic cells and mast cells) are activated when they encounter foreign material or pathogens. The innate immune system does not provide long-lasting immunity to the host. Long-lasting immunity is achieved through the adaptive immune system, also known as the acquired immune system. Through cytokines and the activation of the complement cascade, the cells of innate immune systems activate the adaptive immune system through a process called antigen presentation. The cells of the adaptive immune system are the T and B cell lymphocytes. In Figure 1 the key players in the innate and adaptive immune system are depicted. In short, through a series of cascades, foreign material is recognized by macrophages, ingested and transported to antigen presenting cells (APC). The APC present the foreign material to T helper-cells who then increase the T cell response and activate the B cells to create immunity. The foreign material can be anything, from bacteria and viruses to funghi, or – as is the case of our patients – infused recombinant enzyme.

This immune response was observed by Bijvoet et al. in the Pompe knock-out mouse model in 1999 [5]. The authors found anti-rhGAA antibodies in some of the knock-out mice and argued that neutralizing effects of these antibodies could be a complication in the treatment of patients, but that the immune response might be an overestimation because the mice were infused with human recombinant enzyme. They also concluded that the risk of developing anti-rhGAA antibodies might be lower for children and adults with residual enzyme activity than for the classic infantile patients without any residual activity. But in the first trials by Van den Hout et al., Klinge et al. and Amalfitano et al. all the authors confirmed in patients what Bijvoet et al. had found in mice. In all studies the authors observed the development of anti-rhGAA antibodies in their patients [1, 11-13]. The levels of anti-rhGAA antibody titer varied between the patients. Anti-rhGAA antibody titers ≥1:31,250 were found in five patients in the first clinical trials (Table 2) [12, 13]. Of these patients, two were CRIM-negative.

Figure 1. Immune response to foreign material/pathogens



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Over the years many studies in the CRIM-negative patients followed and results in these patients were poor, as all the CRIM-negative patients described in the literature who were treated with ERT have died [1, 12, 21-24, 26-30, 33-36]. Often this poor clinical outcome has been reported to be related to the development of high anti-rhGAA antibody titers. These studies however also revealed that the development of high anti-rhGAA antibody titers is not limited to the CRIM-negative patients. CRIM-positive patients may also develop high titers [26, 28, 37]. Therefore, CRIM status is not the sole factor that determines immunogenicity. There are several product factors that are known to increase immunogenicity and lead to the development of antibodies [32, 38]. These product factors are (among others): dose, frequency and administration route of the infused enzyme, denaturing properties of the infused enzyme and impurities of the enzyme. The dose required to treat patients is considerably higher in Pompe disease (20 mg/kg eow) compared to for example Fabry disease (0.2- 1.0 mg/kg eow) [38, 39]. This alone could explain why nearly all classic infantile Pompe patients develop an immune response compared to 21-83% of Fabry patients, and why higher anti-rhGAA antibody titers were found in the patients on the higher dose in the AGLU 1602 trial [21, 22]. Denaturing properties of the infused enzyme also play a role. Unfortunately, the ELISA assay used to detect anti-rhGAA antibodies, cannot distinguish between antibodies directed towards native or denatured proteins. Only the active

enzyme is relevant for uptake and correction of the target tissues. This may explain why we found in the studies in **chapter 3** that more GAA protein was antibody-bound using the MS assay than when GAA activity was precipitated using an immunoprecipitation assay. This was further supported by low neutralizing effects found in cells when uptake of enzyme was studied in the presence of patient sera.

Before we can compare immune responses in patients, the discussion of what we call a high anti-rhGAA antibody titer needs to be addressed. One proposition came from Banugaria et al. who defined a high titer as having an anti-rhGAA antibody titer of 1:51,200 on two or more occasions [28]. In this publication the authors looked at the CRIM-positive patients who developed an antibody titer during treatment with ERT, before the titer spontaneously decreased (they called this tolerization). In the CRIM-positive patients who tolerized, the maximum titers found were 1:51,200. Using this as a cut off, the patients were then divided between into three study groups: the low titer CRIM-positive patients with titers below 1:51,200 (LTCP), the high titer CRIM-positive patients with titers above 1:51,200 (HTCP) and the CRIM-negative patients (CN). The clinical outcome in these three groups were then compared, with the patients receiving an ERT dose of either 20 mg/kg eow or 40 mg/kg eow. The LTCP had a better (ventilator-free) survival and motor outcome after 52 weeks than the HTCP and CN patients. Longer follow-up of patients (after the study ended) revealed that all CN and HTCP patients had deceased or had become ventilator dependent by 27 months and 33 months of age, whereas all LTCP were alive and only three required invasive ventilation (at almost 10 years of age).

The cut off value of 1:51,200 is similar to the cut off values found by Van Gelder et al. (1:31,250) where they studied the neutralizing effects of antibodies and related them to clinical outcome [26]. The authors found that the patients who did not learn to walk all had a high titer ≥1:31,250. They also found no inhibitory effects of anti-rhGAA antibody titers in patients with titers < 1:31,250. The authors estimated that (based on an arithmetic method reported by de Vries et al. [40]) a child with a titer of 1:156,250, with a blood volume of 80ml/kg and an ERT dose of 20 mg/kg, could see roughly 50% of the infused enzyme bound to antibodies. In a patient with a titer of 1:6,250 this, using the same arithmetic method, this would only be 5%. Theoretically, when patients receive a higher ERT dose of 40 mg/kg/week more antibody-free rhGAA should be available than in a patient receiving 20 mg/kg eow with the same titer.

Other studies have employed different cut-off values for 'high titers'. These range from 1:1,600 to 1:51,200 [19, 27, 32, 36, 41-51]. These studies make it clear however that we need to come to an agreement what we consider 'high titers' so that we can better interpret the effects of antibody titers on clinical outcome. If we use the cut off values proposed by Van

Gelder et al. that a titer of ≥1:31,250 can have inhibitory effects and apply this in to the AGLU-1602 trial, five patients (28%) had high anti-rhGAA antibody titers at last assessment [21, 22]. The high titers were only observed in patients in the 40 mg/kg eow group. This would suggest that higher titers are observed in patients receiving a higher ERT dose, but unfortunately three of these five patients were CRIM-negative. And the remaining CRIM-negative patient in the AGLU-1602 study (in the 20 mg/kg eow group) initially also had a high titer of 1:102,400 before a decrease in titer was observed at last assessment (titer of 1:25,600).

In **chapters 2 and 5** we described how a higher ERT dose led to a better (ventilator-free) survival and motor outcome and that this was most notable in the CRIM-negative patients. The patients who started in the higher dose did however develop a higher anti-rhGAA antibody titer than patients who started ERT on the standard dose. The median peak anti-rhGAA antibody titer of patients in the 40 mg/kg/week group was 1:156,250; much higher than the median peak titer in patients who started treatment on the 20 mg/kg eow (median titer of 1:6,250). In both groups there were patients with very low titers of 1:50 and 1:250.

As was observed by Banugaria et al. we also found that the development of high titers was not limited to the CRIM-negative patients. Of the 12 patients in the 40mg group, seven patients had a titer ≥1:31,250 at last assessment. Five of these patients were CRIM-positive. In the 20mg group, one CRIM-negative patient had a titer of 1:31,250 at last assessment (at 3 months of treatment, just before the patient died). In both the 20mg and 40mg group one CRIM-positive patient had a peak titer of 1:31,250 before the titer decreased to 1:6,250. After the dose was increased in the 20mg group, an increase in anti-rhGAA antibody titer was not observed, suggesting that tolerization occurs in classic infantile Pompe patients.

This thesis confirms that CRIM-negative patients are at risk to develop high titers, but that this risk is not limited to the CRIM-negative patients as nearly half of the CRIM-positive patients also developed high titers. Despite these high titers the clinical outcome is better in the patients on the higher dose than in our patients on the standard dose, or the patients described in literature. One likely explanation could be that there is an enzyme overload in patients receiving 40 mg/kg/week as was previously suggested by van Gelder et al [26]. But it does not alter the fact that antibodies can have a neutralizing effect on the efficacy of ERT and that strategies such as immunomodulation might improve the clinical outcome further.

IMMUNOMODULATION

Because neutralizing antibodies can have a negative impact on the clinical outcome as was described in the CRIM-negative patients [1, 12, 21-24, 26-30, 33-36], a protocol called immunomodulation to achieve immune tolerance was developed. The definition of immune tolerance is a state of unresponsiveness of the immune system to substances or tissues that have the capacity to elicit an immune response in the given organism. As all CRIM-negative and many CRIM-positive patients develop anti-rhGAA antibodies against the ERT, to achieve a state of immune tolerance could lead to an improvement in clinical outcome. There are two possible ways to achieve immune tolerance and both ways have been applied in classic infantile Pompe patients: to prevent anti-rhGAA antibody formation (**chapter 3**) [23, 42, 45, 47, 50] or to eliminate anti-rhGAA antibodies (**chapter 4**) [19, 42, 46, 48-51].

Prevention of anti-rhGAA antibody formation

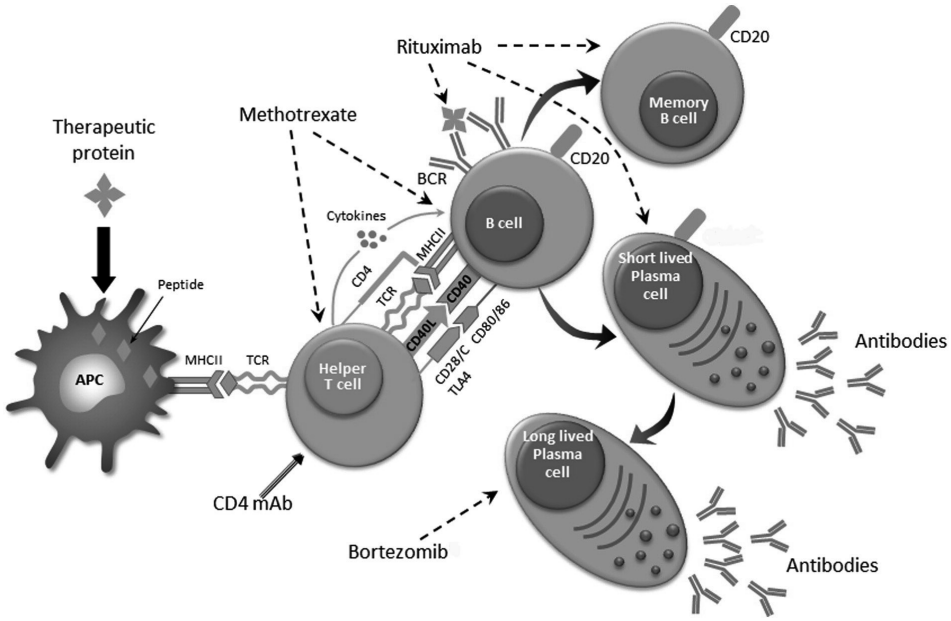
In the first study that tried to prevent anti-rhGAA antibody formation patients are treated with Rituximab (RTX), Methotrexate (MTX) and intravenous immunoglobulins (IVIG) before the first infusion of ERT (immunomodulation in an ERT-naïve setting) [42]. RTX - a chimeric monoclonal antibody - induces apoptosis in B cells expressing CD20 which is found in B cells from the pre-B cell to the mature B cell stage [52]. B cell depletion is rapid, in some patients even within 24 hours after the first dose. The first infusion with ERT should be given 24 hours after the first RTX infusion to let the RTX deplete all the B cells. B cell depletion can be as long as 17 months. In adults with rheumatoid arthritis treated with RTX, time to repopulation of B cells ranged from zero to 17 months [53]. MTX inhibits T cell activation and thus impairs the communication between T cells and B cells [32]. While IVIG was given to prevent infections, IVIG itself has immunomodulatory properties [54]. The mechanism of these medications is shown in Figure 2.

Effect of immunomodulation on antibody formation

The effects of immunomodulation in an ERT-naïve setting on anti-rhGAA antibody formation and clinical outcome was studied in five different studies comprising of 24 patients in total (Table 3) [23, 42, 45, 47, 50]. In two studies, treatment was successful in preventing anti-rhGAA antibody formation [42, 50]. Messinger et al. had one patient that remained antibody-free for up to two years following cessation of temporary RTX treatment, while the other patient developed a titer of 1:400 after 18-24 months [42]. Stenger et al., using a slightly different temporary immunomodulation protocol (see Table 3 for details), reported low antibody titers of 1:3,200 after 12 months of treatment in his patient [50]. In the other three publications, higher titers were observed. Banugaria et al. reported a titer of 1:6,400 in two of the seven patients [47]. This lead to an adaption of their initial immunomodulation protocol so that patients with titers >1:6,400 would receive a second round of immunomodulation.

Broomfield et al. reported that nine patients received temporary immunomodulation and only one developed an antibody titer of 1:12,800 [23]. Finally, Elder et al. found high anti-rh-GAA antibody titers in its first patient that received immunomodulation (titer of 1:500,000). All subsequent patients that were included continued to receive RTX infusions every 12 weeks to suppress antibody formation [45].

Figure 2. Immune response to foreign material/pathogens



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In **chapter 3** the effects of immunomodulation in ERT-naïve patients on anti-rhGAA antibody formation in three patients was studied. The protocol used in our patients (four weekly RTX infusions, weekly MTX and monthly IVIG) led to B cell depletion for at least 6 months. During this time immune suppression was observed. After B cell recovery all three patients developed an antibody titer. In two patients (a CRIM-negative and a CRIM-positive patient) high sustained antibodies titers were observed. The peak titer was 1:800,000 in patient 1 at 13 months of ERT and 1:200,000 in patient 3 at 14 months of ERT. Patient 2 (CRIM-positive) had a peak titer of 1:6,250 at 21 months of ERT. We also studied the effects of the high antibody titers by assessing the neutralizing properties of the antibodies. Immunoprecipitation assays showed that the neutralizing effects of antibodies was mild to moderate (20 %) in patient 1. In the other two patients, no neutralizing

effects were observed. When the same samples analyzed using mass spectrometry, a considerable percentage of rhGAA protein was captured by antibodies during infusion in patient 1 (37%), but only a small fraction (2 %) of enzymatically active GAA was captured. The differences in outcome can be explained by the differences between these assays. The data from the mass spectrometry suggests that infused rhGAA may undergo denaturation upon prolonged exposure to the circulation. The ELISA assay, used to determine the antibody titer, cannot distinguish between antibodies directed towards native or denatured proteins, suggesting that ELISA titers per se are insufficient to predict the potential neutralizing effects of antibodies. Additional analysis using neutralizing assays (by immunoprecipitation) and mass spectrometry are required to more accurately link antibody titer to antibody function.

Effect of immunomodulation on clinical outcome

The overall clinical outcome of the patients receiving immunomodulation in an ERT-naïve setting is described in literature (n=24) is also described in Table 3. In only one patient a high anti-rhGAA antibody titer observed (patient A from Elder et al.), while the clinical outcome was still poor. During follow-up (ranging from 4.2 months to 49.4 months) the overall survival was 79%, ventilator-free survival was 50% and only 6 patients learned to walk (25%). While the results on survival and ventilator-free survival are better than reported in the literature for the whole group of patients treated with ERT, the results on motor function were not better than the results achieved in patients on ERT monotherapy dosed at 20 mg/kg eow (see Table 2). The results in clinical outcome of the patients described in chapter 3, was better than those in literature as all three patients were alive at study end, did not require invasive ventilation and all were persistent walkers at study end (after 24 months of follow-up). This was despite the high anti-rhGAA antibody titer that was found in two patients, one of whom was even CRIM-negative.

There are a number of possible explanations for the differences found in clinical outcome and antibody titers. The first is that there were variations in the immunomodulation protocols: the duration and dosage of MTX treatment varied, and in some cases MTX was replaced by Mycophenolate or Rapamycin. It is unknown to what extent these variations have an effect the immunomodulatory and clinical outcome. Second, the age at which ERT and immunomodulation was started varied between patients. It has been reported that the older patients (older than 2 months of age at start of ERT) are likely to develop higher antibody titers [26]. Third, the majority of patients included were CRIM-negative, who are known to have a higher anti-rhGAA antibody titer and to have a poor clinical outcome [26, 28]. As the anti-rhGAA antibody titer varies between the patients in the different studies, it suggests that other factors play a role in the clinical outcome. This could possibly be the ERT dose. Our patients who received a higher dosage of ERT of 40 mg/kg/week had a

better clinical outcome than the patients who received 20 mg/kg eow. The higher ERT dose is most likely the cause of the good clinical outcome in our patients. This was explained by Van Gelder et al., that the neutralizing effects of antibodies will be less severe in patients on a higher ERT dose compared to a regular dosage, as more antibody-free rhGAA will be available [26]. Concluding, the effects of immunomodulation in an ERT-naïve setting on antibody formation and clinical outcome are heterogeneous. It is important to optimize immunomodulation protocols so that we can provide long-term prevention of antibody formation without compromising general immunity in Pompe patients treated with ERT and improve the clinical outcome. Also, the higher dose applied in our patients did seem to improve clinical outcome. These effects must also be studied n larger cohorts.

Elimination of anti-rhGAA antibodies

In patients who already receive ERT and have developed an immune response, a different approach is required to eliminate the antibodies. B cells develop into (long and short lived) plasma cells that produce antibodies and into memory cells B cells where our immunity is stored. In those patients with an immune response, B cells and memory cells must be eliminated. To achieve this, Bortezomib was added to the immunomodulation treatment protocols to eliminate antibodies (Figure 2) [32, 55]. Bortezomib, a proteasome inhibitor that causes apoptosis of short and long-lived plasma cells by blocking the proteasome pathway. The efficacy of the protocols to eliminate antibodies was studied in six studies comprising of eight patients in total (five were CRIM-negative) [19, 42, 46, 48-51]. The ERT dose varied in these patients between 20 mg/kg eow and 40 mg/kg/week. The results are described in Table 4.

In the study published by Messinger et al. two CRIM-negative patients who had developed an anti-rhGAA antibody titer of 1:1,600 and 1:12,800, received immunomodulation with RTX, MTX and IVIG [42]. Messinger et al. observed B cell recovery after they gave 14 and 15 RTX infusions. After B cell recovery, anti-rhGAA antibodies did not return. Kazi et al. (a longer follow-up study of the two patients previously described by Banugaria et al in 2013 [46]) and Stenger et al. used the Messinger protocol and added Bortezomib to try and eliminate anti-rhGAA antibodies [50, 51]. Their three patients with titers between 1:200,000 – 1:819,200 all showed a decline in titer at their last assessment or were antibody-free (titer ranged from 0 to 1:1,200). Banugaria et al. described the patient that was previously described by Amalfitano et al. and Hunley et al. This patient received Cyclophosphamide instead of Bortezomib and continued to have high titers despite immunomodulation (titer increased from 1:25,600 to 1:204,800; at last assessment the titer was 1:102,400) [19]. Markic et el. (who used the Messinger protocol) and Deodato et al. (who relied on plasmapheresis to eliminate antibodies) applied their immunomodulation protocol in patients with low anti-rhGAA antibody titers (titers of 1:6,400 and 1:3,200). After B cell recovery titer remained low to undetectable [48, 49].

Table 3. Overview of the literature of immunomodulation an ERT-naïve setting

Study	Pt	CRIM	Age start ERT	Follow up	Alive	Vent. free	Pers. Walker	IM treatment
Messinger	3	Neg	16 w	24 m	Yes	Yes	Yes	1A
2012 [42]	4	Neg	15 w	24 m	Yes	Yes	Yes	1A
Elder	A	Neg	8 m	11 m	No	No	No	2A
2013 [45]	B	Neg	8 m	36 m	Yes	No[1]	No	2B
	C	Neg	2.8 m	30 m	Yes	Yes	No	2B
	D	Neg	6 m	24 m	Yes	Yes	No	2B
	E	Pos	3 m	22 m	Yes	Yes	No	2C
Banugaria	1	Neg	3.0 m	101 w	Yes	Yes	No	1E
2013 [47]	2	Neg	4.1 m	92 w	Yes	NIV	No	1E
	3	Neg	2.4 m	89 w	Yes	Yes	Yes	1E
	4	Neg	0.1 m	70 w	Yes	No	No	1E
	5	Neg	0.5 m	59 w	Yes	NIV	No	1E
	6	Neg	1 m	51 w	Yes	NIV	No	1E
	7	Neg	1 m	48 w	No	No	No	1E
Stenger 2015 [50]	1	Pos	1.2 m	17 m	Yes	Yes	Yes	1B
Broomfield	6	Neg	4.7 m	4.2 m	No	No	No	1C
2016 [23]	7	Neg	6.6 m	5.4 m	Yes	Yes	No	1C
	8	Neg	2.4 m	9.6 m	No	No	No	1C
	12	Neg	6.7 m	8.9 m	Yes	Yes	No	1C
	14	Neg	18 d	17.5 m	No	No	No	1C
	16	Neg	36 w#	33 m	Yes	Yes	Yes	1C
	17	Neg	5.2 m	28.4 m	Yes	Yes	Yes	1C
	19	Pos	3.9 m	32.9 m	Yes	NIV	No[2]	1C
	22	Pos	2.2 m	49.4 m	Yes	NIV	No	1C
Chapter 3 2018	1	Neg	5.8 m	~24 m	Yes	Yes	Yes	1D
	2	Pos	4.2 m	~24 m	Yes	Yes	Yes	1D
	3	Pos	3.1 m	~24 m	Yes	Yes	Yes	1D

Pos: Positive; Neg: Negative; IM: immunomodulation; NA: not applicable; ND: not determined; NIV: non-invasive ventilation; RTX: Rituximab intravenous; MTX: Methotrexate.

patient 16 in Broomfield et al was born at 34 weeks gestational age and ERT was initiated at 36 weeks of gestational age. ^ IM treatment is described in table legend.

[1] Patient B became ventilator dependent after follow-up. [2] Patient 19 lost ability to walk 4 months after gaining it, within follow-up period. [3] Ref 15: B cell recovery 4 to 9 months after last RTX; Personal communication, A. Broomfield. [4] Time after B cell recovery in ref 15 was 4-9 months after last RTX, time given here in age when tested.

IM duration	IM repeat	Time since last RTX	B cell recovery[3]	Peak titer (time after B cell recovery)[4]	Neutralizing effects
5 w	No	30 m	Yes	1:1,600 (2m)	ND
5 w	No	17 m	Yes	0 (9m)	ND
5 w	No	10 m	Yes	1:500,000 (5m)	ND
RTX ongoing	No	<12 w	No	NA	ND
RTX ongoing	No	<12 w	No	NA	ND
RTX ongoing	No	<12 w	No	NA	ND
RTX ongoing	No	<12 w	No	NA	ND
5 w	No	96 w	Yes	0 (81w)	ND
5 w	No	87 w	Yes	0 (67w)	ND
5 w	No	84 w	Yes	0 (69w)	ND
5 w	No	65 w	ND	0 (NA)	ND
5 w	Yes	24 w	No	1:6,400 (before)	ND
5 w	Yes	8 w	No	1:6,400 (before)	ND
5 w	No	43 w	Yes	0 (8w)	ND
5 w	No	39 w	Yes	1:3,200 (39w)	ND
5 w	No	~6 m	Yes	0 (>4-9m)	ND
5 w	No	~3 w	Yes	0 (0.6y)	ND
5 w	No	~9 m	Yes	0 (>4-9 m)	ND
5 w	No	~16 m	Yes	0 (0.9y)	ND
5 w	No	~17 m	Yes	0 (>4-9m)	ND
5 w	No	~31 m	Yes	0 (>4-9m)	ND
5 w	No	~28 m	Yes	0 (1.8y)	ND
5 w	No	~32m	Yes	1:12,800 (1.3y)	ND
5 w	No	~49 m	Yes	0 (2.5y)	ND
MTX ongoing	No	23 m	Yes	1:800,000 (6.3m)	mild/ moderate
MTX ongoing	No	23 m	Yes	1:6,250 (7.3m)	No
MTX ongoing	No	23 m	Yes	1:200,000 (6.3m)	No

Immunomodulation (IM) treatment used per study:

1A RTX 375 mg/m^2/dose for 4 doses; MTX 0.4 mg/kg/dose subcutaneous for 9-17 doses; IVIG 500 mg/kg administered once in patient 1.

1B RTX 375 mg/m^2/dose for 4 doses; MTX 15 mg/m^2/dose oral for 9 doses; IVIG 400-500 mg/kg twice.

1C RTX 375 mg/m^2/dose for 1-4 doses; MTX 0.4 mg/kg/dose subcutaneous for 10-18 doses.

1D RTX 375 mg/m^2/dose for 4 doses; MTX 1 mg/kg/wk intravenous for the duration of follow up; IVIG 400 mg/kg monthly.

1E RTX 375 mg/m^2/dose for 4 doses; MTX 0.4 mg/kg/dose subcutaneous for 9 doses; IVIG 400-500 mg/kg 5-6 times.

2A RTX 750 mg/m^2/dose for 2 doses; Mycophenolate 300 mg/m^2/day oral; IVIG 500-1000 mg/kg monthly.

2B RTX 375 mg/m^2/dose for 3 doses + repeat RTX every 12 weeks; Sirolimus 0.6-1 mg/m2/day oral; IVIG 500-1000 mg/kg monthly.

2C RTX 750 mg/m^2/dose for 2 doses+ repeat RTX every 12 weeks; Sirolimus 0.6-1 mg/m2/day oral; IVIG 500-1000 mg/kg monthly.

Table 4. Overview of the literature on immunomodulation in classic infantile Pompe patients after antibodies have formed

Study	Pt	CRIM	Age at start of ERT	Age at start of IM	IM protocol	IM duration	Current ERT dose[1]
Messinger 2012 [42]	1	Neg	7 w	0.5 y	1	40 m	20 eow
	2	Neg	16 d	2 m	1	IVIG ongoing	40 w
Banugaria 2012 [19]	1	Neg	4.2 m	8.8 m	2	RTX twice	variable
Markic 2013 [48]	1	Pos	5 m	17.5 m	1	46 w	20 eow
Deodato 2013 [49]	1	Neg	7 m	13 m	3	3 w	20 eow
Stenger 2015 [50]	1	Pos	23 d	11 m	4	Ongoing	20 eow
Kazi 2016 [46, 51]	1	Pos	6.0 m	2.4 y	5A	3 y	40 w
	2	Neg	4.2 m	2 y	5B	Ongoing	40 eow
Chapter 4 2018	1	Pos	2.4 m	6.6 y	6	Rap/IVIG ongoing	40 w
	2	Neg	5.8 m	3.5 y	6	Rap/IVIG ongoing	40 w
	3	Neg	1.9 m	2.3 y	6	Rap/IVIG ongoing	40 w

[1] Excluding Banugaria 2012 (one patient) and the patients in our study, all patients started ERT dosed at 20 mg/kg every other week. [2] Patient did learn to walk, but lost the ability at the age of 6 years and did not regain the ability when immunomodulation was started. [3] Titer was previously 1:25,400. [4] Titer was previously 1:800,000. Pt: Patient; CRIM: cross-reactive immunologic material; Pos: Positive; Neg: Negative; ERT: enzyme replacement therapy; w: weeks; m: months; y: years; IM: immunomodulation; eow: every other week; RTX: Rituximab; Vent. free: ventilator-free survival; MTX: Methotrexate; IVIG: intravenous immunoglobulin. Rap: Rapamycin

Follow-up since start of IM	Alive	Vent. free	Learned to walk	Titer at start of IM	Number of RTX infusions	B cell recovery	Last known titer (time since B cell recovery)
4.6 y	Yes	No	No	1:1,600	15	Yes	0 (20 m)
3 y	Yes	Yes	Yes	1:12,800	14	Yes	0 (10 m)
2.5 y	No	No	No	1:25,600	6	No	1:102,400 (before recovery)
3 y	Yes	No	No	1:6,400	8	Yes	0 (unknown)
22 m	Yes	No	No	1:3,200[3]	1	Yes	1:100 (16 m)
13 m	Yes	No	No	1:200,000	11	No	1:1,200 (no recovery)
5.5 y	Yes	No	No	1:204,800	19	Yes	0 (2.5 y)
6.9 y	Yes	No	No	1:819,200	52	Yes	0 (4 w)
2.5 y	Yes	Yes	Yes[2]	1:156,250	3	No	1:31,250 (before recovery)
2.1 y	Yes	Yes	Yes	1:156,250[4]	3	Yes	1:31,250 (2 y)
1.5 y	Yes	Yes	Yes	1:781,250	3	Yes	1:156,250 (1.5 y)

Immunomodulation (IM) protocol used per study:

1 RTX 375 mg/m^2/dose for 4 weekly iv doses followed by maintenance doses; MTX 0.5 mg/kg weekly oral doses; IVIG 500 mg/kg/month

2 Cyclophosphamide 15mg/kg iv on day 1 followed by 2mg/kg/day iv for 9 days, IVIG 400 mg/kg day 5 through 9; Plasmapheresis day 1, 3 and 5 in week 20, 34 and 56. Between week 34 and 56 oral Cyclophosphamide 2 mg/kg was given. Followed by iv RTX 375 mg/m^2/week in weeks 99 through 102 and in weeks 140 and 141.

3 Plasmapheresis on days 1, 3 and 5. RTX 375 mg/m^2 iv once on day 7, directly followed by IVIG (dose not mentioned), with 4 extra IVIG doses over the following 8 months.

4 RTX 375 mg/m^2/dose iv followed by 10 maintenance doses; Bortezomib 1.3mg/m^2/dose in 2 sessions of 4 iv doses. MTX 0.5 mg/kg for 27 oral doses; IVIG 500 mg/kg for 5 doses.

5A Cyclophosphamide 250mg/m^2 iv twice; RTX 375 mg/m^2/dose in 2 sessions of 4 doses followed by 11 maintenance doses; Bortezomib 1.3mg/m^2/dose in 3 sessions of 4 iv doses; MTX 15mg/m^2 oral doses; IVIG 400-500mg/kg/month

5B RTX 375 mg/m^2/dose for 4 iv doses followed by RTX maintenance doses 70 weeks later; Bortezomib 1.3mg/m^2/dose in 4 sessions of 4 iv doses; MTX 15mg/m^2 oral doses; IVIG 400-500mg/kg/month

6 RTX 375 mg/m^2/dose for 3 iv doses; Bortezomib 1.3mg/m^2/dose for 6 iv doses. Rapamycin daily according to body weight from week 4 onwards; IVIG 500 mg/kg/month

In **chapter 4** we describe the effects of our immunomodulation protocol in three of our patients with high sustained antibodies. Patients received a three-week treatment protocol with Rituximab and Bortezomib, followed by daily Rapamycin and monthly IVIG. Patients received 40mg/kg/week rhGAA. A substantial decrease in anti-rhGAA antibody titer was observed in only one (CRIM-negative) patient. This was the patient that had previously received immunomodulation in an ERT-naïve setting (patient 1 in **chapter 3**). When it was decided that this patient should start secondary immunomodulation at the age of 3.5 years, his titer was 1:800,000. An additional sample taken at the actual start of secondary immunomodulation was slightly lower (1:156,250) and declined further to 1:6,250. It is unclear whether the decline in titers was due entirely to immunomodulation, or whether a decline was already in progress. At the time secondary immunomodulation was started at the age of 3.5 years the patient still received MTX in a dose of 0.5mg/kg once a week (after ERT infusion). At last assessment, the titer had increased to 1:31.250. In the other two patients, limited effects on anti-rhGAA antibody titer were observed. The effect of immunomodulation to eliminate antibodies was limited in our study compared to the effect in the other reports. In seven of the eight patients RTX maintenance infusions were given (every four to 12 weeks) for a maximum of 52 infusions [42, 48, 50, 51]. Bortezomib was repeated in three patients (eight to 16 infusions) [50, 51]. Cyclophosphamide in two patients (two to 10 infusions and oral maintenance in one patient) [19, 51]. Methotrexate (MTX) was given in six patients. Our patients receive Rapamycin instead of MTX. The differences in the immunomodulatory protocols that were applied could explain the differences in antibody titer. It is possible that longer and/or more frequent dosing of RTX, Bortezomib and/or Rapamycin could be more effective in preventing immune responses.

The clinical outcome of the patients with immunomodulation reported in literature was similar to the clinical outcome of patients on ERT monotherapy. Survival was 87.5%, ventilator-free survival was 12,5% and only one patient (12,5%) learned to walk. The clinical outcome of the patients described in chapter 4 was a lot better, as all were alive at study end, none required invasive ventilation and two were persistent walkers. The persistent walkers were both CRIM-negative patients. The patient that had lost the ability to walk did not regain it but did not lose further motor function. So, while there were slight differences in the immunomodulation protocols, not one particular protocol seems to be better than the rest. Moreover, it seems that the best clinical outcome was more related to ERT dose than immunomodulation as the patients with the best clinical outcome were receiving 40 mg/kg/week from start.

Future studies are required in which higher doses are applied form start and also on how immunomodulation in the broadest sense can be improved both to prevent and to eliminate antibodies.

Additional clinical outcomes

While the main aim of this these was to describe the long-term effects of dosing and immunomodulation on survival, ventilator-free survival, motor outcome and anti-rhGAA antibody formation, some additional clinical outcome parameters of cardiac response and effects of treatment on the central nervous system, which are also relevant for future treatment decision making, are presented in Part 2 of this thesis as well.

Cardiac response

Left ventricle dimensions

There is only limited knowledge in the long-term effects of ERT on the heart. Most studies that described the effects of ERT on the heart either followed patients for 12 months of treatment or included the patients of the initial clinical trials [12, 21, 56, 57]. These studies found an overall reduction in left ventricular mass index (LVMI), a reduction in the left ventricular posterior wall thickness in diastole (LVPWd), or a reduction in the left ventricular mass (LVM) in patients treated with 20 mg/kg eow. Only two studies specifically reported on the effects of ERT on cardiac size and function in patients treated for more than 12 months [22, 30]. In the AGLU-1602 trial Kishnani et al. described the effect of a different dosing strategy (either 20 mg/kg eow or 40 mg/kg eow) on cardiac outcome[22]. After a median follow-up of 2.3 years, a total of seven CRIM-positive patients (39%) had a normal LVM at last assessment. Three of these patients were receiving 40 mg/kg eow, four 20 mg/kg eow. In the remaining 11 patients LVM decreased in nine patients to LVM Z-scores ranging from 2.06 to 4.57. Two CRIM-negative patients from either dose group showed hardly any decline in LVM Z-scores (Z-scores decreased from 6.50 to 5.84 and from 8.23 to 7.02). The authors concluded that they did not observe a benefit of the higher dose on cardiac dimensions.

Prater et al. described effects of ERT on the LVMI in 11 long-term survivors (older than 5 years of age at study end) which was published in 2012 [30] and some patients had been described in other publications with shorter follow-up [11, 20-22]. Prater et al. observed that the LVMI normalized within 5 months after start of treatment in all 11 patients and remained within the normal limits during follow-up. Five patients had had documented cardiac arrhythmias. Patients had supraventricular tachycardia (SVT) defined as Wolff–Parkinson–White syndrome ventricular pre-excitation syndrome, premature ventricular contractions, or arrhythmias that were not otherwise specified. In one patient the SVTs were treated with radio-ablation. On ECG, the PR, QRSD, QT, and QTc interval durations were within normal range during follow-up. While this study shows the efficacy of ERT,

it also underlines the need to monitor classic infantile patients after LVMI normalization due to the fact that rhythm disturbances may occur. Caution must be exercised when interpreting the data of this study, as the authors only included CRIM-positive patients whose age at last assessment was 5 years or more. All the CRIM-negative patients had died before the age of 5 years, the effects on cardiac dimension and rhythm might be different. On the other hand, it can be argued that with a longer follow-up, more studies will observe normalization of cardiac dimensions.

In **chapter 6** of this thesis the long-term effects of ERT on cardiac size, function and rhythm were studied. In 14 patients, the LVMI and LVPWd responded both well to ERT; the LVMI normalized in 13 patients and this effect was preserved for 13 years. In one patient the LVMI did not normalize, but a significant decrease in LVMI Z-score was observed (Z-score from 8.7 to 2.4). This patient was a CRIM-negative. Another CRIM-negative patient was included in the study; his LVMI did normalize.

Rhythm disturbances
Prater et al. described how they found rhythm disturbances despite normalization of the LVMI [30]. Other studies have reported that rhythm disturbances are a frequent finding in classic infantile Pompe disease and can occur in both CRIM-negative and CRIM-positive patients, even after LVMI normalization [58-65]. Three of our patients had supraventricular tachycardias (SVTs). One was a CRIM-negative patient who died at the age of 4 years and two CRIM-positive patients with SVTs were end-stage patients. The two end-stage patients had SVTs due to Wolff-Parkinson-White (WPW) pattern on their ECG and also had a short PR-interval. A delta wave was observed in four CRIM-positive patients, two of these patients had a short PR-interval but none have had SVTs. These patients must be carefully monitored as they remain at risk to develop rhythm disturbances.

The mechanism underlying the short PR interval is not yet completely understood. One hypothesis was raised in 1982 by Bharati et al., who correlated electrophysiological abnormalities with pathological postmortem findings in the conduction system of untreated patients with classic-infantile Pompe disease. The authors found marked glycogen infiltration and vacuolization and an increase in cell size of the Purkinje cells, suggesting that deposition of glycogen enhances conduction [66]. The fact that the electrophysiological finding that the atrium-His interval was shortened, and the His-ventricular interval was normal, while enlarged cells filled with glycogen were found in all parts of the conduction system including the bundle of His, challenges this hypothesis. A second hypothesis was proposed by Arad et al. who studied a mouse model with a human PRKAG2 mutation, a disease that leads to glycogen accumulation in the cytoplasm of cardiomyocytes [67]. A disruption of the annulus fibrosis by glycogen-filled cardiomyocytes was observed, thereby

allowing atrioventricular activation by bypassing the AV node. This disruption might explain the short PR interval and the WPW pattern, but also the fact that some patients were prone to developing SVTs or severe bradycardia. Reduction of the glycogen storage in the cardiomyocytes can possibly result in normalization of the conduction as an increased in PR-interval was observed in some of the patients from **chapter 6** who were treated with ERT. Our patients were not the only patients with an increase in PR-interval after start of treatment, Ansong et al. also found an increase in PR-interval in all his patients [58]. It should be noted that shortening of the PR interval is a characteristic feature in patients with classic infantile Pompe disease, which is observed in many classic infantile patients.

Two patients from the 40 mg/kg/week group had a dilated hypertrophic cardiomyopathy (DCM) at start of treatment. In one of our patients the DCM was so severe that, due to consanguinity, we had to rule out other causes for the DCM (**chapter 3**). We speculate that the DCM could have been a compensatory response to the severe hypertrophy in these children to sustain adequate blood flow before diagnosis. In both patients the DCM resolved completely during ERT treatment and both are off all cardiac medication. As there were no patients with DCM in the 20 mg/kg eow group, we can only speculate that the higher ERT dose was responsible for the resolution of the DCM.

Central nervous system

A major limitation of ERT is that it cannot pass the blood-brain barrier. The white-matter abnormalities that we found in our classic infantile Pompe patients is worrisome. In **chapters 7 and 8** we observed that in our 11 oldest surviving patients, cognitive development ranged from normal to cognitive declines (in two even intellectual disability with an IQ of 64 or lower). In some the development was stable over time, others showed declines. On brain MRIs we observed white-matter abnormalities in different stages. They were progressive over time, but the rate of progression varied between patients. Also, the white-matter abnormalities do not seem to be related to motor outcome. For example, one patient who learned to walk within the normal age limits showed more abnormalities on the MRI than a patient who became tetraplegic before the age of one. Unfortunately, it is difficult to compare our results with other studies as there are only limited studies reporting longer term MRI data. The few studies that have MRI data described predominantly periventricular white-matter abnormalities that are consistent with what we described as our stage one (periventricular white-matter involvement at the level of the centrum semiovale) [68-71]. There is limited data on we called stage 2 (when the white-matter abnormalities expanded to the subcortical areas and internal and external capsule) or stage 3 (when white-matter abnormalities expand to the infratentorial white-matter area), because we only observed this in patients aged nine years or older. There are virtually no reports on patients over 9 years of age.

Autopsy studies are just as rare and limited to relatively older reports of patients with classic infantile Pompe disease who were born before the introduction of ERT. These autopsy studies found glycogen storage in many cells of the central nervous system (CNS) such as the anterior horn cells of the spinal cord, the brain stem, thalamus, cerebellum and to some extent in the cerebral cortex [15, 72-78]. Our studies have shown that the (CNS) indeed is involved in classic infantile Pompe disease and that cognitive development and brain MRIs should be included in the long-term follow-up. Further research is needed to look at the possible relationship between white-matter abnormalities and the cognitive function in classic infantile Pompe patients [70]. And if there is a relationship between the white-matter abnormalities and the cognitive function, new treatment options such as lentiviral gene therapy via hematopoietic stem cells becomes of increasing importance as it has demonstrated to reduce glycogen storage the cerebrum and cerebellum in pre-clinical studies in mice [79].

FUTURE PERSPECTIVES

The introduction of ERT has changed the face of classic infantile Pompe disease forever. This once fatal disease now sees patients reaching adulthood. Unfortunately, there are still unmet needs in the treatment of classic infantile Pompe patients as many patients still have residual muscle weakness including (but not limited to), facial muscle weakness, weakness of the distal muscles of the hand and feet, weakness of the neck flexor muscles and weakness of the hip flexors [22-25, 30, 35, 37, 68, 80-83]. The higher dose of 40 mg/kg/week does not correct all residual muscle weakness.

Another way to improve treatment outcome, is by starting as young as possible with ERT as the best clinical outcome in seen in patients who start ERT at a very young age on a high dose of 40 mg/kg/week [26]. Currently Pompe disease is included in the newborn screening (NBS) in Taiwan and in several states of the United States (US). In the US, NBS has started but no clinical data is available as screening began only in 2015 [84]. From Taiwan, two studies by Chien et al. and Yang et al. have been published describing the clinical outcome in patients diagnosed through NBS [68, 85]. Chien et al. included 10 CRIM-positive patients [68]. Median age at start of ERT was 16.5 days (range 6 to 48 days) and the median age at study end was 63.5 months (range 28-91 months). All started ERT on 20 mg/kg eow, but all were increased to either 40 mg/kg eow or 20 mg/kg/week between the ages of 2 and 6 years. At study end, all patients survived ventilator-free. The LVMI had normalized in most patients and remained stable over time. All 10 patients learned to walk. The maximum anti-rhGAA antibody titer ranged from 0 to 1:12,800. Chien et al. did describe that muscle weakness became prominent beyond the age of two years and that facial muscle weakness and speech disorders were common. They found slight cognitive impairment at 24 months of treatment with IQs between 70 and 91 and found white-matter abnormalities in 7 of the 9 patients that underwent brain MRIs.

Yang et al. included 14 CRIM-positive patients but excluded the first patient from analysis because this patient was diagnosed before 2009 through a slower NBS technique [85]. For the remaining 13 patients mean age at start of ERT was 12 days (range 6 to 23 days) and mean age at study end was 32.7 months (range 13 to 61 months). All received an ERT dose of 20 mg/kg every other week. At study end, all patients survived ventilator-free. The LVMI had normalized within 6 months of treatment and remained within normal limits during follow-up. All had learned to walk between the ages of 10 and 13 months and were persistent walkers one year later. The median maximum anti-rhGAA antibody titer was 1:1,376 (range 0 to 1:6,400). Cognitive development was within normal range after one year of treatment. No brain MRIs were available in this study. The authors concluded that NBS improves clinical outcome and that they found no negative effects. An explanation

why Yang et al. did not find residual muscle weakness or cognitive decline could be the shorter follow-up duration of 32 months (just under three years) as most studies find residual muscle weakness and white matter-abnormalities in patients older than three years [22, 26, 37, 70, 86, 87].

For now, NBS for Pompe disease is currently not performed in the Netherlands as there are some drawbacks of the NBS that prevent its usage. The current assays (Fluorometry and Microfluidics) are enzyme assays only that still have high false-positive rates and more importantly lack the ability to include a second-tier analysis to diagnosis the classic infantile Pompe patients and exclude non classic patients that may be symptom free until late adulthood [84]. This means that a positive result requires additional testing, something that parents might not always agree to because of the high emotional (and financial) burden of being diagnosed with late-onset Pompe disease without being able to predict the disease course in the individual patient. When second tier analysis is improved, making it possible to only diagnose the classic infantile patients without needing additional samples, NBS combined with a higher ERT dose from the start of treatment could then further improve the clinical outcome of classic infantile Pompe patients.

ERT has limitations and is not a curative treatment. Great promise for a curative treatment can be found in lentiviral gene therapy via hematopoietic stem cells. In the mouse model, lentiviral gene therapy has shown to correct glycogen storage in the heart muscle, skeletal muscle but also in the central nervous system which is currently not being reached by ERT [79]. A single transplantation would be the only treatment for these patients; they would no longer be dependent on weekly infusions of alpha-glucosidase and not build an immune response because the genes of the patient will be modified. Because hematopoietic stem cell gene therapy requires pre-transplant conditioning of the patients, classic infantile Pompe patients would need initial treatment with ERT to stabilize their clinical condition before they could undergo the transplant. In Pompe mice who received ERT before the gene therapy, an immune response was seen that targeted the genetically modified hematopoietic stem cells. Before this strategy can be implied in classic infantile patients, some immunogenic problems need to be worked out.

In the coming years these details will be addressed, and gene therapy could be a treatment for Pompe disease in the foreseeable future. Until then the efficacy of enzyme replacement therapy must be enhanced by starting patients as young as possible on a higher and more frequent ERT dose. In addition, improved immunomodulation protocols need to be developed. One of the option could be to extend the current protocols by longer and more frequent treatment with RTX as was proposed by Elder et al. and later by Kishnani et al. [32, 45].

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Chapter 10

Summary
Samenvatting

SUMMARY

Pompe disease, is a rare lysosomal storage disorder, in which an enzyme called *acid α-glucosidase* (GAA) is missing due to variations in the *GAA* gene. GAA is responsible for the degradation of long sugar chains (glycogen) into smaller sugar molecules (glucose). When GAA is deficient, glycogen builds up in the lysosome causing damage to the cells and tissues, which eventually leads to loss of function. Muscle cells are most commonly affected causing muscle weakness, mobility problems, respiratory difficulties, cardiac hypertrophy and cardiac failure and swallowing difficulties

Pompe disease presents as a clinical spectrum. Clinical variation is primarily caused by presence or absence of residual enzyme activity. Patients with childhood onset and adult Pompe disease are at the milder end of the spectrum. In these patients symptoms may present at any age. The delayed onset is the result of some residual GAA activity present in the muscle cells of patients. This opposed to the classic infantile form, the patients studied in this thesis, who have a complete enzyme deficiency and who are at the severe end of the spectrum. They present within the first months of life with hypertrophic cardiomyopathy, generalized muscle weakness, feedings difficulties and respiratory problems. Without treatment these patients die within the first year of life because of cardiac and respiratory failure. In 2006, treatment with enzyme replacement therapy (ERT) became registered for all Pompe patients. Treatment has greatly improved survival and motor function in children with the classic infantile phenotype, but many patients still have residual symptoms of the disease such as residual muscle weakness or respiratory difficulties for which they may require invasive ventilation. In addition, it has been shown that patients may produce antibodies against the ERT with recombinant human alpha-glucosidase (anti-rhGAA antibodies) that pose a serious threat of the efficacy of the treatment.

The aim of this thesis is two-fold. The first part of this thesis describes the steps we took to improve clinical outcome of classic infantile Pompe patients by increasing the ERT dose and starting a treatment called immunomodulation to prevent or eliminate anti-rhGAA antibodies. In the second part of this thesis the long-term effects of ERT on the heart and cognitive development are studied. Studies on cognitive development are of specific interest because ERT cannot reach the brain because it is protected by the blood-brain barrier (BBB). As a consequence, large molecules such as rhGAA cannot enter the brain.

Chapter 1 provides a general introduction into Pompe disease and ERT. Background information is provided on the discovery of Pompe disease, the lysosome and the development of treatment. This chapter also gives an overview of treatment outcomes in classic infantile patients receiving ERT and immunomodulation published so far in the literature.

Chapter 2 describes the effects of a higher and more frequent ERT dose of 40 mg/kg/week from start of treatment in comparison to the regular dose of 20 mg/kg every other week (eow) in cross-reactive immunologic material (CRIM) positive patients. CRIM-positive means that patients produce acid alpha-glucosidase enzyme protein, but that the enzyme is inactive. CRIM-negative patients do not produce any alpha-glucosidase protein at all. The clinical outcome of eight CRIM-positive patients, four in both dose groups, was compared. The survival was 100% in both groups, one patient in the 20mg group required invasive ventilation. The walking abilities was best in the four patients receiving 40 mg/kg/week. Patients receiving 40 mg/kg/week had no loss of motor function during follow-up. These patients also did not require hospitalization for airway infections. Treatment was well tolerated with no apparent differences between the two dose groups in terms of number of infusion associated reactions (IARs). Children in both dose groups developed anti-rhGAA antibodies, but children treated with 40 mg/kg/week did tend to develop a higher anti-rhGAA antibody titer. This warranted further investigation.

In a previous study, it was observed that the highest titers were found in patients who were older than 2 months of age at start of treatment. As anti-rhGAA antibodies can interfere with the efficacy of treatment, additional treatment with immunomodulation was introduced in newly diagnosed patients older than two months of age at start of treatment. The effects of immunomodulation in an ERT-naïve setting on the formation of anti-rhGAA antibody formation was studied in **chapter 3**. Three classic infantile Pompe patients (one CRIM-negative and two CRIM-positive) were included. The ERT dose was 40 mg/kg/week from start and all three received Rituximab (RTX) and Methotrexate (MTX) before the first infusion of ERT to eliminate B cells (RTX) and to mediate T and B-cell interaction (MTX) with the aim to prevent the production of anti-rhGAA antibodies. Patients also received human immunoglobulins (IVIG) to overcome reduced IgG levels and to protect them from infections. The study showed that with this regimen anti-rhGAA antibody formation could not be prevented. Two of the three patients developed high anti-rhGAA antibody titers of 1:200,000 and 1:800,000 despite immunomodulation. These high titers did not seem to elicit neutralizing effects. Despite the high antibody titers, the clinical outcome of patients was good as all were alive at study end and all learned to walk. The patient with the highest titer (a CRIM-negative patient) did temporarily lose the ability to walk but regained this without intervention before study end. We concluded that the protocol applied in this study did not prevent anti-rhGAA antibody formation and that further research is needed to optimize protocols that can provide long-term prevention of antibody formation without compromising general immunity in Pompe patients treated with ERT.

In **chapter 4** we evaluated whether immunomodulation can eliminate high sustained antibody levels. Three patients (two CRIM-negative and one CRIM-positive) who had developed high anti-rhGAA antibody titers and showed a clinical decline (they either lost motor milestones and/or suffered from frequent IARs) were included. The ultimate goal was to eliminate anti-rhGAA antibodies and to improve clinical outcome. The immunomodulation consisted of RTX with Bortezomib, Rapamycin and IVIG. A decline in antibody titer was only observed in one CRIM-negative patient, but it was unclear whether the decrease in antibody titer was the sole effect of the immunomodulation or whether the antibody titer was already declining (on the day immunomodulation was started his titer had spontaneously decreased from 1:781,250 to 1:156,250). In the other patients no substantial decrease in titer was observed. The two CRIM-negative patients who could walk at start maintained their ability to walk; the patient who had lost this ability did not regain it. The immunomodulation protocol used in our study reduced antibody titers to some extent but did not eliminate them. We speculated that the high dose of ERT applied from start was the main reason for the good outcome of our CRIM-negative patients.

Where in chapter 2 the effects of a higher and more frequent dose was studied in CRIM-positive patients, in **chapter 5** we compared the long-term outcome of classic infantile Pompe patients who started ERT on 20 mg/kg eow to those who started on 40 mg/kg/week. For this study we included both CRIM-positive and CRIM-negative patients. In the 40mg group, five children were also treated with immunomodulation. In these patients we also studied the additional effect of immunomodulation. Six patients were treated with 20 mg/kg eow (two were CRIM-negative) and 12 patients with 40 mg/kg/week (three were CRIM-negative). The survival, ventilator-free survival and motor outcome was better in the patients who started ERT on 40 mg/kg/week. The survival was 66% in the 20mg group and 92% in the 40mg group. Both CRIM-negative patients in the 20mg group died, whereas all three are alive in the 40mg group. Ventilator-free survival was 50% in the 20mg group and 92% in the 40mg group. In the 20mg group 67% learned to walk compared to 92% in the 40mg group. At the age of 3 years 33% and 92% were able to walk and at study end this was 17% and 83%. Anti-rhGAA antibody titers were observed in both groups. The highest peak titers were observed in the 40 mg/kg/week group (peak titers ranged from 1:250 to 1:800,000), in the 20mg group it ranged from 1:1,250 to 1:31,250. In this chapter it has become clear that classic infantile patients treated with 40 mg/kg/week from the start have a better (ventilator-free) survival and motor outcome. This is clearly seen in the CRIM-negative patients, as all in the 40mg group were alive and persistent walkers at study end compared to none in 20mg group.

Of the five patients that received immunomodulation, two were CRIM-negative. They were all alive and able to walk at study end, but all of them developed antibodies despite immunomodulation. Because the overall clinical outcome was better in these patients than on ERT monotherapy, immunomodulation may have possibly contributed to the clinical stability of our patients.

In part two of this thesis, additional clinical outcome parameters of cardiac response and effects of treatment on the central nervous system are presented. Cardiac failure is the main cause of death in untreated classic infantile Pompe patients. In **chapter 6** we studied the effects of 14 years of treatment on cardiac dimensions and function, as little is known about long-term effects of ERT on the heart. While an increase in left ventricular mass index (LVMI) was observed in nearly half of the patients in the first four weeks, normalization of the LVMI was observed in 13 of the 14 patients thereafter and this was maintained for more than 13 years of treatment. The LVMI increased again slightly in one patient after clinical deterioration. Delta-waves (that are suggestive for Wolf-Parkinson-White (WPW) pattern) were found in six patients and resulted in supraventricular tachycardias (SVTs) in three children. Two of them required medication and/or ablation to treat the SVTs. Because the risk for rhythm disturbances remains, regular cardiac evaluations should be continued even after a good response to ERT is seen on cardiac dimension.

Because ERT cannot cross the blood-brain barrier, the cognitive development of classic infantile Pompe patients has always been of particular interest. In 2012, a study from our group on the cognitive development of classic infantile Pompe patients reported that cognitive development was normal or mildly delayed in the 10 patients that were followed for a maximum period of 11 years. In **chapter 7** we report on one of our patients that showed a different course than what we had observed before. Until the age of 6 he had a normal cognitive development. He then presented with behavioral problems. At the age of 9 his cognitive development showed a decline towards moderate intellectual disability (IQ 48). Brain MRI showed a progression of the white-matter abnormalities compared to earlier MRIs (this data was previously published by our group in 2012). These findings suggested that the decline in cognitive function in combination with the white-matter abnormalities, might be related and progressive over time. To answer this question, we included the oldest surviving patients (11 patients aged 7 years or older to young adulthood), who had at least two neuropsychological tests and/or brain MRIs, in a new study (**chapter 8**) that evaluated the long-term cognitive development in these patients. The cognitive development was either normal and stable over time, or it showed cognitive declines (in two an intellectual disability with an IQ of 64 or lower was observed). On brain MRI white-matter abnormalities in different stages were observed. These white-matter abnormalities were progressive over time, but the rate of progression varied between patients. There seemed

to be a characteristic pattern of involvement over time (from the periventricular areas at the age of 2 years, to subcortical areas at the age of 8 years, to involvement of the brainstem by the age of 11 years) there were considerable variations between patients, which are reflected by variations in neuropsychological development. This study shows that the central nervous system (CNS) indeed is more involved in classic infantile Pompe disease than was previously thought. Cognitive development and brain MRIs should be included in long term follow-up studies. Future therapies should also include the CNS as a target.

Finally, in **chapter 9** the main findings of the studies, presented in this thesis are discussed, as well as our conclusions and the future perspectives.

SAMENVATTING

De ziekte van Pompe is een zeldzame lysosomale stapelingsziekte. Bij deze ziekte, ontbreekt het enzym *zure alfa-glucosidase* (GAA) doordat er twee variaties zijn in het *GAA* gen. GAA is verantwoordelijk voor de afbraak van lang suikerketens (glycogeen) tot korte suikermoleculen (glucose). Wanneer GAA ontbreekt, stapelt glycogeen zich op in het lysosoom. Dit leidt tot schade aan cellen en weefsels, wat uiteindelijk leidt tot verlies van functie van de cel. Spiercellen zijn met name aangedaan wat resulteert in spierzwakte, mobiliteit problemen, ademhalingsproblemen, een verdikking van de hartspier en hartfalen, en problemen met slikken.

De ziekte van Pompe heeft een breed klinisch spectrum. Dit klinisch spectrum wordt voornamelijk bepaald door de restactiviteit van het enzym. Kinderen en volwassenen hebben een mildere vorm van de ziekte en kunnen zich presenteren op elke leeftijd. Dat de klachten zich pas later uiten komt doordat zij nog enige rest activiteit hebben van GAA in de spiercel. Aan de andere kant van het klinisch spectrum bevinden zich de klassiek infantiele Pompe patiënten, de patiënten waar dit proefschrift over gaat. Zij hebben geen restactiviteit van GAA en presenteren zich in de eerste maanden van hun leven met een hypertrofische cardiomyopathie, algehele spierzwakte, voedingsproblemen, en ademhalingsproblemen. Zonder behandeling zullen zij in hun eerste levensjaar overlijden vanwege cardiorespiratoir falen. Sinds 2006 is vervangingstherapie (ERT) beschikbaar voor alle Pompe patiënten waarbij het ontbrekende enzym via een infuus aan de patient wordt gegeven. Deze behandeling heeft ervoor gezorgd dat de klassiek infantiele patiënten niet meer komen te overlijden in het eerste levensjaar. Helaas hebben veel patiënten ondanks behandeling nog zwakte van de skeletspieren en ademhalingsspieren, en een deel van de kinderen komen aan de beademing. Daarnaast kunnen patiënten antilichamen (anti-rhGAA antilichamen) tegen de ERT vormen die de effectiviteit van de behandeling kan verminderen.

Het doel van dit proefschrift is tweeledig. In het eerste deel beschrijven we welke stappen we hebben genomen om de behandeling te verbeteren, allereerst door de dosis te verhogen en ten tweede door een behandeling genaamd immunomodulatie te introduceren die anti-rhGAA antilichamen moet voorkomen en/of verwijderen. In het tweede deel beschrijven we de lange termijneffecten van ERT op het hart en de cognitieve ontwikkeling. Met name de cognitieve ontwikkeling is van belang, aangezien ERT de bloed-hersen barrière niet over kan.

In **hoofdstuk 1** geven we een algemene introductie over de ontdekking de ziekte van Pompe en van het lysosoom, de ontwikkeling van de behandeling en de eerste uitkomsten van de behandeling met ERT en immunomodulatie.

In **hoofdstuk 2** hebben we onderzocht wat de verschillen zijn van een hogere dosering op de klinische uitkomst. Hierbij werden de klinische uitkomsten van patiënten die een hoge dosering (40 mg/kg/week) vergeleken met patiënten die de standaarddosering (20 mg/kg om de week) kregen. In beide groepen zaten vier van cross-reactive immunological material (CRIM) positieve patiënten. CRIM-positief betekent dat patiënten nog enige productie hebben van *zure alfa-glucosidase*, maar dat het enzym niet actief is. CRIM-negatieve patiënten maken helemaal geen *zure alfa-glucosidase* aan. De overleving was 100% in beide groepen, één patiënt uit de 20mg groep kwam aan de beademing. De motorische uitkomst was het beste in de 40mg groep, zij hadden geen verlies van motorische mijlpalen en werden nooit opgenomen voor luchtweginfecties. De hogere dosering werd goed verdragen, er waren geen verschillen in de frequentie van infusie reacties tussen de beide groepen. In beide groepen ontwikkelden de kinderen een anti-rhGAA antilichamen, waarbij in de 40mg groep een hogere titer werd geobserveerd. Wat het effect van deze hogere titer is moest verder onderzocht worden.

In eerdere studies is aangetoond dat de hoogste anti-rhGAA antilichaam titer werd gevonden in kinderen die bij de start van hun behandeling ouder waren dan 2 maanden. Aangezien anti-rhGAA antilichamen de effectiviteit van ERT kan verminderen, is een behandeling genaamd immunomodulatie geïntroduceerd bij patiënten die bij de start van behandeling ouder waren dan 2 maanden. De effecten van immunomodulatie op de vorming van anti-rhGAA antilichamen bij kinderen die nog ERT-naïef waren, werd bestudeerd in **hoofdstuk 3**. Drie klassiek infantiele Pompe patiënten (één CRIM-negatief en twee CRIM-positief) hebben voorafgaand aan het eerste infuus ERT eerst een behandeling met Rituximab (RTX) en Methotrexaat (MTX) gekregen om B-cellen te vernietigen (RTX) en de om de B- en T-cel interactie te beïnvloeden (MTX) met het doel om antilichaam vorming te voorkomen. Daarnaast kregen zij ook humane immunoglobulines (IVIG) om een periode van lage afweer en infecties te voorkomen. Dit onderzoek liet zien dat met het gebruikte protocol de vorming van anti-rhGAA antilichamen niet wist te voorkomen. Twee van de drie patiënten ontwikkelden een hoge anti-rhGAA antilichaam titer van 1:200.000 en 1:800.000. Deze hoge titer hadden geen neutraliserend effect. Ondanks de hoge titer was de klinische uitkomst heel goed, alle drie de patiënten waren in leven aan het einde van de studie en hebben leren lopen. De patiënt met de hoogste titer (de CRIM-negatieve patiënt) heeft een periode niet kunnen lopen, maar kon aan het einde van de studie weer lopen zonder dat daar een interventie voor nodig is geweest. Wij concludeerden dat het door ons gebruikte protocol een hoge anti-rhGAA antilichaam titer niet voorkomt en dat

aanvullend onderzoek noodzakelijk is om immunomodulatie protocollen te ontwikkelen die wel de vorming van antilichamen kan voorkomen zonder dat dit de gevolgen heeft voor de afweer van patiënten.

In **hoofdstuk 4** is onderzocht of immunomodulatie ook hoge anti-rhGAA antilichamen kan elimineren. Drie patiënten (twee CRIM-negatief en één CRIM-positief) hadden gedurende hun behandeling met ERT hoge anti-rhGAA antilichaam titers ontwikkeld en hadden alle drie een achteruitgang in hun klinisch functioneren (ze gingen motorisch achteruit en/of hadden veel infusie reacties). Het doel was om middels immunomodulatie de antilichamen te verwijderen en de klinische uitkomst te verbeteren. Het protocol bestond uit een behandeling met RTX, Bortezomib, Rapamycine en IVIG. Een daling in antilichaam titer werd alleen gezien in één CRIM-negatieve patiënt, maar het was onduidelijk of deze daling door de immunomodulatie kwam. Op de dag dat deze patiënt startte met immunomodulatie was de titer al spontaan gezakt van 1:781,250 naar 1:156,250. In de andere twee patiënten werd geen substantiële daling gezien van de titer. De twee CRIM-negatieve patiënten die konden lopen bij de start van dit onderzoek, konden dat aan het einde van de studie nog steeds. De patiënt die het lopen verloor (de reden van inclusie in de studie) heeft het lopen niet herwonnen. Het protocol zoals wij die hebben toegepast liet tot op zekere hoogte een daling zien van de titer, maar helemaal verwijderen gebeurde niet. Wij denken dat de goede klinische uitkomst waarschijnlijk komt door de hoge dosis ERT die patiënten kregen en niet vanwege de immunomodulatie

Waar in hoofdstuk 2 de effecten van een hogere dosering werd bestudeerd in CRIM-positieve patiënten, is voor **hoofdstuk 5** vergelijken wat de verschillen zijn op de lange termijn uitkomsten van alle patiënten die startte op de standaarddosering van 20 mg/kg om de week en patiënten die startte op 40 mg/kg/week. Zowel CRIM-positieve als CRIM-negatieve patiënten zijn geïncludeerd in dit onderzoek. In de 40mg groep zijn vijf patiënten aanvullend behandeld met immunomodulatie. Zes patiënten kregen 20 mg/kg om de week (twee waren CRIM-negatief) en 12 patiënten kregen 40 mg/kg/week (drie waren CRIM-negatief). De overleving, de beademingsvrije overleving en motorische uitkomst beter is in kinderen die starten op 40 mg/kg/week. De overleving was 66% in de 20mg groep en 92% in de 40mg groep; beide CRIM-negatieve patiënten in de 20mg groep zijn overleden, terwijl in de 40mg groep leven alle CRIM-negatieve patiënten nog. De beademingsvrije overleving was 50% in de 20mg groep en 92% in de 40mg groep. In de 20mg groep leerde 67% lopen, in de 40mg groep was dit 92%. Bij de leeftijd van 3 jaar kon 33% in de 20mg groep nog lopen, in de 40mg groep was dit 92%. Aan het einde van de studie waren deze percentages 17% en 83%. Anti-rhGAA antilichamen werden in beide groepen gevonden. De hoogste titer werd gevonden in de 40mg groep (piek titers bevonden zich tussen 1:250 en 1:800,000), in de 20mg groep bevonden zij zich tussen 1:1,250 en 1:31,250. Deze studie liet duidelijk zien

dat de kinderen op een hogere dosering een betere klinische uitkomst hadden dan kinderen op een lage dosering. Dit is met name het geval voor de CRIM-negatieve patiënten. De vijf patiënten die naast de hoge dosering ook immunomodulatie gekregen (waarvan twee CRIM-negatief) waren allemaal in leven aan het einde studie en allen konden lopen. Zijn hadden allemaal anti-rhGAA antilichamen ontwikkeld ondanks de immunomodulatie. Maar de klinische uitkomst was beter dan de uitkomst van de kinderen die alleen een hoge dosering ERT kregen, mogelijk heeft immunomodulatie toch een rol gehad in het verbeteren of stabiliseren van de klinische uitkomst

In deel twee van dit proefschrift worden de effecten van ERT op het hart en de cognitieve ontwikkeling besproken. Hartfalen is de belangrijkste doodsoorzaak van onbehandelde klassiek infantiele Pompe patiënten. In **hoofdstuk 6** hebben beschrijven we de lange termijneffecten van ERT op de dikte en functie van het hart, omdat deze nog nooit in kaart waren gebracht. In de eerste vier weken van de behandeling werd in bijna de helft van de patiënten een toename gezien van de dikte (de LVMI) van het hart, waarna de LVMI in 13 van de 14 patiënten normaliseerde en binnen de normaalwaarde bleef gedurende 13 jaar. In één patiënt werd een toename van de LVMI gezien toen deze patiënt klinisch achteruitging. Delta-golven (welke zijn geassocieerd met het Wolf-Parkinson-White (WPW) patroon) werden in zes patiënten gevonden. In drie patiënten leidde dit tot supraventriculaire tachycardie (SVT). Twee van hen zijn hiervoor behandeld met medicatie of een operatie. Deze studie toonde aan dat het risico op hartritmestoornissen ondanks goede respons op ERT lijkt te blijven bestaan en dat patiënten hiervoor onder cardiologisch controle moeten blijven.

Omdat ERT de bloed hersenbarrière niet over kan, is de cognitieve ontwikkeling van de klassiek infantiele Pompe patiënten altijd een belangrijk onderdeel geweest van de klinische studies. In 2012 publiceerde onze groep dat de cognitieve ontwikkeling normaal tot licht vertraagd was in de 10 patiënten die voor maximaal 11 jaar werden gevolgd. In **hoofdstuk 7** beschrijven we echter dat één patiënt een achteruitgang laat zien in zijn cognitieve ontwikkeling. Tot de leeftijd van 6 jaar ontwikkelde hij zich normaal, maar bij de leeftijd van 9 jaar was er sprake van een matige verstandelijke beperking (IQ van 48). Hersen MRI liet uitgebreide witte stof afwijkingen zien, veel meer dan wij in onze eerdere studie hadden gezien. Dit suggereerde dat de witte stof afwijkingen van progressieve aard zouden kunnen zijn. Om dit beter in kaart te brengen werd voor **hoofdstuk 8** de cognitieve ontwikkeling van de 11 oudste patiënten onderzocht (7 jaar en ouder), bij wie er minimaal twee neuropsychologische testen en/of hersen MRIs werden gemaakt. De cognitieve ontwikkeling van de patiënten was normaal en stabiel of liet zien dat er een daling zichtbaar was tot aan het niveau van een lichte verstandelijke beperking (twee patienten hadden een IQ van 64 of lager). Op de MRI werden in verschillende stadia witte stof

afwijkingen gezien. Deze waren progressief in de tijd. Als eerste was de periventriculaire regio aangedaan (vanaf een leeftijd van 2 jaar) daarna de subcorticale regio (vanaf 8 jaar) en later ook de hersenstam (vanaf 11 jaar). De progressie varieerde per patiënt. Met deze studie hebben we aangetoond dat het centraal zenuwstelsel wel degelijk is aangedaan bij kinderen met de klassiek infantiele vorm van de ziekte van Pompe. Bij de zoektocht naar een nieuwe therapie moet gezocht worden naar een therapie dat ook het centraal zenuwstel kan bereiken.

Tot slot, beschrijven we in **hoofdstuk 9** de belangrijkste bevindingen van dit proefschrift. Wij plaatsen onze bevindingen in een breder context en geven advies ten aanzien toekomstige onderzoeken en behandelingen.

Part 3

Addendum

Abbreviations

LIST OF ABBREVIATIONS

4MU	4-methylumbelliferon
6MWT	six-minute walk test
AIMS	Alberta Infant Motor Scale
ALIC	Anterior limb of the internal capsule
ALT	Alanine Transaminase
AST	Aspartate Transaminase
BSID-II	Bayley Scales of Infant Development II
CBCL	Child Behavior Checklist
CHO	Chinese Hamster Ovarium (cells)
CK	Creatine Kinase
CNS	Central nervous system
CRIM	Cross-reactive immunological material
ECG	Electrocardiogram
EOW	Every other week
ELISA	Enzyme-linked immunosorbent assay
ERT	Enzyme replacement therapy
FVC	Forced Vital Capacity
GAA	Acid a-glucosidase
Griffiths	Griffiths Mental Development Scales
HCM	Hypertrophic cardiomyopathy
IAR	Infusion associated reaction
ITI	Immune tolerance induction
IVIG	Intravenous Immunoglobulin
IVSd	Inter-ventricular septum thickness in diastole
IQ	Intelligence Quotient
LDH	Lactate Dehydrogenase
LSD	Lysosomal storage disorder
LVIDd	End-diastolic left ventricular internal cavity dimension in diastole

LVMI	Left-ventricular mass index
LVPEP	Left ventricular pre-ejection period
LVPWd	Left ventricular posterior wall thickness in diastole
NEPSY	A Developmental NEuroPSYchological Assessment
NGT	Nasogastric tube
MRI	Magnetic resonance imaging
MRSA	Meticilline-resistente Staphylococcus aureus
MTX	Methotrexate
MUGlc	4-methylumbelliferyl-a-D-glucopyranoside
PBS	Phosphate Buffered Saline
PK	Pharmacokinetic (analysis)
PIQ	Performance Intelligence Quotient
PSI	Processing-speed index
PLIC	Posterior limb of the internal capsule
PWTDI	Pulsed wave Tissue Doppler Imaging
RAVLT	Rey Auditory-Verbal Learning Test
rhGAA	Recombinant human alpha-glucosidase
RTX	Rituximab
RVPEP	Right ventricular pre-ejection period
RWT	Relative wall thickness
SD	Standard deviation
SF	Shortening fraction
SON-R	Snijders Oomen Nonverbal Intelligence Test-Revised
TIQ	Total Intelligence Quotient
TVIQ	Verbal Intelligence Quotient
WISC-III	Wechsler Intelligence Scales for Children, Third Edition
WPW	Wolff-Parkinson-White syndrome
WNV-NL	Wechsler Non-verbal Scales in Dutch

Addendum

List of publications

PUBLICATIONS

E Poelman, JJA van den Dorpel, M Hoogeveen-Westerveld, JMP van den Hout, LJ van der Giessen, NAME van der Beek, WWM Pijnappel, AT van der Ploeg*; Effects of higher and more frequent dosing of ERT and immunomodulation on long-term clinical outcome of classic infantile Pompe patients.* Submitted in the Journal of Inherited Metabolic Disease

E Poelman, M Hoogeveen-Westerveld, JMP van den Hout, RGM Bredius, AC Lankester, GJA Driessen, SSM Kamphuis, WWM Pijnappel, AT van der Ploeg; *Effects of immunomodulation in classic infantile Pompe patients with high antibody titers.* Submitted in Orphanet Journal of Rare Disease

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BJ Ebbink, **E Poelman**, FK Aarsen, I Plug, L Régal, C Muentjes, NAME van der Beek, MH Lequin, AT van der Ploeg, JMP Van den Hout; *Classic infantile Pompe disease: As pioneering patients approach adulthood, the next puzzle is the brain.* Dev Med Child Neurol. 2018 Jun;60(6):579-586

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Addendum

PhD Portfolio

PHD PORTFOLIO

Name PhD student: Esther Poelman
Erasmus MC Department: Pediatrics, Center for Lysosomal and Metabolic Diseases
PhD period: 01-02-2013 until 01-02-2018
Promotor: Prof. dr. A.T. van der Ploeg

	Year	Workload (Hours/ECTS)
General courses		
- Basiscursus Regelgeving Klinisch Onderzoek (BROK)	2014	1.0
- Integrity in Research	2014	0.3
- CPO-course: design, conductance, analysis and clinical implications	2014	0.3
- Biomedical English Writing and Communication	2016	3.0
Research skills		
- Systematic Literature Retrieval and Endnote	2013	1.0
- Biostatistical Methods I: Basic Principles	2014	5.7
- Journal Club, Center for Lysosomal and Metabolic Disease	2013-2017	1.5
- Biweekly Research Meeting, Center for Lysosomal and Metabolic Disease	2013-2017	2.0
In depth courses		
- 12th International Postgraduate course on Lysosomal Storage Diseases, Nierstein, Germany	2013	1.2
- Steps Forward in Pompe Disease, Turin, Italy	2014	1.0
- Steps Forward in Pompe Disease, Amsterdam, The Netherlands	2016	1.0

	Year	Workload (Hours/ECTS)
Presentations and (Inter)national conferences		
- Sophia Research Day, Rotterdam, The Netherlands (oral presentation)	2013	2.0
- Erfelijke Stofwisselingsziekten Nederland (ESN) Symposium, Rotterdam, The Netherlands	2015	1.0
- Society for the Study of Inborn Errors of Metabolism (SSIEM) Symposium, Lyon, France (poster presentation)	2015	0.5
- World Muscle Society Symposium, Birmingham, United Kingdom (poster presentation)	2015	0.5
- Voorjaarssymposium Vereniging Erfelijke Stofwisselingsziekten (ESN), Groningen The Netherlands (oral presentation)	2016	2.0
- Sophia Research Day 2016, Rotterdam, The Netherlands (SLAM presentation)	2016	1.0
- Patiëntendag klassiek infantiele Pompe patiënten, Rotterdam, The Netherlands	2016	1.0
- Sophia Research Day 2017, Rotterdam, The Netherlands (SLAM presentation)	2017	1.0
- World Muscle Society Congress 2017, Saint Malo, France (poster presentation)	2017	1.0
- Society for the Study of Inborn Errors of Metabolism (SSIEM) Symposium, Rio de Janeiro, Brasil (poster presentation)	2017	0.5
Seminars and Workshops		
- Erasmus MC PhD-day	2014	0.3
- Jonge Onderzoekersdag NVK/TULIPS for Child Health, Veldhoven, The Netherlands	2014	0.5
Awards and Prizes		
- World Muscle Society Fellowship	2017	
Other		
- Organising Committee of the Sophia Research Day 2015	2015	2.0

Addendum

About the Author

ABOUT THE AUTHOR

Esther Poelman was born in Hengelo on March 28th, 1987. She graduated from Lyceum De Grundel in Hengelo in 2005. During her time in secondary school, as an enthusiastic gymnast, she also earned her degree as a Gymnastics coach from the Royal Dutch Gymnastics Federation (KNGU) in 2004. She was a gymnastics coach between 2002 and 2009 in Hengelo and Ouderkerk aan de Amstel and returned to coaching in 2018 for City Gymnastics in Amsterdam. In 2014 she also became a certified Gymnastics judge.

In 2005 she started her medical training at the Vrije Universiteit in Amsterdam. As part of her clinical rotations, she did elective internships at the Department of Rheumatology at the VUmc (which led to her first ever publication) and Child Psychiatry at De Bascule in Amsterdam. After finishing her senior clinical elective at the department of Pediatrics in the Amstelland Ziekenhuis in Amstelveen, she obtained her medical degree in 2011. She traveled for several months in South-east Asia with Sascha, before she started her medical career as a resident (ANIOS) at the department of pediatrics at the Meander Medisch Centrum in Amersfoort. In 2013 she started working at the Center for Lysosomal and Metabolic Diseases at the Erasmus MC under the supervision of Prof. dr. A.T. van der Ploeg and dr. J.M.P. van den Hout, this thesis is the result of all her hard work. In November 2018 she returned to her clinical roots as a resident (ANIOS) at the department of Pedicatrics at the Leids Universitair Medisch Centrum (LUMC). She aspires to become a pediatrician.

Esther lives in Amsterdam with Sascha and their two children Romée and Axel.

Addendum

Acknowledgments | Dankwoord

DANKWOORD

Voordat ik aan het meest gelezen deel van ieder proefschrift begin, wil ik allereerst alle patiënten en hun ouders, grootouders, broers, zussen, ooms, tantes, neefjes, nichtjes en vrienden bedanken voor jullie jarenlange inzet en strijd - samen met ons - tegen deze ziekte. Zonder jullie duizenden kilometers reizen, vertrouwen, geduld, optimisme, ontelbare bezoekjes aan vele specialisten en logeerpartijtjes (met nachtelijks bloedafnames) op 1 Noord met Frozen op de achtergrond, was dit proefschrift er nooit geweest. Ik heb ontzettend veel bewondering voor jullie allemaal.

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Al mijn lieve vriendjes, vriendinnetjes (en jullie kinderen inmiddels) en collega's, te veel om jullie allemaal persoonlijk op te noemen, maar bedankt voor alle etentjes, barbecues, kopjes koffie, taartjes, adviezen voor mijn onderzoek, het arts zijn, de vele playdates en alle andere lieve momenten. Een dikke kus voor jullie allemaal.

Mijn paranimfen Floor en Audrey. Lieve **Floor**, als Poelman en Poelsma waren we jaren lang tot elkaar veroordeeld op de VU. Bij jou mag ik altijd komen juichen, klagen en uithuilen. Met jouw advies help je me weer op weg als ik het zelf even niet meer weet. Ik hou met heel mijn hart van je. Liefste **Audrey**, vanaf de eerste echte sollicitatie voor mijn eerste baan tot aan mijn promotie ben jij er altijd geweest. Wat moet ik nu toch zonder je! Ik heb zo'n bewondering voor jouw wilskracht, zelfvertrouwen en optimisme. Je laat je door niets of niemand uit het veld slaan en dat heeft je gebracht tot waar je nu bent. Ik zou niemand anders naast me willen hebben staan vandaag.

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Mijn allerliefste **papa en mama**, er is geen moment geweest in mijn leven dat ik het gevoel heb gehad dat iets onmogelijk was. Dank voor alle steun en liefde maar bovenal het stimuleren van mijn nieuwsgierigheid. Na alle tegenslagen van 2017 heb ik bewondering voor hoe jullie je er doorheen hebben geslagen en enthousiast de draad weer hebben

opgepakt. Blijf nog heel lang samen de wereld onveilig gaan maken. **Koert**, mijn grote broer, waarvan niemand gelooft dat wij familie zijn totdat ze ons horen praten. Je hebt het maar te stellen gehad met je kleine zusje die het altijd beter weet. Nu er eindelijk weer tijd is moeten gaan wij snel samen wat leuks doen.

Romée, mijn kleine meisje met het geduld van papa en het temperament van mama. Met je lieve kusjes en knuffeltjes maak je altijd alles goed. Blijf altijd omhoog kijken naar die wondere regenboog.
De nieuwste man in mijn leven, mijn liefste **Axel**. Wat een heerlijk vrolijk lachend ventje ben je, ik kan niet wachten om samen met jou de wereld te gaan ontdekken.

Dan tot slot, niemand minder dan de liefde van mijn leven. **Sas**, al gebruik ik 10.000 woorden dan nog schiet ik tekort in mijn dankwoord, maar ik ga een poging wagen voor de man die zonder enige medische kennis gewoon lysosomale stapelingsziekte vloeiend kan uitspreken. Laten we eerlijk zijn, zonder jouw onvoorwaardelijke liefde, steun, duwtjes in de rug, schoppen onder mijn kont, kritische peptalks en heel veel goede koffie was dit boekje nooit tot stand gekomen. Je bent en blijft mijn allerliefste. Op naar die cocktails op een tropische eiland en een heleboel – awesome - Food Ranger momentjes

Esther